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Methanolic Extract Isolated from Root of *Lycoris aurea* Inhibits Cancer Cell Growth and Endothelial Cell Tube Formation *In Vitro*

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In this study, we investigated the effect of methanolic extract isolated from the root of *Lycoris aurea* (LA) on the growth of cancer cells and the tube formation activity of endothelial cells. Various cancer cells were treated with LA at doses of 0.3, 1, 3, 10 or 30 μg/m*l* and LA significantly suppressed the growth of several cancer cell lines, including ACHN, HCT-15, K-562, MCF-7, PC-3 and SK-OV-3, in a dose-dependent manner. We also found that LA induced cell cycle arrest at G2/M phase in ACHN renal cell adenocarcinoma cells. Further study demonstrated that LA concentration-dependently inhibited the tube formation, which is a widely used *in vitro* model of reorganization stage of angiogenesis, in human umbilical vein endothelial cells. Collectively, these results show that LA inhibits the growth of cancer cells and tube formation of endothelial cells and the growth-inhibitory effect of LA might be mediated, at least in part, by blocking cell cycle progression.

***Key words*:** *Lycoris aurea*, Cancer, Growth, Cell cycle, Tube formation

INTRODUCTION

A tumor is an abnormal growth of body tissue. Cancer is a term for a large group of diseases, in which cells become abnormal and divide uncontrollably. Cancer cells may invade nearby parts of the body and also spread to other parts of the body through the lymphatic system or bloodstream. Cancer is one of the major public health problems in the world and it has been reported that cancer is responsible for 25% of all deaths in the United States (Siegel *et al.*, 2011). At present, the most common cancer occurring in men is prostate cancer and in women is breast cancer (Siegel *et al.*, 2011). Both environmental and genetic factors are involved in the development of cancer, but the contribution of environmental factors, such as tobacco smoking, diet, infection, radiation, stress and environmental pollutants, is considered as an important cause of cancer (Anand *et al.*, 2008). A variety of treatment options for cancer patients exist, including chemotherapy, radiation therapy, surgery and immunotherapy, and various alternative treatment methods, such as herbal preparations, acupuncture, electrical stimulation and strict dietary regimens, are also used.

As mentioned above, cancer cells proliferate uncontrollably. As in the case of normal cells, the proliferation of cancer cells also progresses through the four phases of cell cycle, G1, S, G2 and M. The cell cycle progression is controlled by cyclin-dependent kinases (CDK) and associated CDK inhibitors (Banerjee *et al.*, 2011). In normal cells, the cell proliferation and cell cycle progression are strictly controlled and regulated by multiple factors, such as external growth factors and growth-inhibitory signals, to prevent uncontrolled proliferation (Malumbres and Barbacid, 2009). However, cancer cells proliferate in the absence of growth signals and/or loose response to growth-inhibitory signals (Morales *et al.*, 2002). Therefore, the regulation of cell cycle has been considered as a target for cancer therapeutics and a variety of anti-cancer agents targeting cell cycle regulation, including CDK inhibitors, Aurora kinase inhibitors and Polo-like kinase inhibitors, have been developed and evaluated by pre-clinical and clinical testing (Dibb and Ang, 2011).

Angiogenesis is a physiological process involving the growth of blood vessels from the existing vasculature. As

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cancer grows, the development of new blood vessel is essential to maintain an adequate blood supply. Therefore, cancer cells produce various mediators of angiogenesis, such as fibroblast growth factor and vascular endothelial growth factor, to stimulate a formation of new blood vessels (Goto *et al.*, 1993; Itoh and Ornitz, 2011). Angiogenesis is also known as a critical process required for metastasis of cancer cells to other parts of the body (Morales *et al.*, 2002).

Lycoris aurea, also called as a golden spider lily, is a species in the Amaryllidaceae family and found in eastern Asia, mainly Korea, China and Japan. Although other plants in Amaryllidaceae family, such as *Narcissus tazetta* var. chinensis, have been known to exert anti-fungal, anti-cancer and immune-modulating activities, the pharmacological activity of *Lycoris aurea* has not been reported (Chu and Ng, 2004; Liu *et al.*, 2006). In the present study, we examined the effect of methanolic extract isolated from the root of *Lycoris aurea* (LA) on cancer cell growth and endothelial cell tube formation *in vitro*. We also investigated the effect of LA on cell cycle progression in cancer cells.

MATERIALS AND METHODS

Reagents, cell lines and cell culture. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. ACHN (ATCC #: CRL-1611), HCT-15 (ATCC #: CCL-225), K-562 (ATCC #: CCL-243), PC-3 (ATCC #: CRL-1435) and SK-OV-3 (ATCC #: HTB-77) cell lines were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) and MCF-7 cell line was cultured in minimum essential medium (MEM; Gibco BRL). All media were supplemented with 10% fetal bovine serum (Hyclone; Logan, UT, USA), 2 mM L-glutamine, 100 U/m*l* penicillin and 100 mg/m*l* streptomycin. Cells were maintained at 37°C in 5% CO_2 humidified air.

Plant material and preparation of extracts. LA (reference No. 007-004) was provided by Korea Plant Extract Bank in Korea Research Institute of Bioscience and Biotechnology. The roots of *Lycoris aurea* were collected from Cheju Island, Korea. The botanical authentication was carried out by Dr. Chan-Su Kim, a plant taxonomist at Korea Forest Research Institute (KFRI). Samples were washed, dried and cut into pieces. HPLC grade methanol was used as a solvent and the extraction was performed using ultrasonic cleaner (Branson Ultrasonics corporation, Danbury, CT, USA) at room temperature for 3 days. After extraction, the solutions were filtered and the solvents were evaporated under vacuum. Samples were dissolved in dimethyl sulfoxide (DMSO) and diluted using culture media immediately before experiments.

Cell viability assay. Cells were plated at 9×10^3 cells/well in 96-well plates, incubated overnight, and treated with vehicle (DMSO) or LA (0.3, 1, 3, 10 or 30 μg/m*l*) for 48 h. Cell proliferation assays were performed using a Cell Proliferation Kit II (XTT; Roche Applied Science; Mannheim, Germany) according to the manufacturer's instructions. The XTT labeling mixture was prepared by mixing 50 volumes of 1 mg/m*l* sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (in RPMI 1640) with 1 volume of 0.383 mg/m*l* of N-methyldibenzopyrazine methyl sulfate (in PBS). This XTT labeling mixture was added to the cultures and incubated for 2 h at 37°C. Absorbance was measured at 490 nm with a reference wavelength at 650 nm.

Cell cycle analysis. Cell cycle analysis was performed using a previously-described protocol (Krishan, 1975). Briefly, cells were plated at 3×10^6 cells/dish in 100-mm dishes, incubated overnight, and treated with vehicle or LA (1 or 10 μg/m*l*). After 24 h, cells were harvested and washed with PBS. After cell counting with trypan blue staining, 1×10^6 cells were pelleted and fixed in 70% ethanol at 4°C for 1 h. Then, cells were resuspended 1 m*l* of Krishan's buffer (0.1% sodium citrate, 0.02 mg/m*l* RNase A, 0.3% Triton X-100, and 50 μg/m*l* propidium iodide, pH 7.4) for 1 h at 4°C. Samples were centrifuged, resuspended in 1 m*l* of PBS buffer, and analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson; San Jose, CA, USA). Data were collected for 10,000 events. The Modfit LT program (Verity Software House; Maine, ME, USA) was used for cell cycle modeling.

Tube formation assay. The assays were performed as described previously with slight modifications. The wells of 96-well plate were coated with ice-cold BD Matrigel™ matrix gel solution. After polymerizing the matrix at 37°C, human umbilical vein endothelial cells (HUVECs) were seeded onto the polymerized EC matrix at a concentration of 1×10^4 cells in 180 μ*l* of EMB-2 media and 20 μ*l* of diluted LA was immediately added. The tubule branches were photographed after 16 h of incubation.

Statistical analysis. The results are expressed as mean ± S.D. One-way ANOVA and Dunnett's *t*-test was used for multiple comparisons using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). The criterion for statistical significance was set at $p < 0.01$.

RESULTS AND DISCUSSION

Cancer is a major cause of death around the world and a variety of drugs are developed and currently under development to solve this problem. Although most of big pharmaceutical companies are dedicated to the development of synthetic small molecule-based target-specific anti-cancer drug, alternative approaches, such as herbal medicine and

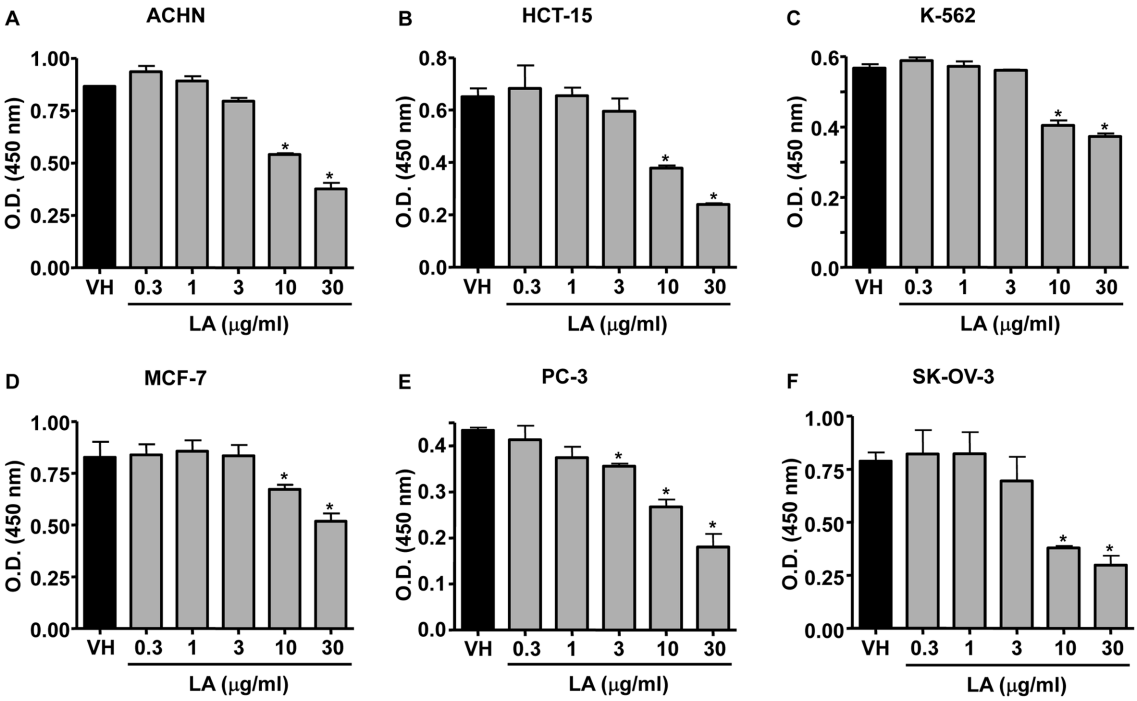

Fig. 1. Effect of LA on the growth of cancer cells. Various cancer cells (ACHN, HCT-15, K-562, MCF-7, PC-3 and SK-OV-3) were treated with vehicle (DMSO) or indicated concentrations of LA (0.3, 1, 3, 10 or 30 μg/m*l*) for 48 h. Cancer cell growth was determined using XTT assay as described in Materials and Methods. Each column shows the mean ± S.D. of triplicate determinations. * $p < 0.05$.

acupuncture, are also being investigated extensively by individual researchers. Herbal medicines and natural compounds have also attracted an increasing attention for the possibility of applying these agents for cancer chemoprevention (Tsuda *et al.*, 2004). In the present study, we investigated the effect of LA, a methanolic extract isolated from the root of *Lycoris aurea*, on the growth of cancer cells and the tube formation activity of endothelial cells.

To investigate the effect of LA on cancer cell growth, various cancer cell lines, including ACHN renal cell adenocarcinoma cell line, HCT-15 colorectal adenocarcinoma cell line, K-562 chronic myelogenous leukemia cell line, MCF-7 breast adenocarcinoma cell line, PC-3 prostatic adenocarcinoma cell line and SK-OV-3 ovarian adenocarcinoma cell line, were used. As shown in Fig. 1, LA suppressed the growth of cancer cells tested in a concentration-dependent manner. According to the GI_{50} values shown in Table 1, ACHN, HCT-15, PC-3 and SK-OV-3 cells were relatively more sensitive to LA treatment than K-562 and MCF-7 cells. In contrast to this result, the extract of *Narcissus tazetta* var. chinensis, another plant in Amaryllidaceae family, has been shown to exert a strong growth-inhibitory activity against leukemia cells, including HL-60 and K562, than other cells (Liu *et al.*, 2006). The GI_{50} values of the extract of *Narcissus tazetta* var. chinensis for these cells were about 1 μg/m*l*, whereas the GI_{50} value of LA for K-562 cells was more than 30 μg/m*l*. These results suggest that the active constituents of *Lycoris aurea* and *Narcissus tazetta* var. chinensis involved in their growth-inhibitory activity might be different although they are in the same family.

Table 1. Effect of LA on the growth of various cancer cell lines

Cell line	Origin	GI_{50} (μg/m*l*)
ACHN	Kidney	20.55
HCT-15	Colon	16.43
K-562	Blood	52.09
MCF-7	Breast	44.13
PC-3	Prostate	19.37
SK-OV-3	Ovary	13.86

Lycorine is a cytotoxic crystalline alkaloid found in *Lycoris* and *Narcissus* species. It has been reported to exert anti-cancer effects against various cancer types, such as glioblastoma, melanoma and non-small cell lung cancer (Lamoral-Theys *et al.*, 2009). However, most of the researches on the anti-cancer effects of lycorine have been focused on their effects against cancers of hematopoietic origin, such as leukemia and multiple myeloma (Liu *et al.*, 2004; Li *et al.*, 2007; Liu *et al.*, 2009; Hayden *et al.*, 2010). The results of these reports are in consistent with the results of *Narcissus tazetta* var. chinensis, suggesting that lycorine might be one of the main constituents contributing to the anti-cancer effect of *Narcissus* species. However, our results demon-

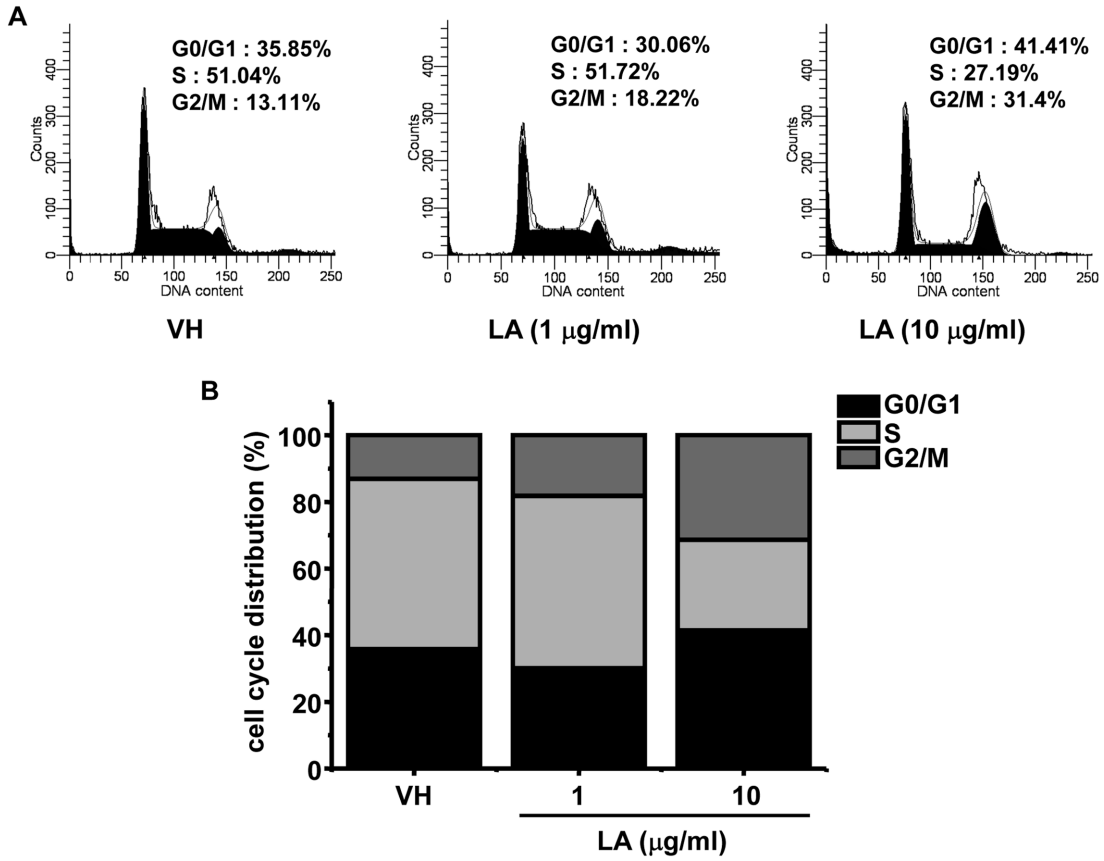

Fig. 2. Effect of LA on cell cycle distribution in ACHN cells. ACHN cells were treated with vehicle (DMSO) or indicated concentrations of LA (1 or 10 μg/m*l*) for 24 h. Cell cycle distribution was determined using flow cytometry.

strated that leukemia is one of the most resistant cell types among various cancers against LA treatment. Collectively, these results suggest that lycorine is not an active constituent involved in the anti-cancer effects of LA and other constituents might be involved in this effect. Therefore, further studies are required to elucidate the active constituents of LA and this might lead to characterization of new compounds with anti-cancer activity.

Anti-cancer activities of many previously reported anti-cancer agents are mostly mediated by induction of cell cycle arrest and/or apoptosis. Cell cycle regulation ensures the maintenance of genomic integrity of cells. Cell cycle is regulated mainly at two major checkpoints, G1 checkpoint and G2 checkpoint. G1 checkpoint makes the key decision of whether the cell should divide or delay division and G2 checkpoint triggers the start of mitosis. It has been well-known that cytotoxic agents and/or DNA-damaging agents induce cell death by arresting the cell cycle (Kaina, 2003; Nakanishi *et al.*, 2006). Therefore, we also investigated the effect of LA on cell cycle regulation. As shown in Fig. 2A and Fig. 2B, LA induced cell cycle arrest at G2/M phase in ACHN renal cell carcinoma cells. Cell cycle distribution of untreated ACHN cells at G2/M phase was about 13%, but this was increased more than two folds after LA (10 μg/m*l*) treatment. These results suggest that the anti-cancer effect of LA might be mediated, at least in part, by arresting cell cycle in cancer cells.

Angiogenesis is a critical process associated with tumor growth and metastasis. It has been reported that tumors cannot grow beyond certain size, about 1~2 mm^3, without angiogenesis because of a lack of oxygen and other essential nutrients (McDougall *et al.*, 2006). Angiogenesis is also required for cancer cells to be carried to a distant site, called metastasis. Therefore, angiogenesis has been an attractive target for cancer treatment. Several anti-angiogenic drugs, including Macugen and Avastin, were developed and approved by FDA for the past decades and numerous drugs targeting angiogenesis are under development for cancer treatment. In the present study, we also examined the effect of LA on tube formation of HUVECs, a well-known *in vitro* model of angiogenesis. As shown in Fig. 3, HUVECs seeded on matrigel-coated plates formed tubular structure. However, LA concentration-dependently suppressed the tube formation of HUVECs. In particular, treatment with 10 μg/m*l* of LA completely abolished cell alignments and tubular structure formation in HUVECs. These results suggest that LA

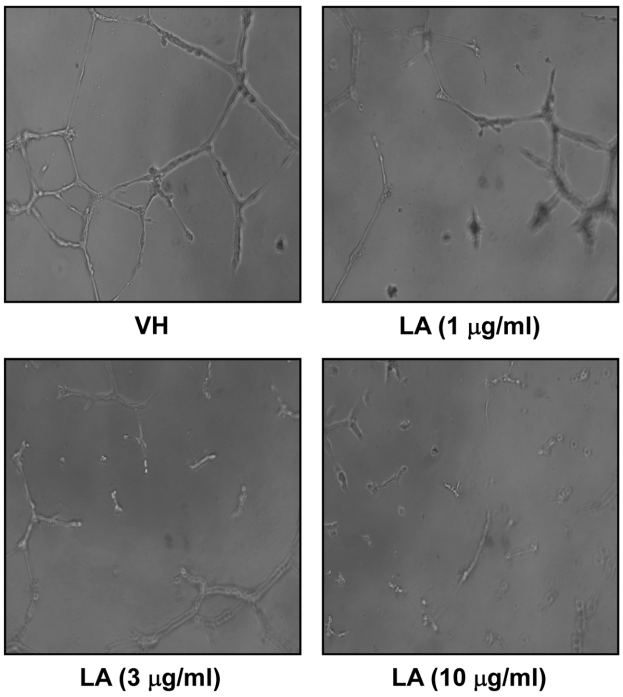

Fig. 3. Effect of LA on tube formation in HUVECs. HUVECs were seeded on matrigel-coated plates and treated with vehicle (DMSO) or indicated concentrations or LA (1, 3 or 10 μg/ml) for 16 h. The capillary tube formation was observed using a light microscope and photographed.

might exert an anti-angiogenic effect and some of the constituents of LA might be developed as anti-angiogenic agents, although active constituents of LA involved in its anti-angiogenic effects need to be further characterized.

In summary, we demonstrated that LA inhibited the growth of cancer cells and tube formation of endothelial cells and the growth-inhibitory effect of LA might be mediated, at least in part, by inducing cell cycle arrest. Our results suggested that LA might be used as an agent for the treatment and chemoprevention of cancer.

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REFERENCES

Anand, P., Kunnumakkara, A.B., Sundaram, C., Harikumar, K.B., Tharakan, S.T., Lai, O.S., Sung, B. and Aggarwal, B.B. (2008). Cancer is a preventable disease that requires major lifestyle changes. *Pharm. Res.*, **25**, 2097-2116.

Banerjee, S., Kong, D., Wang, Z., Bao, B., Hillman, G.G. and Sarkar, F.H. (2011). Attenuation of multi-targeted proliferation-linked signaling by 3,3′-diindolylmethane (DIM): from bench to clinic. *Mutat. Res.*, **728**, 47-66.

Chu, K.T. and Ng, T.B. (2004). First report of a glutamine-rich antifungal peptide with immunomodulatory and antiproliferative activities from family Amaryllidaceae. *Biochem. Biophys. Res. Commun.*, **325**, 167-173.

Dibb, M. and Ang, Y.S. (2011). Targeting the cell cycle in esophageal adenocarcinoma: an adjunct to anticancer treatment. *World J. Gastroenterol.*, **17**, 2063-2069.

Goto, F., Goto, K., Weindel, K. and Folkman, J. (1993). Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels. *Lab. Invest.*, **69**, 508-517.

Hayden, R.E., Pratt, G., Drayson, M.T. and Bunce, C.M. (2010). Lycorine sensitizes CD40 ligand-protected chronic lymphocytic leukemia cells to bezafibrate- and medroxyprogesterone acetate-induced apoptosis but dasatanib does not overcome reported CD40-mediated drug resistance. *Haematologica*, **95**, 1889-1896.

Itoh, N. and Ornitz, D.M. (2011). Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease. *J. Biochem.*, **149**, 121-130.

Kaina, B. (2003). DNA damage-triggered apoptosis: critical role of DNA repair, double-strand breaks, cell proliferation and signaling. *Biochem. Pharmacol.*, **66**, 1547-1554.

Krishan, A. (1975). Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. *J. Cell Biol.*, **66**, 188-193.

Lamoral-Theys, D., Andolfi, A., Van Goietsenoven, G., Cimmino, A., Le Calve, B., Wauthoz, N., Megalizzi, V., Gras, T., Bruyere, C., Dubois, J., Mathieu, V., Kornienko, A., Kiss, R. and Evidente, A. (2009). Lycorine, the main phenanthridine Amaryllidaceae alkaloid, exhibits significant antitumor activity in cancer cells that display resistance to proapoptotic stimuli: an investigation of structure-activity relationship and mechanistic insight. *J. Med. Chem.*, **52**, 6244-6256.

Li, Y., Liu, J., Tang, L.J., Shi, Y.W., Ren, W. and Hu, W.X. (2007). Apoptosis induced by lycorine in KM3 cells is associated with the G0/G1 cell cycle arrest. *Oncol. Rep.*, **17**, 377-384.

Liu, J., Hu, W.X., He, L.F., Ye, M. and Li, Y. (2004). Effects of lycorine on HL-60 cells via arresting cell cycle and inducing apoptosis. *FEBS Lett.*, **578**, 245-250.

Liu, J., Li, Y., Ren, W. and Hu, W.X. (2006). Apoptosis of HL-60 cells induced by extracts from *Narcissus tazetta* var. *chinensis*. *Cancer Lett.*, **242**, 133-140.

Liu, X.S., Jiang, J., Jiao, X.Y., Wu, Y.E., Lin, J.H. and Cai, Y.M. (2009). Lycorine induces apoptosis and down-regulation of Mcl-1 in human leukemia cells. *Cancer Lett.*, **274**, 16-24.

Malumbres, M. and Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm. *Nat. Rev. Cancer*, **9**, 153-166.

McDougall, S.R., Anderson, A.R. and Chaplain, M.A. (2006). Mathematical modelling of dynamic adaptive tumour-induced angiogenesis: clinical implications and therapeutic targeting strategies. *J. Theor. Biol.*, **241**, 564-589.

Morales, C.P., Souza, R.F. and Spechler, S.J. (2002). Hallmarks of cancer progression in Barrett's oesophagus. *Lancet*, **360**, 1587-1589.

Nakanishi, M., Shimada, M. and Niida, H. (2006). Genetic instability in cancer cells by impaired cell cycle checkpoints. *Cancer Sci.*, **97**, 984-989.

Siegel, R., Ward, E., Brawley, O. and Jemal, A. (2011). Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA. Cancer J. Clin.*, **61**, 212-236.

Tsuda, H., Ohshima, Y., Nomoto, H., Fujita, K., Matsuda, E., Iigo, M., Takasuka, N. and Moore, M.A. (2004). Cancer prevention by natural compounds. *Drug Metab. Pharmacokinet.*, **19**, 245-263.