Molecular mechanisms of *Lycoris aurea* agglutinin-induced apoptosis and G₂/M cell cycle arrest in human lung adenocarcinoma A549 cells, both *in vitro* and *in vivo*

C.-Y. Li^{*,a}, Y. Wang^{†,‡,§,a}, H.-L. Wang^{*,§,a}, Z. Shi^{*,¶}, N. An^{*}, Y.-X. Liu^{*}, Y. Liu^{**}, J. Zhang[†], J.-K. Bao^{*} and S.-P. Deng[§]

^{*}School of Life Sciences & Key Laboratory of Bio-resources, Ministry of Education, Sichuan University, Chengdu, China, [†]Institute of Neurology Science, Guangdong Medical University Affiliated Hospital, Zhanjiang, China, [‡]Department of Radiation Oncology, James Comprehensive Cancer Center, Ohio State University, Columbus, OH, USA, [§]Institute of Organ Transplantation, Science & Sichuan Provincial People's Hospital, Chengdu, China, [¶]School of Life Sciences, Guizhou Normal University, Guiyang, China and ^{**}91388 Military hospital, Guangdong, Zhanjiang, China

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Abstract

Objectives: *Lycoris is aurea* agglutinin (LAA) has attracted rising attention due to its remarkable bioactivities. Here, we aimed at investigating its anti-tumor activities.

Material and Methods: *In vitro* methods including MTT, cellular morphology observation, FCM and immunoblotting were performed. *In vivo* methods like detection of tumor volume, body weight and survival ratio, as well as TUNEL staining were performed.

Results and Conclusion: LAA triggers G₂/M phase cell cycle arrest via up-regulating p21 expression as well as down-regulating cdk-1/cyclinA signaling pathway, and induces apoptotic cell death through inhibiting PI3K-Akt survival pathway in human lung adenocarcinoma A549 cells. While LAA has no significant cytotoxic effect toward normal human embryonic lung fibroblast HELF cells, and moreover, LAA could amplify the antineoplastic effects of cisplatin toward A549 cells. Lastly LAA also bears anti-cancer and apoptosis-inducing effects *in vivo*, and it could decrease the volume and weight of subcutaneous tumor mass obviously as well as expand lifespan of mice. These findings

may provide a new perspective for elucidating the complicated molecular mechanisms of LAA-induced cancer cell growth-inhibition and death, providing a new opportunity of LAA as a potential candidate anti-neoplastic drug for future cancer therapeutics.

Introduction

Lectins are carbohydrate-binding proteins that exist widely in animals, plants and microorganisms; as well as binding carbohydrates, they are able to agglutinate cells and precipitate polysaccharides and glycoconjugates (1). Rapid progress has been achieved in isolation and characterization of plant lectins, and recently their classification has been amended from 7 families into 12 families, which are *Agaricus bisporus* agglutinin, amaranthin, chitinase-related agglutinin, cyanovirin, *Euonymus europaeus* agglutinin, *Galanthus nivalis* agglutinin (GNA), hevein, jacalins, legume lectin, LysM (lysine motif), nictaba and ricin B families (2–4). The GNA-related lectin family reveals strict mannose-binding specificity, and the conserved amino acid 'QXDXNXVXY' motif is essential in mannose recognition. Molecular structure studies demonstrate that all GNA-related lectins share a similar three-dimensional structure with GNA, in spite of different compositions of their amino acid sequences. The GNA monomer has a β -barrel structure comprised of three subdomains (I, II, III), each subdomain being made up of three or four strands of antiparallel β sheet interacting by Ω loops (5).

Correspondence: J.-K. Bao, School of Life Sciences & Key Laboratory of Bio-resources, Ministry of Education, Sichuan University, Chengdu 610064, China and S.-P. Deng, Institute of Organ Transplantation, Sichuan Academy of Medical Science & Sichuan Provincial People's Hospital, Chengdu 610072, China. Tel.: +86 28 85415171; Fax: +86 28 85417281; E-mails: jinkubao@yahoo.com and sdeng10@yahoo.com

^aThese authors contributed equally to this work.

Previous reports have made known that lectins from the GNA-related lectin family hold remarkable biological activities, such as anti-tumour properties of *Polygonatum cyrtonema* lectin (PCL) (5–8), *Liparis noversa* lectin (LNL) (7), *Ophiopogon japonicus* lectin (OJL) (7,8) and *Polygonatum odoratum* lectin (POL) (9). GNA (10–12) also display anti-HIV activity and OJL (13) and *Typhonium divaricatum* lectin (14) have anti-HSV-II effects (14). All these bioactivities of plant lectins are intensively associated with their carbohydrate specificities (15).

Lycoris aurea agglutinin (LAA) is a typical representative of the Amaryllidaceae, belonging to a GNA-related lectin family, and has served as an important Chinese traditional herbal medicine for treating skin herpes virus infections, for hundreds of years (16). To date, anti-tumour effects of LAA are still under investigation, and therefore it is of great significance to investigate its anti-cancer properties.

Here, first we report that LAA induces apoptosis through inhibiting the PI3K-Akt pathway in human lung carcinoma A549 cells, and also it initiates G₂/M cell cycle arrest by up-regulating p21 expression and down-regulating the CDK1-cyclin A signalling pathway. Although LAA has no significant cytotoxic effect on normal human embryonic lung fibroblast (HEL F) cells, it amplifies anti-neoplastic effects of cisplatin on A549 cells. In addition, LAA also has anti-cancer and apoptosis-inducing effects *in vivo*, and reduces volume and mass of subcutaneous tumours, as well as extending lifespan of host mice.

Materials and methods

Reagents

Lycoris aurea agglutinin was purified and maintained in our laboratory (16), and cisplatin was purchased from Sigma Chemicals (St. Louis, MO, USA). Human lung carcinoma A549 cells and HEL F cells were purchased from American Type Culture Collection (Manassas, VA, USA). Foetal bovine serum (FBS) was purchased from Gibco BRL (Grand Island, NY, USA), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), z-VAD-fmk (pan-caspase inhibitor), z-DEVD-fmk (caspase-3 inhibitor) and z-IETD-fmk (caspase-8 inhibitor) were purchased from Sigma Chemicals. All other chemicals used in this study were of the highest purity available.

Molecular modelling

MODELLERv9 (17) implemented in Insight II (Accelrys, San Diego, CA, USA) was utilized to build molecular modelling of LAA with *Narcissus pseudonarcissus*

agglutinin as a template (PDB code: 3DZW). Docking experiments were carried out using UCSF DOCK.

Cell culture

Human lung carcinoma A549 cells were cultured in RPMI-1640 medium (Gibco), containing 10% FBS, 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin (Invitrogen) and were maintained at 37 °C and 5% CO₂ in a humidified atmosphere. HEL F cells, used as the corresponding control group, were cultured in Dulbecco's minimal essential medium (Gibco) containing the same additional components.

MTT colorimetric assay

A549 cells in RPMI-1640 (Gibco) containing 10% FBS were seeded in 96-well plates and cultured for 24 h; cytotoxic effects of LAA and cisplatin were performed as previously described (18). In addition, cytotoxic effects of serial doses of cisplatin in combination of 20 µg/ml LAA, were also detected. Absorbance at 570 nm was measured with a spectrophotometer (Model 3550 Microplate Reader; Bio-Rad, Hercules, CA, USA):

$$\text{Cell viability(\%)} = (\text{OD}_{570\text{nm}}(\text{drug}) / \text{OD}_{570\text{nm}}(\text{control})) \times 100\%.$$

Observations of cell morphological changes

A549 cells in RPMI-1640 (Gibco) and HEL F cells in Dulbecco's minimal essential medium (Gibco) containing 10% FBS were seeded in 96-well plates and cultured for 24 h. Control groups, LAA groups and cisplatin groups were treated with PBS, 20 µg/ml LAA and 16 µg/ml cisplatin, respectively. After further 24-h incubation in PBS, 20 µg/ml LAA and 16 µg/ml cisplatin, as well as 24-h incubation HEL F cells in PBS and 20 µg/ml LAA, cell morphology was observed using phase-contrast microscopy (Leica, Wetzlar, Germany). In addition, Hoechst 33258 (5 µg/ml) staining was applied for 15 min, and both cell types incubated with LAA, cisplatin or PBS were fixed in 4% paraformaldehyde for 30 min at room temperature; they were then washed twice in PBS. Cells were then analysed immediately using fluorescence microscopy (Olympus, Tokyo, Japan).

Measurement of cell cycle and sub-G₁ cells

A549 cells treated with LAA or PBS at 37 °C for 12, 24, 36 and 48 h were harvested and FACScan flowcytometry assay was performed as previously described (19). Percentages of cells at different phases of the cell

cycle or undergoing apoptosis were evaluated by Calibur FACScan flowcytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Caspase assay

A549 cells were seeded into wells of 96-well plates for 24 h incubation after which they were treated with 200 μ M caspase3, 8 and pan-caspase inhibitor, or not treated, for a further 2 h incubation at 37 °C. Subsequently, 20 μ g/ml LAA for 24 h incubation was performed, and MTT was assayed as described above.

Detection of mitochondrial membrane potential

Mitochondrial membrane potential was measured using fluorescent dye rhodamine-123 as previously mentioned (18). Fluorescence intensity of cells was analysed using Calibur FACScan flow cytometry (Becton Dickinson).

Western blot analysis

A549 cells were treated with 20 μ g/ml LAA for 12, 24, 36 and 48 h, then both adherent and floating cells were collected. Western blotting was performed as previously described (20,21)

Acute toxicity testing

Acute toxicity testing was performed to determine median lethal dosing (LD_{50}) of LAA using the method of Abdel-Barry *et al.* (22). After 16-h fasting, 80 male nude mice were randomly divided into 8 groups of 10 mice each. Graded doses of LAA, dissolved in PBS, (20, 50, 100, 200, 400, 600, 1000 and 2000 mg/kg) were separately administered intraperitoneally to the mice; average volume injected was 0.3 ml. All mice were allowed free access to food and water, then mortality in each group was assessed after 24, 48 and 72 h administration of LAA. Percentage mortality in each group was calculated and plotted against $\log_{10}$ of LAA dose. A regression line was fitted by the method of least squares, and confidence limits for lethal dose (LD_{50}) values were calculated by the method of Abdel-Barry *et al.* (22).

In vivo study design

Fifty 3 month-old male nude mice were randomly divided into five groups:

Blank control group, mice administered PBS after A549 cells injection 100 mg/kg LAA group; mice administered high-dose (100 mg/kg) LAA after A549 cells

injection 50 mg/kg LAA group; mice administered medium-dose (50 mg/kg) LAA after A549 cells injection 10 mg/kg LAA group; mice administered low-dose (10 mg/kg) LAA after A549 cells injection; positive control group, mice administered 1 mg/kg cisplatin after A549 cells injection (23). 100 mg/kg, 50 mg/kg and 10 mg/kg of LAA were injected intraperitoneally into mice, and therapy lasted for 2 weeks. Animal handling was in accordance with Ethics Committee of Sichuan University, and all animals were kept in 12-h light/dark cycle with free access to water and food, which is in accordance with IVC requirement in Sichuan University.

Relative tumour volume, survival rate, rate of inhibition and body weight determination

Tumour volume was determined by calliper measurements according to the formula:

$$T_{vol} = \text{length} \times \text{width} \times \text{depth} \times 0.5.$$

Relative tumour volume (RTV) was calculated as relative increase or decrease in mean tumour volume from initiation of treatment (V_0) up to value at a given time (V_t) and $RTV = V_t/V_0$.

Inhibitory rate of tumour volume

$$= (V_{\text{control}} - V_t)/V_{\text{control}} \times 100\%.$$

After 14 days treatment, mice were killed by cervical dislocation, and subcutaneous tumour masses were determined.

Inhibitory level of tumour weight

$$= (W_{\text{control}} - W_t)/W_{\text{control}} \times 100\%.$$

Histological staining

After 2 weeks injection of LAA, mice were killed by cervical dislocation, and the subcutaneous tumours were harvested. Tissues were fixed in 10% formalin for 24 h at room temperature, dehydrated, embedded in paraffin wax and sectioned. To detect whether LAA could induce apoptosis *in vivo*, transferase-mediated dUTP nick-end labelling (TUNEL) staining was performed using Roche fluorescence Dead End kit according to the manufacturer's instructions. Cells were analysed and captured using fluorescence microscopy (Nikon, Tokyo, Japan).

Statistical analysis

All results presented here were confirmed in at least three independent experiments. Data were expressed as

mean $\pm$ SEM. Statistical comparisons were made using Student's *t*-test and two-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Molecular modelling and docking experiments

Molecular modelling of LAA was carried out by MO-DELLERv9; it was noteworthy that structure of LAA is predominantly comprised of β -sheets connected by Ω turns and loops (as shown in Fig. 1). Docking results show that LAA bears affinity to mannose, and also three conserved carbohydrate-binding domains; the LAA monomer also possesses an additional mannose-binding site near its tryptophan cluster (16).

Cytotoxic effects of LAA on A549 cells

Both LAA and cisplatin induced A549 cell death in a dose-dependent manner. LAA from 0 to 100 $\mu\text{g/ml}$ exerted a potent inhibitory effect on population growth of A549 cells (Fig. 2a) and after 24 h incubation in

Figure 1. Molecular modelling of *Lycoris aurea* agglutinin monomer. The monomer exhibits a β -barrel structure comprising four mannose-binding subdomains.

Figure 2. *Lycoris aurea* agglutinin induced apoptosis in A549 cells while leaving human embryonic lung fibroblast cells intact, and LAA could amplify the anti-neoplastic effects of cisplatin on A549 cells. (a) Inhibitory ratio of A549 cells with different concentrations of LAA and cisplatin. (b) A549 cells were incubated with serial doses of cisplatin in combination of 20 $\mu\text{g/ml}$ LAA, and LAA treatment enhanced cisplatin-induced apoptosis in A549 cells. (c) The apoptotic morphological observations of A549 cells treated with PBS, 20 $\mu\text{g/ml}$ LAA and 16 $\mu\text{g/ml}$ cisplatin under the phase-contrast microscopy and fluorescence microscopy (200 $\times$). (d) Presence of 20 $\mu\text{g/ml}$ LAA did not significantly lead to apoptotic morphology in human embryonic lung fibroblast cells. (e) Different phase percentages of A549 cells that interfered with 20 $\mu\text{g/ml}$ LAA were measured by flowcytometry, and the cell cycle was represented by a bar diagram (mean $\pm$ SD, $n = 3$).

20 µg/ml LAA, the inhibitory rate reached also 50%. For cisplatin, from 0 to 100 µg/ml exerted a potent inhibitory effect on population growth of A549 cells, and 16 µg/ml cisplatin lead to almost 50% population growth inhibition (Fig. 2a). It has previously been demonstrated that cisplatin has more potent growth inhibitory activity on A549 cells. Then, a series of doses of cisplatin in combination with 20 µg/ml LAA (designated as cisplatin + 20 µg/ml LAA) were used to treat A549 cells, and viability of the cells was assessed by MTT assay, at different time points. Cisplatin + 20 µg/ml LAA synergistically inhibited population expansion of A549 cells, and as shown in Fig. 2b, almost 9 µg/ml cisplatin in combination of 20 µg/ml LAA resulted in 50% inhibitory ratio of A549s, indicating that LAA treatment amplified anti-neoplastic effects of cisplatin on them.

Cell morphology

Marked apoptotic morphological changes such as membrane blebbing, cell volume reduction and rounding were obvious in LAA- and cisplatin-treated cells, when viewed by phase contrast microscopy (Fig. 2c). Hoechst 33258 staining demonstrated that LAA- and cisplatin-treated cells presented manifestly fragmented DNA in nuclei, while in control groups, DNA resided in round nuclei, homogeneously stained (Fig. 2c). However, presence of 20 µg/ml LAA did not significantly lead to typical apoptotic morphology of HELF cells (Fig. 2d). These results suggest that LAA selectively induced death in human lung adenocarcinoma A549, but not in normal HELF cells.

LAA induced phase changes in A549 cells

As shown in Fig. 2e, LAA markedly induced increase in sub-G₁ proportions of A549 cells, in a time-dependent manner (control group: 4%, 12 h group: 7%, 24 h group: 18%, 36 h group: 24%, 48 h group: 31%, $P < 0.05$, $n = 3$), indicating that LAA induced apoptosis in A549 cells. At the same time, LAA also increased G₂/M portion enhancement (control group: 6%, 12 h group: 23%, 24 h group: 30%, 36 h group: 38%, 48 h group: 49%, $P < 0.05$, $n = 3$), demonstrating that LAA initiated G₂/M cycle arrest in A549 cells. Altogether, these results indicate that LAA induced both apoptosis and G₂/M cycle arrest in A549 cells.

LAA induced apoptosis in a caspase-dependent manner

After 24 h incubation in LAA, pancaspase, caspase-3 and -8 inhibitors almost completely suppressed LAA-

induced cell population expansion inhibition (as shown in Fig. 3a). This indicates that LAA induced apoptosis in a caspase-dependent manner. Moreover, we further examined activities of caspases-3 and -8 by western blot analysis. As shown in Fig. 3b, when A549 cells were treated with LAA for 12, 24, 36 and 48 h, expressions of procaspase-8 and procaspase-3 were reduced, but expressions of caspase-8 and caspase-3 were elevated with culturing time, suggesting that LAA increased activities of caspase-3 and -8 in a time-dependent manner. As poly ADP-ribose polymerase (PARP) and inhibitor of caspase-dependent DNase (ICAD) were caspase-3 substrates, subsequently, ICAD and PARP expressions were examined. After exposure to LAA, ICAD was reduced, and 116-kDa protein expression declined, while 85-kDa protein was degraded, indicating cleavage of PARP.

LAA induced apoptosis via the mitochondrial pathway

As shown in Fig. 3c, LAA reduced rhodamine 123 fluorescence intensity in a time-dependent manner (control group: 93%, 12 h group: 82%, 24 h group: 57%, 36 h group: 37%, 48 h group: 21%, $P < 0.05$, $n = 3$). In addition, quantity of cytochrome *c* in cell mitochondria was reduced, but amount of cytochrome *c* in cell cytoplasm was elevated, suggesting that cytochrome *c* was released from mitochondria (Fig. 3d) into the cytoplasm. These results clearly indicate that LAA induced apoptosis in A549 cells was mediated by the mitochondrial pathway.

LAA induced apoptosis in A549 cells by inhibiting the PI3K-Akt pathway

A549 cells were pre-treated with the PI3K inhibitor wortmannin, Akt inhibitor KP372-1, and LAA-induced cell cytotoxicity was measured. As shown in Fig. 4a, these inhibitors significantly increased LAA-induced cytotoxicity, indicating that both PI3K and Akt were playing protective roles in LAA-induced A549 cell apoptosis. Western blot data showed that treatment of A549 cells with LAA resulted in down-regulation of PI3K and p-Akt proteins levels (Fig. 4b). Western blot data demonstrated that levels of Akt were not obviously changed, while p-Akt level was markedly reduced in a time-dependent manner after LAA administration (Fig. 4b).

LAA induced G₂/M cell-cycle arrest by down-regulating CDK1-cyclin A signaling pathway

A549 cells were treated with 20 µg/ml LAA for different time periods and cell cycle distribution was subse-

Figure 3. *Lycoris aurea* agglutinin induced apoptosis through a caspase-mediated mitochondrial pathway in A549 cells. (a) After pre-treatment with or without 200 μ M z-VAD-fmk (pan-caspase inhibitor), z-DEVD-fmk (caspase-3 inhibitor) and z-IETD-fmk (caspase-8 inhibitor) for 2 h, the growth inhibition of A549 cells treated with 20 μ g/ml LAA for 24 h was determined (mean $\pm$ SD, $n = 3$). (b) LAA-induced procaspase-8, procaspase-3 cleaving and inhibitor of caspase-dependent Dnase, poly ADPribose polymerase expressing. β -Actin was used as an equal loading control. (c) The integrity of mitochondrial membranes was measured by rhodamine 123 staining, and fluorescence intensity was represented by a bar diagram (mean $\pm$ SD, $n = 3$). (d) A549 cells were treated with 20 μ g/ml LAA for various time periods, cytochrome *c* in mitochondria and cytosol were detected by western blot analysis, and β -Actin was used as an equal loading control.

quently analysed by flow cytometry. As shown in Fig. 2e, compared to control groups, treatment of A549 cells with LAA resulted in significant increase in percentage of G₂/M cells (control group: 6%, 12 h group: 23%, 24 h group: 30%, 36 h group: 38%, 48 h group: 49%, $P < 0.05$, $n = 3$). This result indicates that LAA also induced G₂/M cell cycle arrest. Subsequently, effects of LAA on cell cycle-regulatory molecules, including p21, cyclin A and CDK1, were determined. As shown in Fig. 4c, treatment of A549 cells with LAA resulted in increased level of p21, while levels of cyclin A and CDK1 were down-regulated. Thus, LAA induced G₂/M phase cell cycle arrest by up-regulating expression of p21 and down-regulating activities of CDK1 and cyclin A.

Tumour volume, body weight and survival ratio detection

Acute toxicity testing indicated that high, minimal and low doses of LAA were 100 mg/kg, 50 mg/kg and 10 mg/kg, respectively. Volumes of tumours *in vivo*

were determined in three dimensions with vernier callipers, then relative tumour volumes were calculated by the above equation (Fig. 5a). After 14 days treatment, all mice were sacrificed, and subcutaneous tumours were peeled off and weighed. As shown in Table 1, after 14 days 100 mg/kg LAA treatment, tumour volumes decreased to 0.13 ± 0.08 cm³, almost reducing by 70% compared to the blank control group, while inhibitory ratio of volume treated with cisplatin reached almost 80% (also shown in Fig 5b). Meanwhile, weight of tumours of 100 mg/kg LAA decreased to 0.36 ± 0.13 g, reducing almost by 60%, while inhibitory ratio of positive control groups reached almost 75% (presented in Table 1). After seven days (designated as day 6 to day 0) A549 cell inoculation, tumours formed, and weights of mice increased. Treatment with various dosages of LAA for 14 days, caused weights of mice to decrease markedly (shown in Fig. 5c). As shown in Fig. 5d, compared to the blank control group, LAA could prolong life span of A549-bearing mice, thus indicating that LAA bore remarkable anti-tumour effects *in vivo*.

Figure 4. The molecular mechanisms of *Lycoris aurea* agglutinin induced G₂/M phase cell cycle arrest and apoptosis in A549 cells.

(a) A549 cells were pre-treated with PI3K inhibitor wortmannin and Akt inhibitor KP372-1, and LAA-induced cell growth inhibitory ratio was measured (mean $\pm$ SD, $n = 3$). (b) A549 cells were treated with 20 μ g/ml LAA for various time periods, and the expressions of Akt, p-Akt and PI3K were detected by western blot analysis. β -Actin was used as an equal loading control. (c) A549 cells were treated with 20 μ g/ml LAA for various time periods, and the levels of cell cycle-related proteins including p21, cyclin B and cdk1 were detected by western blot analysis. β -Actin was used as an equal loading control.

LAA induced apoptotic cell death in vivo

Transferase-mediated dUTP nick-end labelling assays of subcutaneous tumour tissue sections demonstrated that LAA produced apoptosis in tumour masses (Fig. 6), whereas little apoptosis was observed in control groups.

Discussion

Recently, the GNA-related lectin family has been attracting rising attention due to its remarkable biological activities, including anti-tumour, anti-viral and anti-fungal properties. Lectins from this family potentially have medical applications and have been reported to be 'ideal' anti-cancer candidate agents due to their capability to induce apoptosis in susceptible cancer cells. LAA, a typical representative of the GNA-related lectin family, has served as an important traditional Chinese herbal medicine for treating skin herpes virus infection, for hundreds of years (24). However, until now, anti-tumour

effects of LAA were unknown, and investigating its anti-cancer activity is of great significance. In this study, LAA was reported for the first time to induce apoptosis by inhibiting the PI3K-Akt pathway in human lung carcinoma A549 cells. LAA also initiated G₂/M phase cell-cycle arrest by up-regulating p21 expression and down-regulating the CDK1-cyclin A signalling pathway. While LAA had no significant cytotoxic effect on normal HELF cells, it amplified anti-neoplastic effects of cisplatin on A549 cells. In addition, LAA has anti-cancer and apoptosis-inducing effects *in vivo*, and it was able to reduce volume and weight of subcutaneous tumour mass, as well as able to extend lifespan of mouse hosts.

In this study, 24 h respective incubation of A549 cells with 20 μ g/ml LAA and 16 μ g/ml cisplatin led to 50% growth inhibition of A549 cells, demonstrating that cisplatin had more potent growth inhibitory activity towards A549 cells. Then, a series of different doses of cisplatin in combination with 20 μ g/ml LAA were used to treat A549 cells; almost 9 μ g/ml cisplatin in combination with 20 μ g/ml LAA resulted in 50% inhibitory ratio of A549 cells. Compared to 16 μ g/ml cisplatin treatment alone, only 9 μ g/ml cisplatin in combination with 20 μ g/ml LAA lead to 50% growth inhibition of A549 cells, indicating that in the presence of LAA, lower concentrations of cisplatin result in 50% death of A549 cells. Thus, it is demonstrated that LAA treatment could amplify anti-neoplastic effects of cisplatin on A549 cells.

In addition, ratio of G₂/M cell cycle of A549 cells was increased in a time-dependent manner after LAA treatment, indicating that LAA could induce G₂/M cell cycle arrest in them. After using 20 μ g/ml LAA for 12, 24, 36 and 48 h, expressions of CDK1 and cyclin A were reduced. However, levels of p21, a cyclin-dependent kinase inhibitor hampering cell cycle progression, was elevated, which would amplify growth-inhibitory effects of LAA. During transition from second gap (G₂) phase to mitosis (M), CDK1-cyclin A complex is abruptly activated to facilitate commencement of mitosis, by regulation of chromosome condensation and microtubule dynamics (25). Thus, suppressing expression of CDK1-cyclin A complex could inhibit initiation of mitosis, further leading to cell cycle arrest.

In addition to inhibition of cell proliferation, induction of cell death such as by apoptosis is a further way to treat cancer. Here, LAA was induced intrinsic mitochondria-mediated apoptosis in a caspase 3- and caspase 8-dependent manner, accompanying collapse of mitochondrial membrane potential and cytochrome *c* release, from mitochondria into cytoplasm. This indicates that mitochondrial dysfunction occurred during LAA-induced

Figure 5. *Lycoris aurea* agglutinin decreased the volume and weight of subcutaneous tumour mass *in vivo* as well as increased survival ratio and weight of A549-bearing mice. (a) Relative tumour volume of each group after 20 µg/ml LAA treatment ($n = 10$, $P < 0.05$). (b) The comparison of the anti-tumour effects among various dosage of LAA ($n = 10$, $P < 0.05$). (c) Weight variety of A549-bearing mice ($n = 10$, $P < 0.05$) (d) Survival curve of A549-bearing mice after various dosage of LAA treatment ($n = 10$, $P < 0.05$).

Table 1. Inhibitory of *Lycoris aurea* agglutinin on A549-bearing mice at the 14th day ($n = 10$, $P < 0.05$)

Group	Volume/cm ³	Weight/g	Inhibitory ratio of Volume (%)	Inhibitory ratio of Weight (%)
Blank control	0.43 ± 0.32	0.89 ± 0.58		
10 mg/kg	0.33 ± 0.13	0.73 ± 0.36	23.3	17.9
50 mg/kg	0.22 ± 0.08	0.52 ± 0.28	48.8	41.6
100 mg/kg	0.13 ± 0.08	0.36 ± 0.13	69.8	59.6
Positive control	0.08 ± 0.04	0.22 ± 0.09	81.4	75.3

A549 apoptosis. Release of cytochrome *c* appears to be a central event in apoptosis, as it is critical for initiation of caspase family activation. Our results are in agreement with the findings of other studies with regard to apoptosis-inducing effects of GNA-related lectins such as OJL, PCL, LNL and POL. Further detection of molecular mechanisms of LAA-induced apoptosis indicated that PI3K and p-Akt protein levels were markedly

reduced in a time-dependent manner, while Akt expression was not obviously changed, indicating that LAA could induce apoptotic cell death by hampering the PI3K-Akt survival pathway in human lung carcinoma A549 cells. A negative feedback loop, such as the PI3K-Akt pathway, exerting apoptosis-inhibitory effects mainly through blocking caspase-activated DNase and inhibiting chromatin condensation, has been shown to be needed to be suppressed in several cancer treatments.

Previous reports have demonstrated that OJL, PCL and LNL, representative GNA-related lectins, can induce apoptosis in human breast adenocarcinoma MCF-7 cells (7). Among them, PCL has been reported to induce apoptosis and autophagy in human melanoma A375 cells *via* mitochondrial-mediated ROS-p38-p53 (6), and also that PCL can induce apoptosis and autophagy *via* blocking Ras-Raf and the PI3K-Akt signalling pathways in murine fibrosarcoma L929 cells (26). Additionally, POL has been demonstrated to induce apoptosis through both death receptor and mitochondrial pathways, as well as to potentiate activity of TNF α -induced apoptosis in murine fibrosarcoma L929 cells (9). Due to carbohydrate-binding activity of plant lectins, it is possible that they could reversibly bind to glycoproteins of cell sur-

Figure. 6. *Lycoris aurea* agglutinin induced apoptotic cell death in A549-bearing mice. Apoptotic cells in xenograft tumour tissue under the treatment of PBS, cisplatin and various concentrations of LAA were detected using the transferase-mediated dUTP nick-end labelling assay (200 $\times$). Nucleus was stained with DAPI and represented as blue fluorescence, while apoptotic cell nucleus represented as green fluorescence.

faces similar to interactions between antibody and antigen. Some receptors residing on cancer cell surfaces bear very high mannose-type glycans and thus has specificity to various cell membrane surface glycoproteins enabling lectins to induce apoptosis and autophagy specifically in tumour cells while leaving the normal cells intact (27).

Here, we have also demonstrated that LAA visibly reduced volume and weight of subcutaneous tumour masses. After 14 days treatment with highest dosage of LAA (100 mg/kg), tumour volume and weight decreased nearly 70% and 60%, respectively, and highest dosage of LAA had almost similar anti-tumour effects to those of cisplatin, an accepted effective anti-tumour agent. To detect whether LAA could also induce apoptotic cell death *in vivo*, TUNEL staining was performed, and TUNEL assay of subcutaneous tumour tissue sections demonstrated that LAA produced apoptosis in tumour masses, and with the reduction in dosage, numbers of apoptotic nuclei also reduced. All these results indicate that LAA has the capability of inhibiting human lung carcinoma A549 cell growth and to induce apoptosis *in vivo*.

Hitherto, two types of plant lectin, mistletoe and ricin, have been widely utilized in early clinical studies. Mistletoe-I induces apoptosis *via* both the caspase-8/FLICE pathway and the p53-independent pathway (28), whereas other studies have demonstrated that Korean mistletoe lectin-induced apoptotic mechanisms were death-receptor and mitochondrial pathways, as well as remarkable generation of ROS by activation of caspase and by activation of the SEK/JNK pathway (29). Extracts of *Viscum album coloratum*, Korean mistletoe, have been shown to have prophylactic effects on tumour metastasis of colon carcinomas, melanoma, and lymphoma cells in mice (30), and extracts from *Viscum album* (mistletoe) have been widely used in cancer therapies in Europe for almost 80 years. Additionally, ricin has been demonstrated to induce apoptosis *via* a caspase-dependent pathway by activating caspase-8, probably responsible for following downstream activation of caspase 3/7 (31). Phase I clinical studies on ricin indicate that ricin may induce antibody formation and reduce tumour metastasis; however, treatment with ricin would lead to clinical side-effects (32). Lectins from the GNA-related lectin family, such as LAA studied here, demonstrated no toxicity towards normal cells, making GNA-related lectins 'ideal' anti-cancer candidate drugs with no side-effects.

In summary, we report for the first time that LAA triggered G₂/M phase cell-cycle arrest *via* up-regulating p21 expression as well as by down-regulating CDK1-cyclin A signalling pathway and inducing apoptotic cell death by inhibiting the PI3K-Akt pathway in human

lung carcinoma A549 cells. In addition, LAA had anti-cancer and apoptosis-inducing effects *in vivo*, and it could visibly reduce volume and weight of subcutaneous tumour masses, as well as extend lifespan of mouse hosts. However, whether the PI3K-Akt survival pathway is also involved in LAA-induced apoptosis *in vivo* was not detected, due to insufficient experimental techniques and environment. Subsequent intricate molecular mechanisms implicated in LAA-induced apoptosis *in vivo* urgently need to be investigated, but much more study and experimentation still needed to be completed. Further investigations into the molecular mechanisms of LAA-induced apoptosis *in vivo* would pave the way for developing LAA into an effective anti-neoplastic drug. Study of anti-neoplastic activities of LAA are still preliminary, and strict as well as robust pre-clinical and clinical examinations should be performed to ensure the safety and efficacy for its use. With the molecular mechanisms of LAA-induced anti-tumour activities in both *in vitro* and *in vivo* gradually clarified, the GNA-related lectin family can become a potential anti-neoplastic drug for future cancer therapeutics.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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