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 exhibit a potent ability to improve the antitumor effects of lentinan (LNT). In the present study, the effects of oridonin, LNT, and the combination of these treatments were assessed on the normal human fetal lung fibroblast cell line MRC-5, as well as the non-small cell lung cancer cell line A549. Next, their effects on metastasis and survival *in vivo* were assessed in a mouse model of lung cancer. The effects of the treatments on the mRNA and protein expression levels of several regulatory factors in A549 cells and lung tissues were determined using reverse transcription-quantitative PCR and western blotting. The results showed that the viability 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 reduced the viability of A549 cells. Thus, 20 μg/ml oridonin and 100 or 300 μg/ml LNT were used in the subsequent experiments. Treatment with oridonin and LNT, alone or combined, had no effect on MRC-5 cell viability. Oridonin treatment had no effect on A549 cell viability; however, LNT suppressed A549 cell viability, and oridonin promoted the suppressive effects of LNT on A549 cells. *In vivo* analysis showed that oridonin alone had no effect on metastasis and survival, but LNT decreased metastasis and survival in mice. Oridonin augmented the effects of LNT against metastasis and further improved the survival rates of mice. In both A549 cells and lung tissues, LNT increased the mRNA and protein expression levels of caspase-3, caspase-8, caspase-9, Bax, p53, p21 and inhibitor of nuclear factor-κB (NF-κB)-α, and reduced the mRNA and protein expression levels of Bcl-2 and NF-κB. Oridonin augmented all the effects of LNT on expression of these proteins in the cells. Together, the results showed that oridonin enhanced the antitumor effects of LNT, and may thus serve as an adjuvant alongside LNT as a novel anticancer regimen for treatment of lung cancer.

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Abbreviations: LNT, lentinan; NSCLC, non-small cell lung cancer; Bax, Bcl-2 associated X protein; Bcl-2, B-cell lymphoma-2; NF-κB, nuclear factor-κB; IκB-α, inhibitor of NF-κB-α

Key words: oridonin, lentinan, lung cancer, growth inhibition

Introduction

Lung cancer (both small cell and non-small cell lung cancer) is the second most common type of cancer diagnosed in both men and women (1). In 2019, ~13% of all new cases of cancer were lung cancer, including >228,000 new cases of lung cancer in the United States (1). Lung cancer led to >142,000 deaths each year, making it, by far, the leading cause of cancer-associated death amongst both men and women; accounting for more deaths than colon, breast and prostate cancer-associated deaths combined (1). Non-small cell lung cancer (NSCLC) accounts for ~85% of all lung cancer cases (1). In clinical treatments, NSCLCs are relatively insensitive to chemotherapy, compared with small cell carcinoma. Therefore, improving the efficiency of chemotherapy against NSCLCs is of utmost importance for clinical lung cancer treatments.

In previous studies, the clinical value of traditional Chinese medicines have been assessed, due to the lower incidence of side effects (2,3). Increasing attention has been given to a fungal polysaccharide, lentinan (LNT), due to its strong antitumor activity (4,5). It was reported to effectively inhibit proliferation, differentiation, growth and senescence of cells (6). LNT has also been reported to effectively prevent development of cancers caused by chemical or viral carcinogens (4,5). Clinically, LNT enhanced the effects chemotherapy and improved the survival of patients with several types of cancer, including gastric, colon, breast and lung cancer (7). Current evidence has also shown LNT can target small-cell lung cancer cells (7). Although it has a relatively weak effect on cancer, the results of the present study have demonstrated the effects of LNT, indicating its potential to act as an adjuvant for use alongside chemotherapy.

Previously, a compound obtained from *Rabdosia rubescens*, called oridonin, has been shown to possess potential as an anticancer treatment. Studies have shown that oridonin can

suppress the growth of breast (8) and pancreatic cancer (9). It was also found to inhibit gene mutations induced by chemical carcinogens (10) and may exert its effects by blocking sodium pumps in cancer cells, decreasing nutrient uptake (11), as well as through regulation of the apoptotic pathways via modulation of caspase activity/expression (12). A previous study showed that oridonin enhances the anticancer effects of LNT in SMMC-7721 human hepatoma cells (13) and HepG2 human hepatoblastoma cells (14). However, its effect on lung cancer have not been studied previously, to the best of our knowledge.

Several traditional Chinese medicines have been studied extensively (6,7,15). Clinical findings from our hospital showed that the use of oridonin enhanced the beneficial effects of LNT. In the present study, the human fetal lung fibroblast cell line MRC-5, lung cancer cell line A549, and Lewis lung carcinoma mouse model were used to evaluate and validate the adjuvant effects of oridonin on the therapeutic effects of LNT in lung cancer.

Materials and methods

Cell lines and cell culture. The Human fetal lung fibroblast cell line MRC-5, the NSCLC cell line A549, and the Lewis lung carcinoma cells were obtained from the Conservation Genetics CAS Kunming Cell Bank. RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) was used to culture the cells. The cells were cultivated in culture flasks in a humidified incubator with 5% CO_2 at 37˚C. In the experiments, the cells were exposed to 0-20 μg/ml oridonin, 0-300 μg/ml LNT or a combination of both for 24 h.

MTT assay. The MTT assays were performed as described previously (16). A549 cells ($4x10^4$ cells/well) were seeded into 96-well plates in 100 μl supplemented media per a well the night before treatments. Different concentration of LNT (Shanghai Yuanye Biological Technology Co., Ltd.) and oridonin (Shanghai Yuanye Biological Technology Co., Ltd.) were added to the plates and cultured for 72 h. Subsequently, the culture medium was discarded, and 100 μl fresh culture media containing 0.5 mg/ml MTT was added (Nanjing Aoduofuni Biology Technology, Co. Ltd.), and the plates were further incubated for 4 h. The solutions were discarded and 100 μl DMSO was added to dissolve the crystals, Finally, the optical density of each well was measured at a wavelength of 540 nm using a microplate reader (Imar; Bio-Rad Laboratories, Inc.) and the ratio of suppression of viability induced by the different treatments was calculated. Based on preliminary results, 300 μg/ml LNT was used for the subsequent experiments as the high concentration group (LNT-H), as it was the concentration that had the most potent effect on the viability of cancer cells, whilst having little effect on the viability of normal lung cells. As a comparison, a low concentration group (LNT-L) was also included, in which cells were treated with 100 μg/ml LNT.

Reverse transcription-quantitative (RT-q)PCR. RT-qPCR was performed as described previously (17). Total RNA was extracted from A549 cells or lung tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). A Takara Reverse Transcription system was used to synthesize target cDNAs, and RT was performed according to the manufacturer's protocol (Takara Bio, Inc.). Primers were purchased from Beijing Genomics Institute. The following primers were used for the RT-qPCR: GAPDH forward, 5'-GTCTCC TCTGACTTCAACAGCG and reverse, 5'-ACCACCCTG TTGCTGTAGCCAA; caspase-3 forward, 5'-AGAGGGGAT CGTTGTAGAAG and reverse, 5'-GTTGCCACCTTTCGG TTAAC; caspase-8 forward, 5'-GCATTAGGGACAGGA ATGGA and reverse, 5'-CCCCTGACAAGCCTGAATAA; caspase-9 forward, 5'-AGCCAGATGCTGTCCCATAC and reverse, 5'-CAGGAACCGCTCTTCTTGTC; Bax forward, 5'-ACCAAGAAGCTGAGCGAGTGTC and reverse, 5'-TGT CCAGCCCATGATGGTTC; Bcl-2 forward, 5'-CTACGA GTGGGATGCGGGAGATG and reverse, 5'-GGTTCAGGT ACTCAGTCATCCACAG; Bcl-xL forward, 5'-GGATGG CCACTTACCTGA and reverse, 5'-CGGTTGAAGCGT TCCTG; p53 forward, 5'-TTGCTTTATCTGTTCACTTGT G and reverse, 5'-TCCTTCCACTCGGATAAG; p21 forward, 5'-GTGAGCGATGGAACTTCGACT and reverse, 5'-CGA GGCACAAGGGTACAAGAC; NF-κB forward, 5' TGTAAA ACGACGGCCAGT and reverse, 5'CAGGAAACAGCTATG ACC; and inhibitor of NF-κB-α (IκB-α) forward, 5'-GGC TGAAAGAACATGGACTTG and reverse, 5'-GTACACCAT TTACAGGAGGG-3' (Tiangen Biotech Co. Ltd.) A Takara one step RT-PCR kit was adopted for PCR (Takara Bio, Inc.). The reaction system included 0.8 μl cDNA template, 5 μl SYBR Premix ExTaq II (2X) (Takara Bio, Inc.), 0.4 μl each forward and reverse primers (10 μmol/l), and 3.4 μl dH_2O. The thermocycling conditions included pre-heating for 30 sec at 95˚C; followed by 39 cycles of degeneration at 95˚C for 5 sec, annealing at 50˚C for 30 sec and extension at 68˚C for 45 sec with a final extension step of 5 min at 65˚C.

Western blotting. Western blotting was performed as described previously (18). Total protein was extracted from A549 cells or lung tissues using RIPA Buffer (Invitrogen; Thermo Fisher Scientific, Inc.). The concentration of proteins was determined using a BCA assay. A total of 30 μg of each protein was loaded per lane on a 12% SDS-gel (Nanjing KeyGen Biotech Co., Ltd.), resolved using SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked in 5% milk in TBS with 0.1% Tween-20 at room temperature for 1 h. The membrane was subsequently incubated with the primary antibodies at 4˚C overnight, followed by incubation with the secondary antibodies: Goat anti-rabbit IgG H&L (HRP; cat. no. ab6721; Abcam; 1:5,000) or goat anti-mouse IgG H&L (HRP; cat. no. ab6789; Abcam; 1:5,000) at room temperature for 1 h. Signals were visualized using ECL; β-actin was used as the loading control. Antibodies included: Anti-caspase-3 antibody (E87; cat. no. ab32351; Abcam; 1:2,000); anti-caspase-8 antibody (E7; cat. no. ab32397; Abcam; 1:2,000); anti-caspase-9 antibody (E23; cat. no. ab32539; Abcam; 1:5,000); anti-β-actin antibody (cat. no. ab8227; Abcam; 1:3,000); anti-Bax antibody (E63; cat. no. ab32503; Abcam; 1:2,000); Anti-Bcl-2 antibody (EPR17509; cat. no. ab182858; Abcam; 1:2,000); anti-Bcl-XL antibody (E18; cat. no. ab32370; Abcam; 1:3,000); anti-p53 antibody (E26; cat. no. ab32389; Abcam; 1:6,000); anti-p21 antibody (EPR362; cat. no. ab109520; Abcam; 1:1,000); anti-NF-kB p65 (phospho S276) antibody (EPR17622;

cat. no. ab183559; Abcam, 1:2,000); and anti-IkB alpha antibody (EP697; cat. no. ab76429; Abcam; 1:2,000).

Experimental animal model and Lewis lung carcinoma in vivo experiments. A total of 120 C57BL/6J male mice (7-weeks old) were purchased from the Animal Center of the Wuhan University, were fed standard mouse chow and housed at 23±1˚C with a 50±5% humidity and a 12 h light/dark cycle. The animal experiments were approved by the Animal Ethics Committee of Wuhan University. The modeling method was performed as described previously (19). Anesthesia was induced by placing the animals into a clear plastic box containing 2-3% isoflurane in a 50-50% mixture of O_2 and air. After induction, the animals received a 50-50% mixture of O_2 and air administered via a face mask with spontaneous ventilation. The method of euthanasia used at the endpoint was CO_2 inhalation by using a gradual 10 to 30% vol/min displacement rate.

Lewis lung carcinoma cells; mouse lung cancer cells that are widely used as a model for metastasis and are useful for studying the mechanisms of cancer chemotherapeutic agents, were used in this study. A total of 0.2 ml tumor cell suspension ($5x10^3$ cells) 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 Lewis lung cancer was induced in mice after 21 days, the mice were randomly divided into six groups (n=18 per a group) as follows: Control group (with cancer, without treatment); LNT-L group (0.2 ml/day 100 μg/ml LNT); oridonin group (0.2 ml/day 20 μg/ml oridonin); oridonin + LNT-L group (0.2 ml/day 20 μg/ml oridonin and 0.2 ml/day 100 μg/ml LNT); LNT-H group (0.2 ml/day 300 μg/ml LNT); oridonin + LNT-H group (0.2 ml/day 20 μg/ml oridonin and 0.2 ml/day 300 μg/ml LNT). 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 treatments, and an increase in bust size >50% compared with the respective 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 CO_2 inhalation as described above. The euthanasia chamber enabled animals to be readily visible and provided a minimum purity for CO_2 of at least 99.0%. This allowed induction of unconsciousness with minimal distress to the animals. The lung tissues were used for mRNA and protein extraction for use in the RT-qPCR and the western blotting experiments. For the second set of experiments, the mice were fed until the endpoint to obtain the survival rate. The criteria for the endpoint were: i) Tumor growth that impedes the ability to ingest food or water; ii) tumor pain or distress that could not be relieved with palliative measures; or iii) 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 least three times. A one-way ANOVA followed by a post-hoc Tukey's test was used to compare differences between multiple groups. The survival was analyzed using the Kaplan-Meier (KM) method. SAS version 9.1 statistical software package (SAS Institute Inc.) was used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of oridonin and LNT on the growth of MRC-5 and A549 cells. Cell viability was determined using MTT assays. The viability of MRC-5 and A549 cells were not affected by 0-20 μg/ml oridonin. Additionally, 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. Oridonin (0-20 μg/ml); LNT (LNT-L, 100 μg/ml; LNT-H, 300 μg/ml); or the combination of both (LNT-L, 20 μg/ml oridonin + 100 μg/ml LNT; LNT-H, 20 μg/ml oridonin + 300 μg/ml LNT) did not affect MRC-5 cell viability. When compared with the 0 μg/ml oridonin group, oridonin had no effect on A549 cell viability; however, LNT significantly suppressed A549 cell viability, and when combined with oridonin, the reduction in viability was further increased (Fig. 1).

In subsequent studies, 20 μg/ml oridonin was used, as it was the highest concentration that had no significant effect on cell viability. Based on preliminary results, 300 μg/ml LNT was used for the subsequent experiments as the high concentration group (LNT-H), as it was the concentration that had the most potent effect on the viability of cancer cells, whilst having little effect on the viability of normal lung cells. As a comparison, a low concentration group (LNT-L) was also included, in which cells were treated with 100 μg/ml LNT.

Effect of oridonin and LNT on the mRNA and protein expression levels of apoptosis associated genes and proteins in A549 cells. At both concentrations assessed, LNT increased the mRNA and protein expression levels of caspase-3, caspase-8 and caspase-9 in A549 cells, with a significantly larger increase in the LNT-H group compared with the control group. Compared with the LNT groups, LNT with oridonin further increased the mRNA and protein expression levels of caspase-3, caspase-8 and caspase-9 when combined with LNT in A549 cells (Fig. 2A and B). Additionally, compared with the groups that received no treatment, LNT with LNT at both concentrations assessed increased the mRNA and protein expression of Bax in A549 cells, and the increase was greater in the LNT-H group. Compared with the LNT groups, LNT with oridonin further increased the mRNA and protein expression levels of Bax in LNT treated A549 cells. Conversely, compared with the no treatment group, LNT at both concentrations assessed decreased the mRNA and protein expression levels of Bcl-2 and Bcl-xL in A549 cells, and the decrease was greater in the LNT-H group. Compared with the LNT groups, oridonin combined with LNT further decreased the mRNA and protein expression levels of Bcl-2 and Bcl-xL in the A549 cells (Fig. 2C and D). These results suggested that LNT may increase apoptosis of cancer cells, and that oridonin may augment these effects.

Effect of oridonin and LNT on the mRNA and protein expression levels of the p53/p21 pathway proteins in A549 cells. It was hypothesized that the effect of LNT on the viability of the cells was associated with p53/p21 signaling, thus, their expression was assessed at the mRNA and protein

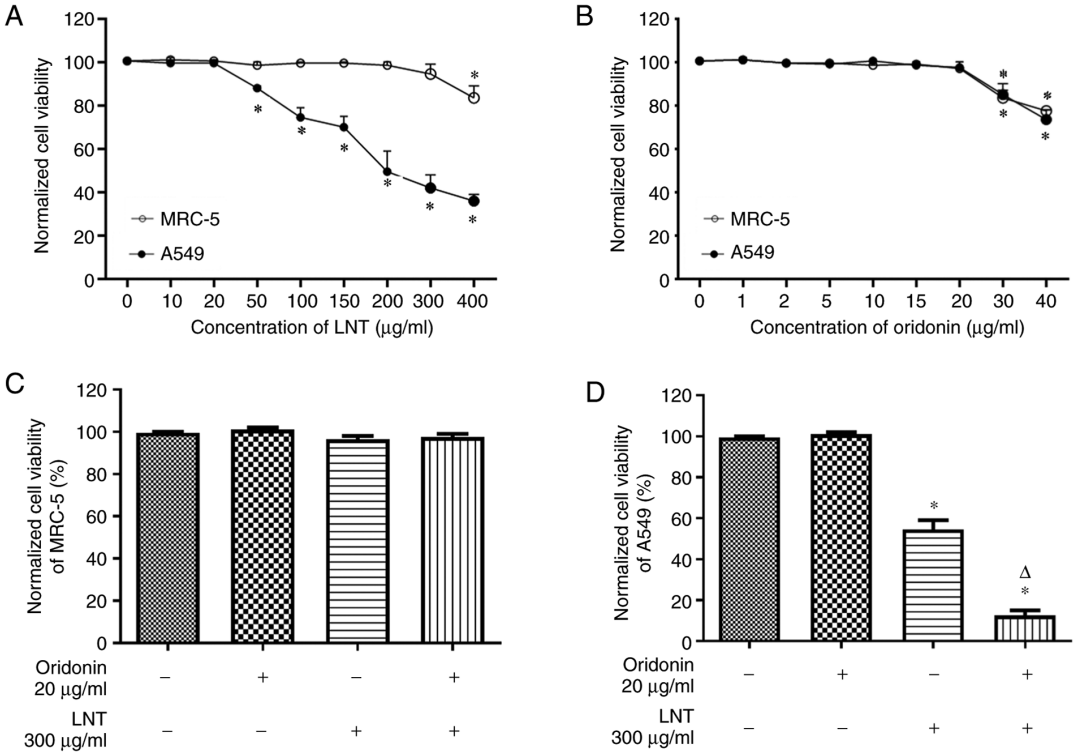

Figure 1. Effect of oridonin and LNT on the viability of MRC-5 and A549 cells. Cell viability was determined using an MTT assay. Effects of different concentrations of (A) LNT or (B) oridonin on the viability of MRC-5 and A549 cells. Effect of 20 μg/ml oridonin, 300 μg/ml LNT, or a combination of these on cell viability of (C) MRC-5 and (D) A549. *P<0.05 vs. vehicle; $^{\Delta}$P<0.05 vs. LNT alone. LNT, lentinan.

level. Compared with the no treatment group, LNT at both concentrations assessed increased the mRNA and protein expression levels of p53 and p21 in A549 cells. Compared with the LNT groups, LNT with oridonin further increased the mRNA and protein expression levels of p53 and p21 when combined with LNT in A549 cells (Fig. 2E and F). These results suggested that LNT exerted its effects via modulation of the p53/p21 pathway.

Effect of oridonin and LNT on the mRNA and protein expression levels of NF-κB and IκB-α in A549 cells. Compared with the no treatment group, LNT at both concentrations tested, increased the mRNA and protein expression levels of NF-κB in A549 cells, and the increase was greater in the LNT-H group. Compared with the LNT groups, LNT with oridonin further increased the mRNA and protein expression levels of NF-κB in LNT treated A549 cells. Conversely, compared with the no treatment group, both concentrations of LNT assessed decreased the mRNA and protein expression levels of IκB-α in A549 cells, and the decrease was greater in the LNT-H group. Compared with the LNT groups, LNT with oridonin further decreased the mRNA and protein expression levels of IκB-α when combined with LNT (Fig. 2G and H). These results suggested the involvement of NF-κB and IκB-α signaling in the effects of oridonin and LNT.

Effects of oridonin and LNT on lung tumor metastasis in mice. A mouse model of lung cancer metastasis was established, and the mice were treated with either a low or high dose of LNT (LNT-L and LNT-H, respectively), oridonin, oridonin + LNT-L, LNT-H, or oridonin + LNT-H. Mice without cancer or any treatments and mice with lung cancer that received no treatments were used as controls. As demonstrated in Table I, after 10 days, oridonin alone had no effect on short term lung cancer metastasis when compared with the control. LNT treatment decreased the metastasis with a higher inhibitory rate in LNT-H group compared with the LNT-L group. Oridonin augmented the suppression of LNT against lung cancer metastasis at both doses. These results suggested that oridonin may serve as an adjuvant, to augment the effects of LNT on lung tumor metastasis in mice.

Effects of oridonin and LNT on the overall survival of mice with lung cancer. A survival assay was used to assess the effects of oridonin and LNT. The results revealed that when compared with the control, oridonin alone had almost no effect on the survival of the animals. Both LNT-L and LNT-H improved the survival, and LNT-H exhibited improved effects on outcomes compared with LNT-L. Compare with LNT groups, oridonin combined with LNT notably improved survival at both doses of LNT assessed, and the survival was greatest in the mice treated with a high dose of LNT combined with oridonin (Fig. 3). These results suggested that the combined use of both oridonin and LNT most notably improved survival.

Effects of oridonin and LNT on the mRNA and protein expression levels of caspase-3, caspase-8 and caspase-9 in the mice lung cancer tissues. The mRNA and protein expression levels of caspase-3, caspase-8, and caspase-9 were significantly decreased in the lung tissue samples of all treatment

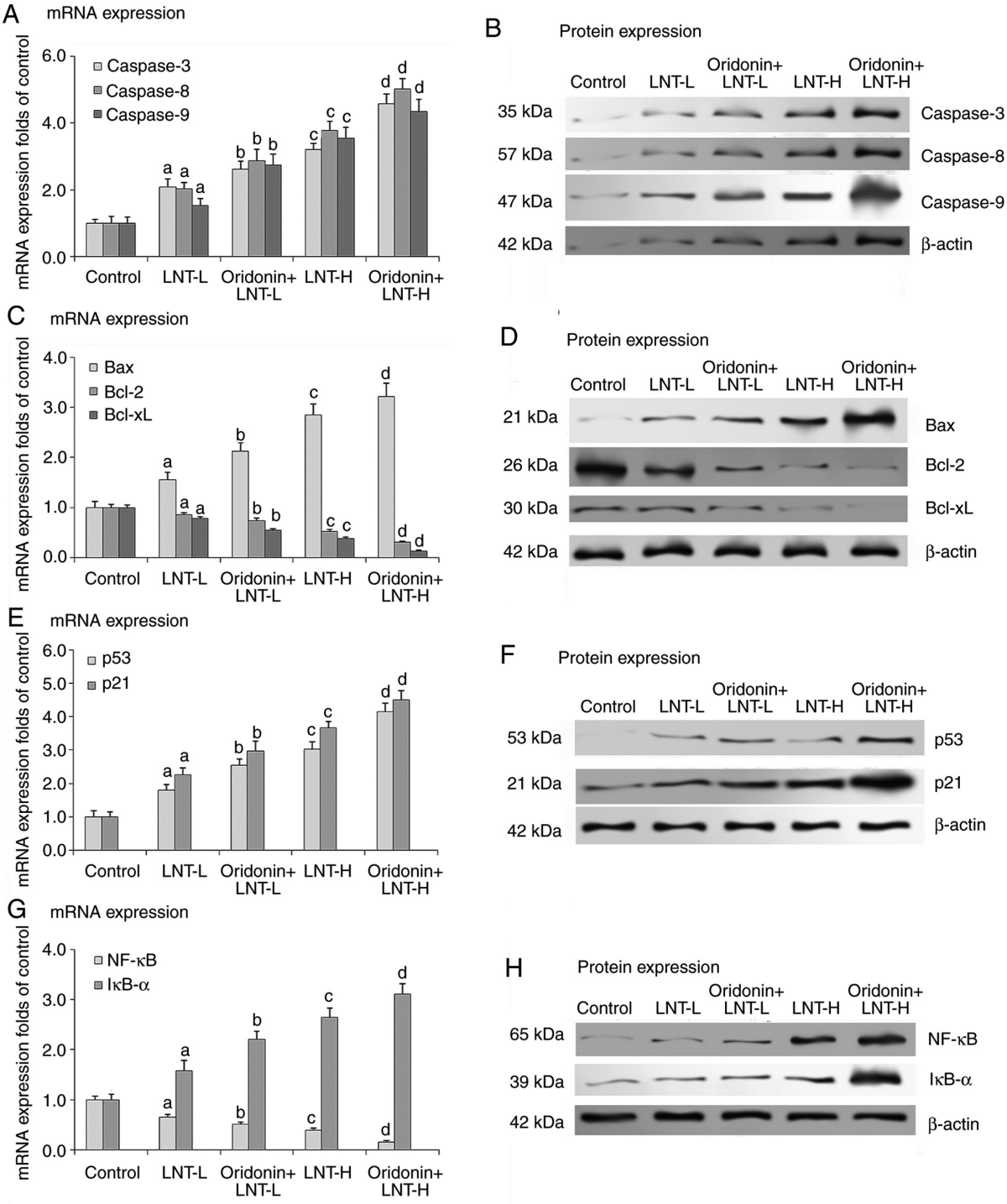

Figure 2. Effect of oridonin and LNT on the mRNA and protein expression levels of viability-associated proteins in A549 cells. The mRNA and protein expression levels were determined by reverse transcription-quantitative PCR and western blotting, respectively. mRNA and protein expression levels of (A and B) caspase-3, caspase-8 and caspase-9, (C and D) Bax, Bcl-2 and Bcl-xL, (E and F) p53 and p21, and (G and H) NF-κB and IκB-α in A549 cells. [a]P<0.05 vs. control; [b]P<0.05 vs. LNT-L; [c]P<0.05 vs. oridonin + LNT-L; [d]P<0.05 vs. LNT-H. LNT, lentinan; NF-κB, nuclear factor-κB; IκB-α, inhibitor of NF-κB-α.

groups compared with the control. Treatment with LNT at both concentrations assessed increased the mRNA and protein expression levels of caspase-3, caspase-8 and caspase-9 in the mice lung cancer tissue samples, and the effect was more potent in the mice treated with a high dose of LNT. Oridonin further increased the mRNA and protein expression levels of caspase-3, caspase-8 and caspase-9 in the lung cancer tissues of the LNT treated animals (Fig. 4A and B).

Effects of oridonin and LNT on the mRNA and protein expression levels of Bax, Bcl-2 and Bcl-xL in the mice lung cancer tissues. Compared with the control, the mRNA and protein expression levels of Bax were significantly increased, whereas those of Bcl-2 and Bcl-xL were significantly decreased in the lung cancer tissue samples in treatment groups. Compared with the control, LNT at both concentrations assessed increased the mRNA and protein expression levels

Table I. Inhibitory effect of oridonin and LNT on lung tumor metastasis in mice.

Group	Total, n	Lung tumor metastasis, n	Inhibitory rate, %
Normal	10	0	0
Control	18	18	100
Oridonin	18	18	100
LNT-L	18	15	16.7
Oridonin + LNT-L	18	13	27.8
LNT-H	18	9	50.0
Oridonin + LNT-H	18	7	61.1

LNT, lentinan; LNT-L, 0.2 ml/day 100 μg/ml LNT; LNT-H, 0.2 ml/day 300 μg/ml LNT.

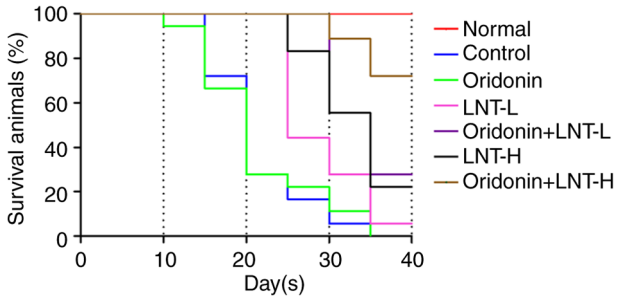

Figure 3. Effects of oridonin and LNT on the survival of mice with lung cancer. Animal survival assays were used to assess the effects of the different treatment combinations. Oridonin alone had little effect on the survival of the animals. Both LNT-L and LNT-H improved the survival of mice, with LNT-H exhibiting improved outcomes compared with than LNT-L. The use of oridonin increased the effects of LNT on survival. LNT, lentinan; LNT-L, 0.2 ml/day 100 μg/ml LNT; LNT-H, 0.2 ml/day 300 μg/ml LNT.

of Bax, and the effect of LNT-H was more potent than that of LNT-L. Compared with LNT groups, LNT with oridonin further increased the mRNA and protein expression levels of Bax in the lung cancer tissue samples of mice treated with LNT. Conversely, compared with the control, LNT at both concentrations assessed decreased the mRNA and protein expression levels of Bcl-2 and Bcl-xL and the decrease was greater in the LNT-H group. Compared with the LNT groups, LNT with oridonin further decreased the mRNA and protein expressions of Bcl-2 and Bcl-xL (Fig. 4C and D).

Effects of oridonin and LNT on the mRNA and protein expression levels of p53 and p21 in mice lung cancer tissues. The mRNA and protein expression levels of p53 and p21 in lung tissue samples were significantly decreased in the mice with cancer compared with non-cancerous control mice. Treatment with LNT at both concentrations increased the mRNA and protein expression levels of p53 and p21 in the lung cancer tissues, and the increase was greater in the LNT-H treated group. Oridonin further increased the mRNA and protein expression levels of p53 and p21 in the lung cancer tissues when combined with LNT-H (Fig. 4E and F).

Effects of oridonin and LNT on the mRNA and protein expression levels of NF-κB and IκB-α in lung tissues. The mRNA and protein expression levels of NF-κB were significantly increased, whereas those of IκB-α were significantly decreased in lung tissue samples of mice with cancer compared with those without. LNT at both concentrations tested decreased the mRNA and protein expression levels of NF-κB, and the decrease was greater in the LNT-H group. Oridonin further decreased the mRNA and protein expression levels of NF-κB in the lung cancer tissue samples of the LNT treated animals. Conversely, LNT at both concentrations assessed increased the mRNA and protein expression levels of IκB-α, and the decrease was larger in the LNT-H treated group. Oridonin further increased the mRNA and protein expression levels of IκB-α in the LNT treated mice with lung cancer (Fig. 4G and H). The *in vivo* results were similar to those observed in the A549 cells, confirming the effects and mechanisms of oridonin and LNT on lung cancer.

Discussion

Oridonin has been reported to increase the anticancer effects of LNT in HepG2 human hepatoblastoma cells (14), and also to enhance the *in vitro* anticancer effects of LNT in SMMC-7721 human hepatoma cells through regulation of the expression of genes associated with apoptosis (13). However, there are no studies that have investigated the effects of LNT on lung cancer, to the best of our knowledge. Lung cancer cells differ from liver cancer due to their differing natures of the cells they are derived from; however, it was hypothesized that LNT would exhibit beneficial effects for the treatment of lung cancer, similar to its effects in liver cancer cells. In addition, oridonin was also shown to inhibit human pancreatic cancer migration (9). The aim of the present study was to assess the effects of the combined treatment of oridonin and LNT *in vivo*.

The results showed that the A549 lung cancer cell line was considerably more sensitive than the normal human fetal lung fibroblast cell line MRC-5 to LNT. This suggested that LNT can be used as a potential cancer medicine with few side effects. LNT has been previously shown to exhibit a suppressive effect on cell growth in certain cancer cell lines (20), as well as an immunomodulatory effect in patients with lung cancer (21). A retrospective study showed that LNT improved the quality of life of patients with multiple types of cancers, including: Lung cancer (3,469 cases); gastric cancer (3,039 cases); colorectal cancer (1,646 cases); ovarian cancer (183cases);cervicalcancer(130cases);pancreaticcancer(15cases);

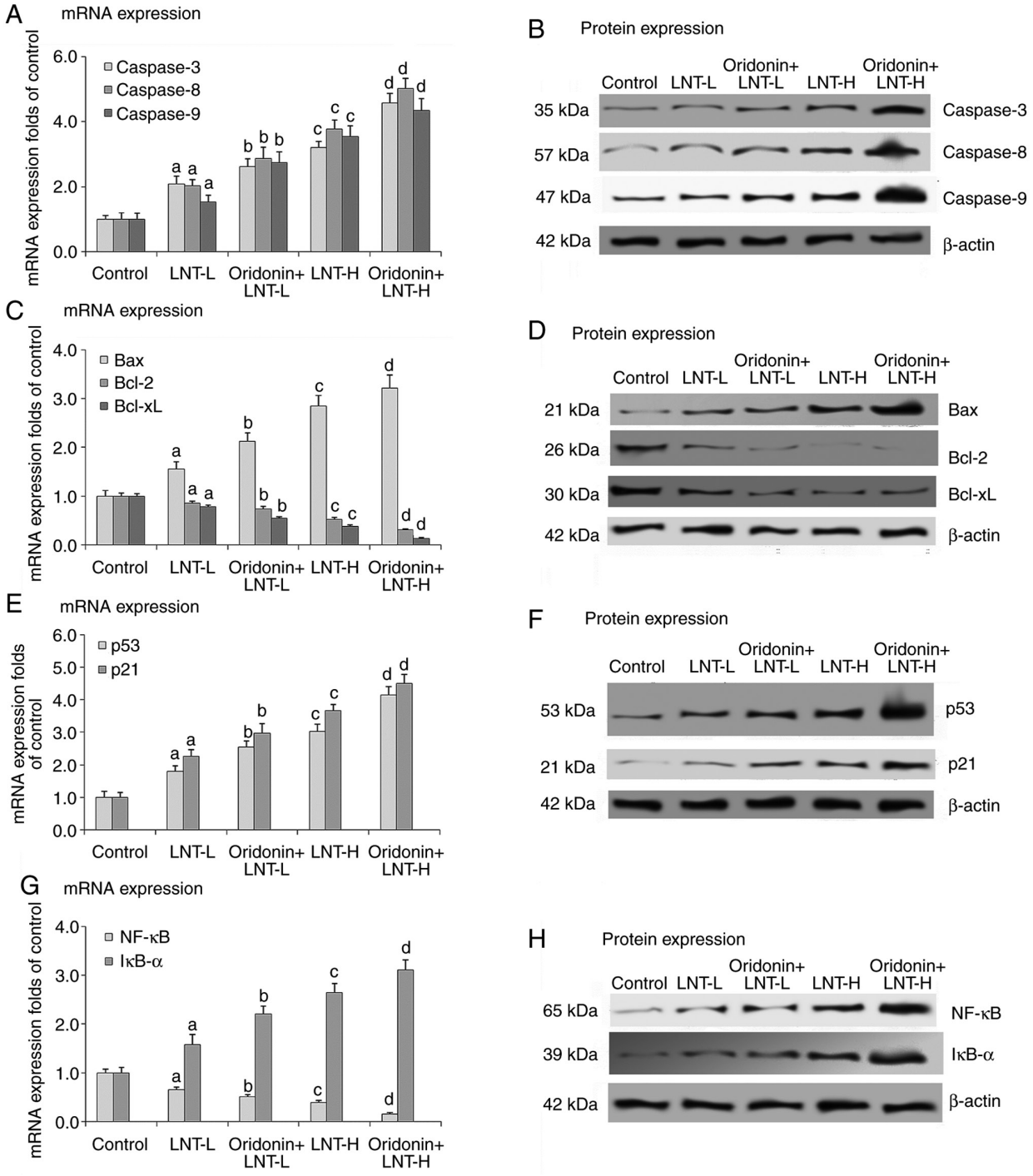

Figure 4. Effects of oridonin and LNT on the mRNA and protein expression levels of viability-associated proteins in lung tissue samples. The mRNA and protein expression levels were determined using reverse transcription-quantitative PCR and western blotting, respectively. mRNA and protein expression levels of (A and B) caspase-3, caspase-8 and caspase-9, (C and D) Bax, Bcl-2 and Bcl-xL, (E and F) p53 and p21, and (G and H) NF-κB and IκB-α in lung tissues. [a]P<0.05 vs. control; [b]P<0.05 vs. LNT-L; [c]P<0.05 vs. oridonin + LNT-L; [d]P<0.05 vs. LNT-H. LNT, lentinan; NF-κB, nuclear factor-κB; IκB-α, inhibitor of NF-κB-α.

cardiac cancer (15 cases); nasopharyngeal cancer (14 cases); duodenal cancer (1 case); Non-Hodgkin lymphoma (70 cases); and 110 cancer cases with no classifying patient information (4). LNT significantly promoted the efficacy of chemotherapy and radiation therapy during these cancer treatment regimens (4). An *in vivo* study also showed that LNT exhibited therapeutic potential for colitis-associated cancer. Additionally, it has been shown that 36 μg/ml (0.1 mmol/l) oridonin inhibited breast cancer growth and metastasis through inhibition of the Notch signaling pathway (8).

In the present study, MTT assays were used to assess the viability of lung cancer cells. MTT assays are widely used in cancer pharmacological studies (22). The results showed that 0-300 μg/ml oridonin had little effect on both MRC-5 and A549, indicating that lung cancer cells may have a lower sensitivity to oridonin than breast cancer cells. The survival rate is

a critical indicator of the effectiveness of cancer therapies in different types of cancer (23). Thus, the clinical treatment with LNT and oridonin were mimicked using a mouse model, and survival of the mice was assessed as described previously (24). The *in vivo* experiments performed in the present 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, oridonin, when combined with LNT, promoted the effects of LNT with regard to both the viability of A549 cells and the metastasis and the survival of mice. These results suggest that oridonin may activate pathways that facilitate the actions of LNT. However, in the survival experiments, mice were anesthetized, and this procedure may potentially affect the induced cancer (23,25), which may have potentially affected the results.

To explore the underlying mechanisms modulated by LNT, the expression of several potential targets of LNT in both A549 and lung tissue samples were assessed. A previous study showed that proliferation and apoptosis affect cancer cell viability (26). Thus, it was hypothesized that apoptosis may be involved in the effects of oridonin and LNT. Firstly, the caspase signaling pathway was assessed. Caspases (cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases) are a family of protease enzymes that serve essential roles in programmed cell death and inflammation (27). A previous study showed that co-treatment with paclitaxel and LNT enhanced cell apoptosis rates by inducing caspase-3 activation (28). In the present study, it was shown that LNT reduced A549 cell viability by increasing the expression of the apoptosis executioner caspase-3, and oridonin further promoted this increase in expression. Moreover, it was also shown that there was a potential negative feedback of the caspase signals, as the expression of the apoptosis initiators of caspases, caspases 8 and 9, increased with alongside caspase-3.

LNT has been reported to exert synergistic apoptotic effects when combined with paclitaxel in A549 cells (28). The apoptosis-inducing effects of LNT have also been reported in a study using the human bladder cancer cell line T24 (29). In hepatoma cells, oridonin was shown to promote the effects of LNT through regulating the expression of apoptotic genes (13). To confirm that the effects observed in the present study were mediated by apoptosis (28), the activity of the Bax signaling pathway, an apoptosis regulatory pathway, was assessed. Bcl2 family members act as anti- or pro-apoptotic regulators in 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 (30). In the present study, the expression of Bax, Bcl-2 and Bcl-xL were affected by LNT and oridonin, suggesting that their effects were mediated by regulation of apoptosis.

The expression of the Bcl2 family of genes is regulated by the tumor suppressor p53 and has been shown to be involved in p53-mediated apoptosis (31,32). In was hypothesized that p53 and p21 may be the upstream targets of LNT and oridonin. Hence, the expression of p53 and p21 in A549 and lung tissues were determined, and the results showed that their expression was increased by treatment with LNT and oridonin. NF-κB is a protein complex that controls transcription of DNA, cytokine production and cell survival, whereas its inhibitor, IκBα, functions to inhibit the transcriptional activity of NF-κB transcription factors (33). In the present study, it was shown that NF-κB and IκBα were involved in the effects of LNT and oridonin on cancer development, and their expression was altered in the treated cells, and may have served a role in the decrease in cell viability observed in the treated cells. In the lung tissues, the results were similar to that of the *in vitro* experiments.

In conclusion, it was shown that oridonin enhanced the antitumor effects of LNT. Additionally, several potential regulatory mechanisms by which oridonin and LNT exerted their effects were determined. However, there are other mechanisms that may also be involved, such as cancer stem cells, which might be targeted by oridonin and LNT (34). Thus, more work is required to confirm these results and obtain a more in-depth understanding of the specific mechanisms modulated by oridonin and LNT. Several compounds derived from traditional Chinese medicines have been explored for their potential clinical use in the treatment of various diseases (35-37). In cancer treatment, although traditional medicines are not able to cure cancer alone, when applied in combination with traditional treatment approaches, they may reduce the adverse effects caused by chemotherapy or radiotherapy, thus improving therapeutic outcomes and quality of life for patients, or may act as adjuvants (38). In addition, some ion channels might also be involved in the drug action of cancer cell proliferation (39).

In the present study, a constituent compound of a traditional herbal medicine was shown to augment the effects of a more traditional treatment. These results support the notion of the further study of oridonin and LNT as a novel cancer drug regimen, and contributes to the application of traditional medicines as clinical treatments.

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Availability of data and materials

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

Authors' contributions

YG, JC, and ZC made substantial contributions to conception and design, acquisition of data, and the analysis and interpretation of data. JC and ZC were involved in drafting the manuscript and revising it critically for important intellectual content. YG and JC confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the Animal Ethics Committee of Wuhan University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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