Oridonin Improves the Therapeutic Effect of Lentinan on Lung Cancer

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Abstract

Oridonin, a compound from *Rabdosia rubescens*, has been shown to have a potency for the improvement of the antitumor effect of lentinan (LNT). In this study, we tested the effect of oridonin, LNT, and the combination of them on a normal human fetal lung fibroblast cell line MRC-5 and non-small cell lung cancer cell line A549. Then we tested their effects on metastasis and survival with a lung cancer mice model. The effects of them on the mRNA and protein expression of several regulatory factors in A549 and lung tissue were determined by QPCR and western blotting. Results showed that the viability of MRC-5 and A549 were not affected by 0-20 µg/ml oridonin. 0-300 µg/ml LNT did not affect the viability of MRC-5, but 50-400 µg/ml LNT inhibited the viability of A549. 20 µg/ml oridonin and 100 or 300 µg/ml LNT were used in the subsequent study. The oridonin, LNT, or the combination of both had no effect on MRC-5 cell viability. The oridonin had no effect on A549 cell viability but LNT suppressed A549 cell viability, and oridonin promoted the suppression of LNT on A549 cells. *In vivo* study showed that oridonin alone had no effect on metastasis and survival but LNT decreased metastasis and survival in mice. Oridonin improved the suppression of LNT against metastasis and further improved the survival rate. In both A549 and lung tissues, LNT increased the mRNA and protein expression of caspase-3, caspase-8, caspase-9, Bax, p53, p21, and IκB-α, reduced mRNA and protein expressions of Bcl-2 and NF-κB. Oridonin enhanced all the effects of LNT on cells. Our study demonstrated that oridonin enhanced the antitumor effects of LNT and is conducive to the development of oridonin and LNT as a novel cancer drug regimen.

Introduction

Lung cancer (both small cell and non-small cell) is the second most common cancer in both men and women (1). In 2019, about 13% of all new cancers are lung cancers, including more than 228,000 new cases of lung cancer in the United States (1). Lung cancer led to over 142,000 deaths each year, which is by far the leading cause of cancer death among both men and women, even more than the sum of the number of colon, breast, and prostate cancers (1). Among all the lung cancer, non-small cell lung cancer (NSCLC) accounts for about 85% of all lung cancers. In clinical treatments, NSCLCs are relatively insensitive to chemotherapy, compared to small cell carcinoma. Therefore, to improve the theoretic efficiency of chemotherapy toward NSCLCs is of utmost importance for clinical lung cancer treatments.

In recent years, traditional medicine has been studied for clinical treatment for their low side effects (2, 3). To achieve fewer side effects, increasing attention has been given to a fungal polysaccharide, lentinan (LNT), for its strong antitumor activity (4, 5). It was reported to effectively affect division, differentiation, growth, and senescence of cells (6). LNT was reported to effectively prevent patients from cancers caused by chemical or viral carcinogens. In clinical, LNT enhances chemotherapy and improves the survival of patients in several cancer types, including gastric, colon, breast, and lung cancers. Current evidence also showed that it targeted small-cell lung cancer cells (7). Although it has a relatively weak effect on cancers, it is a promising chemotherapy synergist.

Recently, a compound from *Rabdosia rubescens*, oridonin, has been associated with cancer treatments. Studies showed that it suppressed breast cancer (8) and pancreatic cancer (9). It was also found to inhibit gene mutations induced by chemical carcinogens (10). It was also shown to block sodium pumps in cancer cells, decreased their nutrient intake (11), and regulated apoptosis and caspases pathways (12). A previous study showed that oridonin enhances the anticancer effects of lentinan in SMMC-7721 human hepatoma cells(13) and HepG2 human hepatoblastoma cells (14). However, its effect on lung cancer has not been studied.

Traditional medicine was used and studied previously (15). Some clinical findings in our hospital proposed that the use of oridonin enhances the treatment effects of LNT. In this study, human fetal lung fibroblast cell line MRC-5, lung cancer cell line A549, and Lewis lung carcinoma mice model were used to evaluate and validate the improvement of oridonin on the therapeutic effects of LNT on lung cancer.

Materials And Methods

Cell lines

Human fetal lung fibroblast cell line MRC-5, NSCLC cell line A549, and the Lewis lung carcinoma cells were obtained from the Conservation Genetics CAS Kunming Cell Bank (Kunming, Guangxi, China). RPMI1640 medium (Gibco BRL, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Gibco Co., Birmingham, MI, USA) was used to culture the cells. The cells were cultivated in the culture flasks under a humidified atmosphere containing 5% CO₂ at 37°C.

MTT assay

The MTT method was described elsewhere (16). A549 cells were seeded into a 96-well plate with a volume of 100 µL/well and a concentration of 4×10^4 cells/ml one night before the treatment. LNT and oridonin (Shanghai Yuanye Biological Technology Co., Ltd., Shanghai, China) with different concentrations were added into the plate and cultured for 72 h. The culture medium was discarded, followed by the addition of 100 µL fresh culture medium containing 0.5 mg/ml MTT (Nanjing Aoduofuni Biology Technology Co. Ltd., Nanjing, Jiangsu, China), then the plate was incubated for another 4 h. Afterward, the solution was discarded followed by the addition of 100 µL DMSO to crystallized and completely dissolved, finally, OD value under the wavelength of 540 nm was detected by a microplate reader (Imark, Bio-Rad, Hercules, CA, USA) and the ratio of suppression by drugs on cells was calculated thereby.

Real-time PCR assay

The PCR method was described elsewhere (17). Total RNA was extracted from A549 cells or lung tissues with Trizol agent (Invitrogen, Carlsbad, CA, USA). TaKaRa Reverse Transcription System was used to synthesize target cDNAs. The primers were purchased from Beijing Genomics Institute (Beijing, China). The primers included GAPDH (household), caspase-3, caspase-8, caspase-9, Bax, Bcl-2, Bcl-xL, p53, p21,

NF- κ B, and I κ B- α (Tiangen Biotech Co. Ltd., Beijing, China). TaKaRa System was adopted for the PCR assay. The reaction system included 0.8 μ L cDNA template, 5 μ L SYBRPremix ExTaq II (2X) (TaKaRa, Beijing, China), forward and reverse primers (10 μ mol $\cdot$ L⁻¹) each of 0.4 μ L, dH₂O 3.4 μ L. Reaction program: pre-heat for 30 s under 95°C; degeneration for 5 s under 95°C, annealing temperature for 30 s and amplification for 39 circles; finally, did the extension for 5 s under 65°C.

Western blotting

The Western blotting method was described elsewhere (18). Total protein was extracted from A549 cells or lung tissues. The concentration of protein was determined by the BCA assay. Then the protein was isolated with the 12% SDS-PAGE (Keygen Biotech, Nanjing, Jiangsu, China). Then the protein was transferred to a PVDF membrane. The membrane was incubated with primary and secondary antibodies subsequently. The bands were photographed after reaction with the ECL agent. The β -actin was used as an internal reference.

Experimental animal and Lewis lung carcinoma in vivo experiment

C57BL/6J male mice at 7-weeks old were purchased from the Animal Center of the Wuhan University, which were fed at 23 $\pm$ 1°C and 50 $\pm$ 5% humidity with a 12-h light/dark cycle. The animal trials were approved by the Animal Ethics Committee of Wuhan University (Wuhan, Hubei, China). The modeling method was described elsewhere (19). Anesthesia was induced by placing the animals into a clear plastic box containing 2–3% isoflurane in a 50–50% mixture of O₂ and air. After induction, the animals received a 50–50% mixture of O₂ and air administered via a face mask with spontaneous ventilation. The method of euthanasia used at the endpoint was CO₂ inhalation.

Lewis lung carcinoma was taken from liquid nitrogen and quickly put into 37°C water for melting, and then was centrifugated for 5 min at 14000 r/min; the supernatant was abandoned, the remaining was diluted to 1 $\times$ 10⁷/ml. 0.2 ml tumor cells suspension was subcutaneously injected into the right axillary of each C57BL/6J mouse. There were two sets of animal experiments, in each set, 10 mice without Lewis lung cancer were used as the normal group (without any cancer or treatment,). After the lewis lung cancer was induced in mice after 21 days, the mice were randomly divided into six groups, 18 mice for each group, control group (with cancer without treatment); LNT-L group (0.2 ml/day of 100 μ g/ml LNT); Oridonin group (0.2 ml/day of 20 μ g/ml oridonin); oridonin + LNT-L group (0.2 ml/day of 20 μ g/ml oridonin and 0.2 ml/day of 100 μ g/ml LNT); LNT-H group (0.2 ml/day of 300 μ g/ml LNT); oridonin + LNT-H group (0.2 ml/day of 20 μ g/ml oridonin and 0.2 ml/day of 300 μ g/ml LNT). The dose was converted from the clinical dose for human patients. For the first set of animal experiments, after 10 days, the busts of the mice were measured with a mini tape measure before and after the treatment, and the bust increased over 50% compared with the size before injection was defined as significant lung cancer metastasis. All the mice were euthanized followed by the collection of lung tissues. The method of euthanasia used at end point was CO₂ inhalation. The euthanasia chamber enabled animals to be readily visible which provided a minimum purity for CO₂ of 99.0%. This allowed unconsciousness with minimal

distress to the animals. The lung tissues were used to extract mRNA and protein for the PCR and the Western Blotting assay. For the other set of experiments, the mice were fed until the endpoint to obtain the survival rate. The criteria for the endpoint were: 1) tumor growth that impedes the ability to ingest food or water; 2) the tumors pain or distress that cannot be relieved with palliative measures; 3) Solid tumors estimated to exceed 20% of normal body weight. The death of animals was recorded every 5 days.

Statistical analysis

All experiments were repeated at less three times. One-way ANOVA with post-hoc Tukey's tests was used to analyze the difference, $P < 0.05$ was considered to indicate a statistically significant difference. SAS v9.1 statistical software package (SAS Institute Inc., Cary, NC, USA) was used for the statistics.

Results

The effect of oridonin and LNT on the growth of MRC-5 and A549 cells

Cell viabilities were determined using MTT assay. The viabilities of MRC-5 and A549 cells were not affected by 0-20 µg/ml oridonin. 0-300 µg/ml LNT did not affect the viability of MRC-5 cells, but 50-400 µg/ml LNT significantly inhibited the viability of A549 cells. The oridonin, LNT, or the combination of both did not affect MRC-5 cell viability. The oridonin had no effect on A549 cell viability but LNT significantly suppressed A549 cell viability, and the used of oridonin increased the suppression effect of LNT on A549 cells. (Fig. 1)

20 µg/ml oridonin was used in the subsequent study because it was the highest concentration that had no significant effect on cell viability. In addition, 300 µg/ml LNT was selected for the subsequent study as a high concentration group (LNT-H) because it had the strongest potency in the viability of cancer cells while had no effect on the viability of normal cells. As a comparison, we also used a low concentration group (LNT-L) which was 100 µg/ml LNT.

The effect of oridonin and LNT on mRNA and protein expression of apoptosis-associated proteins in A549

LNT at both concentrations tested increased the mRNA and protein expressions of caspase-3, caspase-8, and caspase-9 in A549 cells with a larger increase in LNT-H. Oridonin further increased the mRNA and protein expression of caspase-3, caspase-8, and caspase-9 in LNT treated A549 cells. (Fig. 2 AB) Besides, LNT at both concentrations tested increased the mRNA and protein expression of Bax in A549 cells with more increase in LNT-H (LNT high concentration group). Oridonin further increased the mRNA and protein expression of Bax in LNT treated A549 cells. On the other hand, LNT at both concentrations tested decreased the mRNA and protein expression of Bcl-2 and Bcl-xL in A549 cells with more decrease in LNT-H. Oridonin further decreased the mRNA and protein expression of Bcl-2 and Bcl-xL in LNT treated A549 cells. (Fig. 2 CD) These results revealed that apoptosis might mediate the effects.

The effect of oridonin and LNT on mRNA and protein expression of the p53/p21 pathway proteins in A549

We suggested the viability effect was associated with p53/p21 signaling, thus we also tested them. LNT at both concentrations tested increased the mRNA and protein expression of p53 and p21 in A549 cells. Oridonin further increased the mRNA and protein expression of p53 and p21 in LNT treated A549 cells (Fig. 2 EF). These results suggested that the p53/p21 pathway was involved.

The effect of oridonin and LNT on mRNA and protein expression of NF- κ B and I κ B- α in A549

Additionally, LNT at both concentrations tested increased the mRNA and protein expression of NF- κ B in A549 cells with a more dramatic increase in LNT-H. Oridonin further increased the mRNA and protein expression of NF- κ B in LNT treated A549 cells. On the other hand, LNT at both concentrations tested decreased the mRNA and protein expression of I κ B- α in A549 cells with more decrease in LNT-H. Oridonin further decreased the mRNA and protein expression of I κ B- α in LNT treated A549 cells (Fig. 2 GH). These results suggested that the NF- κ B and I κ B- α signaling was involved.

The effects of oridonin and LNT on lung tumor metastasis in mice

We established lung cancer metastasis mice model and treatment them with LNT-L, oridonin, oridonin + LNT-L, LNT-H, and oridonin + LNT-H. Mice without cancer or any treatment and mice with lung cancer but any treatment were used for comparison. As showed in Table 1, after 10 days, the oridonin alone had no effect on short time lung cancer metastasis. LNT treatment decreased the metastasis with a higher inhibitory rate in LNT-H group than in LNT-L group. Oridonin improved the suppression of LNT against lung cancer metastasis in both groups. These results suggested that oridonin help LNT decrease lung tumor metastasis in mice.

The effects of oridonin and LNT on the overall survival of mouse with lung tumor cells

We also conducted a survival assay to test the effect of oridonin and LNT. Results showed that oridonin alone had almost no effect on the survival of the animals. Both LNT-L and LNT-H improved the survival and LNT-H had a better outcome than LNT-L. Oridonin increased the improvement effect of LNT on the survival rate (Fig. 3). Results indicated that the combined use of both oridonin and LNT optimized survival.

The effects of oridonin and LNT on the mRNA and protein expression of caspase-3, caspase-8, and caspase-9 in mice lung tissues

After the lung cancer cell injection, the mRNA and protein expression of caspase-3, caspase-8, and caspase-9 significantly decreased in lung tissue samples. The LNT at both concentrations tested increased the mRNA and protein expression of caspase-3, caspase-8, and caspase-9 in lung tissue samples with a more significant increase in LNT-H. Oridonin further increased the mRNA and protein

expression of caspase-3, caspase-8, and caspase-9 in the lung of LNT treated animals (Fig. 4 AB). These results were similar to those in A549, which confirmed the effect of oridonin and LNT on lung cancer.

The effects of oridonin and LNT on the mRNA and protein expression of Bax, Bcl-2, and Bcl-xL in lung tissues

In addition, after cancer induction, the mRNA and protein expressions of Bax significantly increased while Bcl-2 and Bcl-xL significantly decreased in lung tissue samples. LNT at both concentrations tested increased the mRNA and protein expressions of Bax while LNT-H increased more than LNT-L. Oridonin further increased the mRNA and protein expression of the expression of Bax in the lung. On the other hand, LNT at both concentrations tested decreased the mRNA and protein expressions of Bcl-2 and Bcl-xL with more decrease in the LNT-H group. Oridonin further decreased the mRNA and protein expressions of Bcl-2 and Bcl-xL in LNT treated groups (Fig. 4 CD). These results were similar to those in A549, which confirmed the effect of oridonin and LNT on lung cancer.

The effects of oridonin and LNT on the mRNA and protein expression of p53 and p21 in mice lung tissues

Besides, the cancer induction significantly decreased the mRNA and protein expressions of p53 and p21 in lung tissue samples. The LNT at both concentrations tested increased the mRNA and protein expressions of p53 and p21 in lung tissue samples with a more increased rate in LNT-H. Oridonin further increased the mRNA and protein expressions of p53 and p21 in the lung (Fig. 4 EF). These results were similar to those in A549, which confirmed the effect of oridonin and LNT on lung cancer.

The effects of oridonin and LNT on the mRNA and protein expression of NF-κB and IκB-α in lung tissues

Additionally, the mRNA and protein expression of NF-κB significantly increased while IκB-α significantly decreased in lung tissue samples. LNT at both concentrations tested decreased the mRNA and protein expression of NF-κB with more decrease in LNT-H. Oridonin further decreased the mRNA and protein expression of NF-κB in the lung of LNT treated animals. On the other hand, LNT at both concentrations tested increased the mRNA and protein expression of IκB-α with a larger increase in LNT-H. Oridonin further increased the mRNA and protein expression of IκB-α in LNT treated lung tissues (Fig. 4 GH). These results were similar to those in A549, which confirmed the effect of oridonin and LNT on lung cancer.

Discussion

Oridonin has been reported to increase anticancer effects of lentinan in HepG2 human hepatoblastoma cells (14), and also to enhance in vitro anticancer effects of lentinan in SMMC-7721 human hepatoma cells through apoptotic genes (13). However, no study has been done to investigate the improvement of anti-lung cancer effects of lentinan. Lung cancer cells are different from liver cancer, but we hypothesized that it works in lung cancer cells as in liver cancer cells and we validated the hypothesis. The significance

of this work is to help explain the treatment effect of oridonin and LNT in clinical lung cancer patients in our hospital.

Our results showed that lung cancer cell line A549 was much more sensitive than normal human fetal lung fibroblast cell line MRC-5 toward LNT. This suggested that LNT can be a potential cancer medicine with low side effects. LNT was proved to have a suppression effect on some cancer cells (20) as well as an immunomodulatory effect on cancer patients (21). A retrospective study demonstrated that LNT improved the quality of cancer patients' life and remarkably promoted the efficacy of chemotherapy and radiation therapy during the cancer treatment (22). An *in vivo* study also revealed that LNT showed therapeutic potential for colitis-associated cancer. While for the oridonin, previous studies showed that 36 µg/ml (0.1 mmol/l) oridonin inhibited breast cancer growth and metastasis through blocking the Notch signaling (8).

In this study, we used the MTT assay to test the viability of lung cancer cells. The MTT assay was widely used in cancer pharmacological studies (23). Surprisingly, our results show that 0-300 µg/ml oridonin had little effect on both MRC-5 and A549, indicating that lung cancer might have lower sensitivity than breast cancer toward oridonin. In addition, oridonin was also shown to inhibit human pancreatic cancer migration (9). The survival rate is a critical indicator of cancer therapy in different cancer types (24). Thus, we mimic the clinical treatment and survival in the mice as a previous study (25). The *in vivo* evidence of this study showed that oridonin alone failed to suppress the migration of lung cancer cells and did not affect the survival of mice with lung cancer. However, we found that oridonin promoted the effects of LNT both in the viability of A549 and the migration and the survival of mice. These results indicated that oridonin might trigger pathways that facilitate the actions of LNT. However, in survival experiment, we applied inhalation anesthesia. The anesthesia was reported to potentially affect cancers (24, 26), this might potentially affect the results.

To explore the underlying mechanisms, we tested several potential targets of LNT in both A549 and lung tissue samples. Studies show that proliferation and apoptosis affected cancer viability (27). We suggested that apoptosis might be involved in the effects of oridonin and LNT. Firstly, we looked at the caspase signaling pathway. Caspases, cysteine-aspartic proteases, cysteine-dependent aspartate-directed proteases, are a family of protease enzymes playing essential roles in programmed cell death and inflammation (28). A previous study showed that the co-treatment with paclitaxel and lentinan enhanced cell apoptosis rate by inducing caspase-3 activation (29). Here we discovered that LNT suppressed A549 cell viability by improving the expression of apoptosis executioner caspase-3 and the oridonin could further promote this improvement. Moreover, we also found potential negative feedback of the Caspases signal, since the expression of apoptosis initiator in Caspases, the Caspases-8 and 9, increased with Caspases-3.

In fact, lentinan was reported to exert synergistic apoptotic effects with paclitaxel in A549 cells (29). The apoptosis-inducing effect of lentinan was also reported in a study in human bladder cancer T24 cells (30). In hepatoma cells, oridonin was proved to promote the effects of lentinan through regulating

apoptotic genes. To confirm that these effects are mediated by apoptosis (29), we further tested an apoptosis regulatory pathway, the Bax pathway. BCL2 family members act as anti- or pro-apoptotic regulators for cancer cells. Bcl-xL acts as an anti-apoptotic protein by preventing the release of mitochondrial contents such as cytochrome c, which leads to caspase activation and ultimately, programmed cell death (31). In the present study, the expressions of Bax, Bcl-2, and Bcl-xL were affected by LNT and oridonin, indicating that the effect of them was mediated by apoptosis.

The expression of the BCL2 family genes is regulated by the tumor suppressor p53 and has been shown to be involved in p53-mediated apoptosis (32, 33). We proposed that p53 and p21 might be the upstream targets of LNT and oridonin. Hence, the expression of p53 and p21 in A549 and lung tissues were determined, and it came out that the expressions of p53 and p21 were improved by LNT and oridonin. NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells, is a protein complex that controls transcription of DNA, cytokine production and cell survival while I κ B α , nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha, is cellular proteins that function to inhibit the NF- κ B transcription factors (34). The present study proved that NF- κ B and I κ B α are involved in the effects of LNT and oridonin on cancer, and their expression changed and played a role in the decrease of the cell viability. In the lung tissues, the result was similar to that of cancer cells, which confirmed the conclusion under *in vivo* condition.

Our study demonstrated that oridonin enhanced the antitumor effects of LNT. We also found several potential regulatory targets of the effect of oridonin and LNT. However, there are other mechanisms that might be involved, such as cancer stem cells (35), ion channels in cancers(36, 37), etc. Besides, studies have showed that traditional medicines(38) and anesthetics(24, 25, 39) might affect cancer treatments. Hence, more work is required in the future. Recently, many compounds derived from traditional medicines were explored for potential clinical use (40-42). In Cancer treatment, although traditional medicines were not able to cure cancer alone, they are applied in the combination of medical therapy for reducing the adverse effects caused by chemotherapy or radiotherapy, thus improving therapeutic outcome and quality of life for patients (43). Here we tried to develop a compound from a traditional herb medicine to be a potential chemotherapy synergist. This study is conducive to the development of oridonin and LNT as a novel cancer drug regimen and contributes to the application of traditional medicine in clinical treatments.

Abbreviations

LNT: lentinan,

NSCLC: non-small cell lung cancer,

Bax: Bcl-2 associated X protein,

Bcl-2: B-cell lymphoma-2,

NF- κ B: nuclear factor kappaB,

I κ B- α : inhibitor kappaB- α .

Declarations

Conflict of Interest

There is no conflict of interest.

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Table

Table 1. The inhibition of oridonin and LNT against lung tumor metastasis in mice.

Group	Total	Lung tumor metastasis number	Inhibitory rate (%)
Normal	10	0	0 Δ
Control	18	18 [*]	100 Δ
Oridonin	18	18 [*]	100 Δ
LNT-L	18	15 [#]	16.7 [@]
Oridonin + LNT-L	18	13 ^{&}	27.8 ^{&}
LNT-H	18	9 [@]	50.0 [#]
Oridonin + LNT-H	18	7 Δ	61.1 [*]

Figures

Figure 1

Effect of oridonin and LNT on the viability of MRC-5 and A549. Cell viabilities were determined using MTT assay. Effect of different concentrations of LNT (A) or oridonin (B) on the viability of MRC-5 and A549 cells. Effect of 20 $\mu\text{g/mL}$ oridonin, 300 $\mu\text{g/mL}$ LNT, or the combination of them on cell viabilities of MRC-5 (C) and A549 (D). * represent the significant difference compared to vehicle, Δ represent the significant difference compared to LNT alone group.

Figure 2

The effect of oridonin and LNT on mRNA and protein expression of viability-associated proteins in A549. The mRNA and protein expression were determined by QPCR and Western blotting. (AB) The mRNA and protein expression of caspase-3, caspase-8, and caspase-9 in A549 cells. (CD) The mRNA and protein expression of Bax, Bcl-2, and Bcl-xL in A549 cells. (EF) The mRNA and protein expression of p53 and p21 in A549 cells. (GH) The mRNA and protein expression of NF- κ B and I κ B- α in A549 cells. “*”, “&”, “#”, “@”,

and “△” marked values with significant difference compared to each other ($P<0.05$) according to post-hoc Tukey’s tests. (B) representative images.

Figure 3

The effects of oridonin and LNT on the survival of mice with lung tumor cells. Animal survival assay was used to test the effect of the drug. Oridonin alone had little effect on the survival of the animals. Both LNT-L and LNT-H improved the survival and LNT-L had a better outcome than LNT-L. The use of oridonin promoted the improvement of LNT on the survival rate.

Figure 4

The effect of oridonin and LNT on mRNA and protein expression of viability-associated proteins in lung tissue samples. The mRNA and protein expression were determined by QPCR and Western blotting. (AB) The mRNA and protein expression of caspase-3, caspase-8, and caspase-9 in lung tissue. (CD) The mRNA and protein expression of Bax, Bcl-2, and Bcl-xL in lung tissue. (EF) The mRNA and protein expression of p53 and p21 in lung tissue. (GH) The mRNA and protein expression of NF- κ B and I κ B- α in lung tissue. “*”,

"&", "#", "@", and "△" marked values with significant difference compared to each other ($P < 0.05$) according to post-hoc Tukey's tests. (B) representative images.