

ANTI-CANCER EFFECT OF LIRIODENINE ON HUMAN LUNG CANCER CELLS

Hui-Chiu Chang, Fang-Rong Chang,¹ Yang-Chang Wu,¹ and Yung-Hsiung Lai²
Departments of Physiology and ²Internal Medicine and ¹Graduate Institute of
Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan.

Our previous work demonstrated that lirioidenine, an isoquinoline alkaloid isolated from plant species of many genera, exhibits a potent cytotoxic effect on various types of human cancer cells. In this study, we investigated the effect of lirioidenine on the growth and viability of human lung cancer cells and addressed the underlying mechanism of action. We found that lirioidenine suppressed proliferation of A549 human lung adenocarcinoma cells in a dose- and time-dependent manner. Flow cytometric analysis showed that lirioidenine blocked cell cycle progression at the G2/M phase. Induction of G2/M arrest by lirioidenine was accompanied by reduction of G1 cyclin (D1) and accumulation of G2 cyclin (B1). *In vitro* kinase activity assay demonstrated that the enzymatic activity of the cyclin B1/cyclin-dependent kinase 1 complex was reduced in lirioidenine-treated cells. More importantly, incubation with lirioidenine led to activation of caspases and apoptosis in A549 cells. The apoptosis-inducing activity of lirioidenine was more apparent when cells were cultured under serum-free conditions. Our results indicate that lirioidenine exerts potent anti-proliferative and apoptosis-inducing effects on human lung cancer cells.

Key Words: lirioidenine, cyclin, cyclin-dependent kinase, poly(ADP-ribose) polymerase, apoptosis
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Lirioidenine (8H-benzo[g]-1,3-benzodioxolo[6,5,4-de]-quinolin-8-one), a cytotoxic isoquinoline alkaloid, has been isolated from plant species of many genera [1]. It has many biologic activities, including anti-platelet, anti-fungal, and anti-microbial actions [2-4]. Additionally, it has anti-arrhythmic efficacy in cardiac tissues and exerts a growth-inhibitory effect on cultured cancer cells [5-8]. Our previous study demonstrated that lirioidenine has potent cytotoxicity against a number of cancer cell lines [9]. However, the molecular mechanism by which it suppresses cancer cell proliferation is not totally understood.

Lung cancer is one of the most lethal malignancies in many countries. In addition, many lung cancer cells show significant resistance to chemotherapeutic drugs. Therefore, development of new therapeutic drugs for lung cancer is clinically important. A recent study has demonstrated that deregulation of cell cycle control is frequently found in cancer cells [10]. Three distinct classes of proteins, cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CDKIs), are involved in the regulation of cell cycle progression [11,12]. In mammalian cells, G1 cyclins (D1, D2, D3, and E) and their partner CDKs control the G1/S transition of the cell cycle. On the other hand, mitotic cyclins (mainly B-type cyclins) regulate the progression of the cell cycle from the G2 to M phase. Cyclins and CDKs are positive cell cycle regulators, and up-regulation of these two gene families is frequently found in human cancers. For example, amplification or over-expression of cyclin D1 or E has been reported in esophageal, breast, lung, and colon

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Address correspondence and reprint requests to: Dr. Yung-Hsiung Lai, Department of Internal Medicine, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung 807, Taiwan.
E-mail: yhlai@kmu.edu.tw

cancers [13–16]. Deregulation of CDK2 and 4 was detected in colon cancer and glioma [17,18]. Conversely, CDKIs are negative cell cycle regulators, and down-regulation of these inhibitory proteins is a general phenomenon observed in human cancers [19,20].

Many natural products and Chinese medicinal herbs are promising biologic modifiers in cancer treatment. In the present study, we tested the anti-cancer activity of liriodenine on lung cancer cells and clarified the molecular mechanism of action.

MATERIALS AND METHODS

Drug and agents

Liriodenine was prepared according to the procedures described previously [21]. Propidium iodide (PI) and histone H1 were obtained from Sigma Chemical Co (St Louis, MO, USA). Annexin-V was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Anti-human cyclin D1 polyclonal antibody was purchased from Calbiochem (San Diego, CA, USA). Anti-human cyclin B1 and CDK1 antibodies were from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Poly(ADP-ribose) polymerase (PARP) monoclonal antibody was purchased from Clontech (Palo Alto, CA, USA).

Cell culture

A549 human lung adenocarcinoma cells were cultured in Dulbecco's modified Eagle medium/F12 medium, supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 µg/mL streptomycin, and 100 IU/mL penicillin in 5% CO₂ at 37°C. Cells were incubated in 10% FCS or serum-free medium containing various doses of liriodenine for different time intervals and were harvested for analysis.

Western blot analysis

After various treatments, cells were washed with ice-cold phosphate-buffered saline (PBS), and harvested in a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mg/mL aprotinin, 2 µg/mL pepstatin A, and 1 mM phenylmethylsulfonyl fluoride) for 20 minutes on ice, and centrifuged at 12,000g for 20 minutes at 4°C. Protein concentration in cell lysates was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Company, Rockford, IL, USA). The lysates (20 µg protein/sample) were subjected to 12% or 7.5% sodium dodecyl sulfate-polyacrylamide gel elec-

trophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose filters, and the blots were blocked overnight in 5% non-fat milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 0.05% Tween 20) at 4°C. The blots were then probed with various antibodies for 1 hour at room temperature. After extensive washing in TBST buffer, peroxidase-conjugated secondary antibody was added with the blocking buffer for another hour. The blots were developed using the enhanced chemiluminescence (ECL) system (Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, UK) as described previously [22].

Trypan blue exclusion assays

Control or liriodenine-treated cells were harvested after 48 hours' incubation with drug, and subjected to trypan blue exclusion assay to evaluate cell viability, as described previously [23].

Measurement of cell cycle distribution and apoptosis

Measurement of cell cycle distribution was performed as described previously [24]. Translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer is found in the early stage of apoptosis and is a useful biomarker for the detection of apoptosis [25]. We investigated the appearance of PS on the outer leaflet of cell membranes by annexin-V staining. In addition, cells were also stained with PI to differentiate apoptotic cells from necrotic cells. PI-positive and annexin-V-positive stained cells (necrotic cells) were gated out in flow cytometric analysis. Control or liriodenine-treated cells were washed with ice-cold buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl₂) and pelleted by centrifugation at 400g for 10 minutes. Cell pellets were fixed in 4% paraformaldehyde and resuspended in 100 µL of staining solution (2 µL annexin-V and 2 µL of 50 µg/mL PI in 100 µL HEPES buffer), and incubated for 10 to 15 minutes at room temperature in darkness. Annexin-V or PI fluorescence intensities were investigated using fluorescence-activated cell sorter (FACSort) flow cytometry (Becton Dickinson, San Jose, CA, USA), and the results were analyzed using CellFIT™ (Becton Dickinson) software. Ten thousand cells were evaluated in each sample.

In vitro kinase activity assays

CDK1 was immunoprecipitated from control or liriodenine-treated cells, and an *in vitro* kinase assay was performed according to the procedures of our previous study using histone H1 as a substrate [24].

RESULTS

Effect of liriodenine on cell cycle distribution of lung cancer cells

As shown in Table 1, there were fewer cells in the G0/G1 phase after liriodenine treatment for 24 hours, while the number of cells in the G2/M phase increased. These results suggest that liriodenine induces G2/M arrest in A549 cells. Since cell cycle progression is controlled via periodic changes in the cyclin level in cells, we investigated whether liriodenine might modulate the expression of cyclins to block proliferation of A549 cells. As shown in Figure 1, a persistent reduction in G1 cyclin (D1) expression was observed in liriodenine-treated cells. This is consistent with the results of flow cytometric analysis, which indicated that the number of cells in the G0/G1 phase was reduced after liriodenine treatment. Conversely, liriodenine induced accumulation of G2 cyclin (B1) under the same experimental conditions. The major partner of cyclin B1 in mammalian cells is CDK1 (or CDC2), so we performed an *in vitro* kinase assay to study CDK1 activity. Liriodenine inhibited CDK1 activity in a time-dependent manner (Figure 2). These results indicate that, although cyclin B1 expression was increased in liriodenine-treated cells, the enzymatic activity of the cyclin B1/CDK1 complex was suppressed, leading to G2/M arrest.

Apoptosis-inducing activity of liriodenine

In addition to growth inhibition, we found that liriodenine exhibited cytotoxicity on A549 cells. As shown in Table 2, there was a dose-dependent increase in the number of dead cells after liriodenine treatment. Additionally, cells cultured under serum-free conditions were more susceptible to liriodenine treatment. We next addressed whether liriodenine caused cell death via induction of apoptosis.

Table 1. Effect of liriodenine on cell cycle distribution of A549 human lung cancer cells assayed by flow cytometric analysis*

	Cell cycle distribution, %		
	G0/G1	S	G2/M
Control	50.8 ± 3.2	28.1 ± 2.1	21.1 ± 2.5
2 µM	41.9 ± 4.1	35.9 ± 3.5	22.2 ± 1.8
20 µM	29.1 ± 3.4	36.0 ± 3.5	34.9 ± 1.2

*Cells were cultured in 10% fetal calf serum medium and treated with liriodenine for 24 hours. Results are expressed as mean ± standard error ($n = 3$).

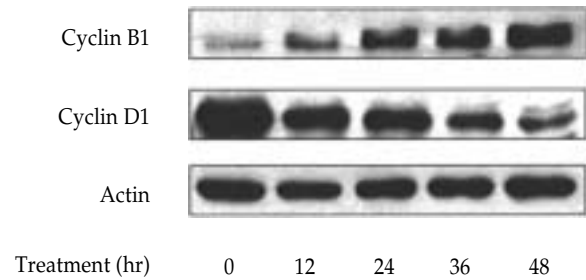


Figure 1. Modulation of the expression of cyclin B1 and D1 by liriodenine in A549 human lung cancer cells. Cells were cultured in 10% fetal calf serum medium and incubated with 20 µM liriodenine for different times. Cells were harvested in a lysis buffer, and equal amounts of cellular proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the expressions of cyclin B1 and D1 were examined by Western blot analysis.

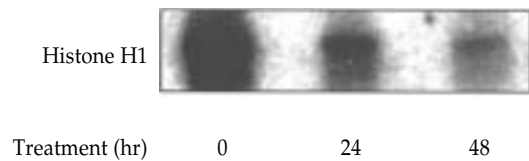


Figure 2. Liriodenine suppresses cyclin B1/cyclin-dependent kinase (CDK) 1 activity. Cells were cultured in 10% fetal calf serum medium and incubated with 20 µM liriodenine for different times. Cells were harvested in a lysis buffer, and equal amounts of cellular proteins were subjected to immunoprecipitation by CDK1 antibody. The *in vitro* kinase assay was performed using histone H1 as substrate.

The number of PI-negative and annexin-V-positive cells (apoptotic cells) was increased after liriodenine treatment (Figure 3). Liriodenine treatment at 20 µM and 50 µM cells caused apoptosis in 67% and 80% of cells, respectively. We conclude that the cytotoxic activity of liriodenine on human lung cancer cells is mediated mainly via induction of apoptosis.

To further confirm our conclusion, we studied the involvement of caspases in liriodenine-induced apoptosis by investigating the expression and degradation of a nuclear protein, PARP. This protein is the first identified *in vivo* substrate for various caspases; proteolytic cleavage of PARP from a 116 kDa polypeptide to an 85 kDa fragment is a typical marker for the onset of apoptosis. As shown in Figure 4, degradation of PARP was observed in a time-

Table 2. Cytotoxic effect of liriodenine on A549 human lung cancer cells evaluated by trypan blue exclusion assay*

	10% FCS		Serum-free	
	24 hr, n = 3	48 hr, n = 3	24 hr, n = 3	48 hr, n = 3
Control	3.8%	3.7%	5.5%	7.4%
2 μ M	7.8%	12.9%	14.6%	29.0%
20 μ M	22.7%	33.3%	32.9%	73.8%
50 μ M	30.2%	42.9%	76.5%	82.5%

*Results are expressed as the mean of two independent experiments, and are shown as the percentage of dead cells.

dependent fashion in liriodenine-treated A549 cells. These results support the notion that liriodenine induces caspase activation and apoptosis in A549 cells.

DISCUSSION

In this study, we provide the first evidence that liriodenine, an isoquinoline alkaloid isolated from a variety of plant species, may inhibit proliferation of lung cancer cells via inhibition of cell cycle progression and induction of apoptosis. The first novel finding is that liriodenine induces

G2/M arrest via inhibition of the kinase activity of the cyclin B1/CDK1 complex. Regulation of the G2 to M phase transition of the cell cycle is complicated. In mammalian cells, cyclin B1 synthesis is increased from the end of the S phase due to cell cycle-regulated transcription. Cyclin B1 then associates with CDK1 to form a protein complex that participates in the control of the G2/M phase. However, it should be noted that the cyclin B1/CDK1 complex is not active, and the enzymatic activity of this complex is regulated by a number of phosphorylation and dephosphorylation events. Phosphorylation of threonine 161 is required for the activation of the cyclin B1/CDK1 complex and is mediated by CDK activating kinase [26]. Moreover, dephosphorylation of two negative regulatory sites, threonine 14 and tyrosine 15, by CDC25 protein phosphatase is also needed for cyclin B1/CDK1 activation [27,28]. Some drugs or apoptotic stimuli may inhibit cyclin B1 expression and CDK1 activity to induce G2/M arrest [29,30]. On the contrary, other external signals may stimulate cyclin B1/CDK1 activity to trigger G2/M arrest [31,32]. Our current results demonstrate that

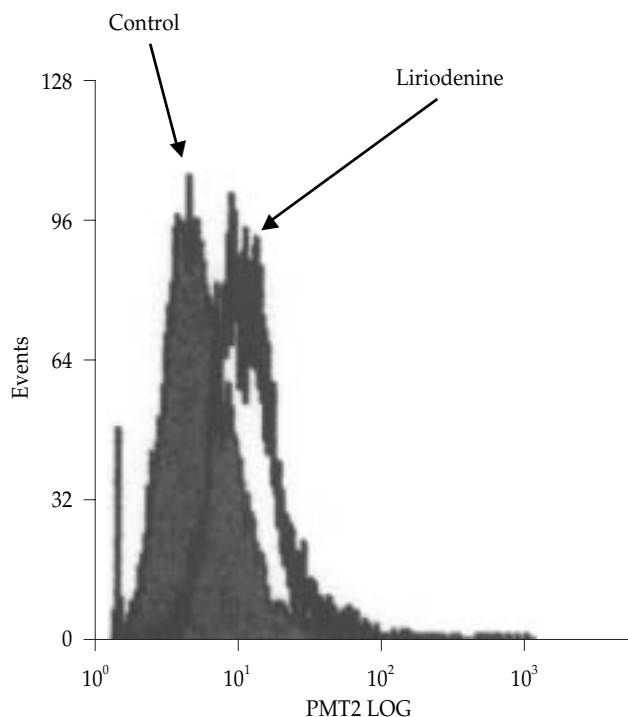


Figure 3. Induction of apoptosis by liriodenine. Cells were cultured in serum-free medium and incubated with 50 μ M liriodenine for 48 hours and then harvested. Annexin-V staining shows apoptotic cells. A typical figure for flow cytometric analysis is shown.

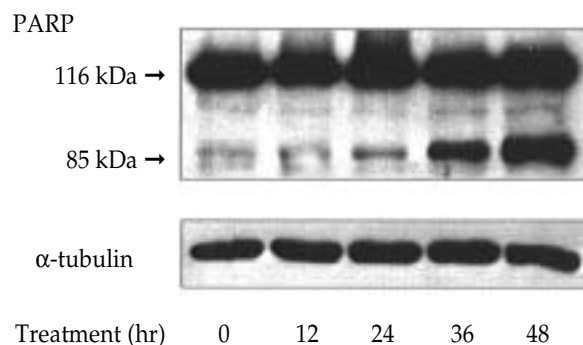


Figure 4. Induction of poly(ADP-ribose) polymerase (PARP) degradation by liriodenine. Cells were cultured in serum-free medium and incubated with 50 μ M liriodenine for different times. Equal amounts of cellular proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and PARP degradation from its native form (116 kDa) to the degraded form (85 kDa) was detected by Western blot analysis.

cyclin B1 accumulated after liriodenine treatment. However, the kinase activity of the cyclin B1/CDK1 complex was potently inhibited. It is possible that liriodenine may affect the phosphorylation and dephosphorylation of this protein complex to induce G2/M arrest. Further work is needed to clarify this issue.

Another critical finding of this study is that liriodenine may induce caspase activation and apoptosis in human lung cancer cells. Apoptosis is an evolutionarily conserved process by which cells systematically inactivate and degrade their own structural and functional components to complete their own demise. It is activated by different extracellular stimuli through a genetically defined program. Apoptosis may be divided into three distinct stages: commitment, in which cells receive a potentially lethal apoptotic stimulus and become irreversibly committed to death; execution, during which the major structural changes occur; and clearance, when cellular remnants are removed by phagocytosis. Since cells that have undergone apoptosis are recognized and removed by macrophages without induction of a severe inflammatory reaction, agents that can specifically activate the apoptotic signaling pathways in cancer cells are expected to be ideal anti-tumor agents. According to our results, liriodenine could effectively trigger caspase activation and PARP degradation and induce apoptosis in A549 lung cancer cells. These results suggest that liriodenine may be useful for the treatment or chemoprevention of lung cancer.

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Liriodenine 對人類肺癌細胞抗癌效果之研究

張慧秋¹ 張芳榮² 吳永昌² 賴永勳³

高雄醫學大學 ¹生理學科 ²天然藥物研究所 ³內科學

我們先前的研究發現多種植物中所含的生物鹼 **liriodenine** 可毒殺多種人類癌細胞，本研究中我們探討 **liriodenine** 對人類肺癌細胞生長與存活之影響及其作用之機制，我們發現 **liriodenine** 可明顯抑制 **A549** 肺癌細胞之生長，流式細胞儀分析顯示細胞週期進行被阻斷在 **G2/M** 期，同時 **liriodenine** 處理降低 **G1** 週期素 (**D1**) 的表現，但 **G2** 週期素 (**B1**) 則明顯增加。試管內蛋白激酶活性分析發現 **cyclin B1/CDK1** 活性被 **liriodenine** 顯著抑制。更重要的，**liriodenine** 會誘發 **A549** 細胞凋亡，當細胞培養於低濃度血清時，其毒殺作用更明顯。綜言之，我們結果顯示 **liriodenine** 對肺癌細胞具有抑制生長與誘發凋亡之作用。

關鍵詞：liriodenine，細胞週期素，細胞週期素依賴型蛋白激酶，
poly(ADP-ribose) polymerase，細胞凋亡
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通訊作者：賴永勳醫師

高雄醫學大學內科學

高雄市 807 十全一路 100 號