Liriodenine enhances radiosensitivity in esophageal cancer ECA-109 cells by inducing apoptosis and G₂/M arrest

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Received July 9, 2016; Accepted February 19, 2018
DOI: 10.3892/ol.2018.9253

Abstract. At present, chemotherapy and radiotherapy represent the primary modalities of treatment for patients with unresectable esophageal squamous cell carcinoma (ESCC). However, the outcome of patients remains poor owing to radioresistance. The present study aimed to determine the radiosensitizing effect of liriodenine, an aporphine alkaloid derived from Enicosanthellum pulchrum, and investigating the underlying mechanisms in ESCC, using the esophageal cancer ECA-109 cell line. Cellular proliferation was evaluated using the Cell Counting kit-8 assay. Colony formation assay was performed to characterize the radiosensitive effects of liriodenine on ECA-109 cells, and flow cytometry was used to detect the percentage of cells undergoing apoptosis. An immunofluorescence assay was utilized to evaluate the DNA damage repair ability. Western blotting was used to assess the protein levels of caspase-3, B cell lymphoma-2 (Bcl-2) and Bcl-2-associated X (Bax). Liriodenine dose-dependently inhibited ECA-109 cell viability. The clonogenic survival assay demonstrated that liriodenine increased the radiosensitivity of ESCC cells, with a sensitization enhancement ratio of 1.11-1.69. The results of flow cytometry demonstrated that liriodenine induced apoptosis and G₂/M arrest. The immunofluorescence assay revealed that liriodenine delays DNA damage repair. The upregulation of Bax and Caspase-3, and the suppression of Bcl-2 confirmed that apoptosis was occurring. Liriodenine radiosensitizes ECA-109 cells by inducing apoptosis and G₂/M arrest. The findings of the present study indicated that liriodenine may represent an anticancer agent with promising potential for the treatment of ESCC.

Introduction

Esophageal cancer is the eighth most common malignant tumor and the sixth leading cause of cancer-associated mortalities in the world (1-3). Esophageal squamous cell carcinoma (ESCC) is the major histological subtype of esophageal cancer, representing 80% of the incidence of this disease (2). The 5-year survival rate remains poor, at 15-25% following diagnosis (4,5). Although radiotherapy remains the primary treatment modality for unresectable ESCC, this approach is limited by the significant radioresistance exhibited by the neoplasm. As such, the identification of a novel effective anticancer agent may be essential for improving treatment in ESCC by radiation therapy.

8H-benzo (g), 3-benzoxololo (6,5,4-de)-quinolin-8-one (liriodenine) is a cytotoxic isoquinoline alkaloid that has been isolated from plant species of numerous genera, including Fissistigma glaucescens, Annona glabra and Liriodendron tulipifera (6-8). Previous studies have highlighted the diverse biological properties of liriodenine, including anti-platelet, mutagenic, anti-microbial, anti-fungal and anti-arrhythmic effects (9-11). In addition, in vitro studies have identified that liriodenine exhibits growth inhibition on cancer cell lines (12,13). Specifically, Li et al (12) demonstrated that liriodenine could significantly enhance antitumor effects via upregulation of tumor protein p53 expression, and that this compound could significantly inhibit migration and induce apoptosis in laryngocarcinoma HEP-2 cells. Furthermore, liriodenine led to the suppression of proliferation and concomitant induction of apoptosis in human lung adenocarcinoma A549 cells (13). Liriodenine also induced a G₁ cell cycle arrest and repression of DNA synthesis in HepG2 and SK-HeP-1 cells (14).

Taken together, these findings highlight the significant therapeutic potential of liriodenine. However, to the best of our knowledge, no study has yet evaluated the combinational effects of liriodenine with radiation therapy. The present study evaluated the use of liriodenine as an anticancer agent, particularly for human ESCC, and characterized the molecular mechanism through which liriodenine acts.
Materials and methods

Cell culture and reagents. The ESCC ECA-109 cell line was obtained from the Type Culture Collection of the Chinese Academy of Science (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Little Chalfont, UK), in an incubator with an atmosphere of 5% CO\(_2\) at 37°C. Liriodenine powder was obtained from SenBeiJia Biological Technology Co., Ltd. (Nanjing, China) dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 1x10\(^{-6}\)μM and subsequently added to cell culture media for in vivo experiments. B cell lymphoma-2 (Bcl-2; 15071; dilution, 1:500), Bcl-2-associated X protein (Bax; 5023; dilution, 1:500) and Caspase-3 (9665; dilution, 1:500) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), while antibodies targeting GAPDH (5174; dilution, 1:5000) were purchased from BioWorld Technology, Inc. (St. Louis Park, MN, USA). Biotinylated goat anti-rabbit IgGs (111-035-003), biotinylated anti-mouse IgG (715-097-003), fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (715-097-003), biotinylated anti-mouse IgG (715-035-003) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (715-097-003), biotinylated anti-mouse IgG (715-035-003) were purchased from Jackson ImmunoResearch Laboratories, Inc.

Cell proliferation assay. A total of 5x10\(^4\) cells/well were seeded on 96-well culture plates. After 24 h, the cells were treated with liriodenine at 37°C at various concentrations (0, 0.1, 0.5, 1, 2.5, 5, 7.5, 10 and 20 μM). A Cell Counting kit-8 (OBI Technology, Corp., Ltd., Shanghai, China) was added to wells after 24 and 48 h according to manufacturer's protocol. Living cells were detected at a wavelength of 450 nm using a microplate reader. All experiments were repeated three times.

Clonogenic assay. ECA-109 cells were plated onto 6-well plates at a density of 1x10\(^3\) cells/well and treated with liriodenine (0.1 or 1 μM) or control (DMSO) for 24 h. A medical linear accelerator (ElektaPrecise; Elekta Instrument AB, Stockholm, Sweden) was used to irradiate cells with 0, 2, 4, 6 and 8 Gy. Following irradiation, the cells were cultured for 10-14 days. The cells were fixed with methanol (99.99%) for 0.5 h at 37°C and Giemsa-stained for 15 min at 37°C. The numbers of colonies were counted using the TS100 microscope at magnification x10 (Nikon Corporation, Tokyo, Japan). Each effective colony was greater than 50 cells. All experiments were repeated three times. SER (sensitization enhancement ratio) was calculated according to \(D_0\) (mean lethal dose of control group)/\(D_0\) (mean lethal dose of Liriodenine treatment group). SF was calculated according to SF=1-(1-e\(^{-D/D_0}\)).

Apoptosis assay. ECA-109 cells were plated on 6-well plates at a density of 1x10\(^5\) cells/well and divided into one of following groups: Control, liriodenine treatment group, 8 Gy X-rays group, combination group. The numbers of cells undergoing apoptosis were evaluated using annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining using an Annexin V-FITC Apoptosis kit according to the manufacturer's protocol (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) for 15 min in the dark at 37°C. The treated cells were detected by FlowJo V10 (FlowJo LLC, Ashland, OR, USA) using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell cycle assay. ECA-109 cells were cultured on 6-well plates at a density of 1x10\(^5\) cells/well and treated with liriodenine (0.1 and 1 μM) or 8 Gy X-rays. Cells were collected and fixed in 75% ice-cold ethanol overnight at 4°C. Cells were stained with 5% PI for 15 min in the dark at 37°C and the cell cycle distribution was assessed using a FACSCalibur flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Immunofluorescence assay. ECA-109 cells were plated onto confocal laser small dishes at a density of 5x10\(^4\) cells/well and harvested at 2, 8 and 24 h post-irradiation (8 Gy) with or without liriodenine (1 μM). Cells were then fixed in 4% paraformaldehyde at 37°C for 1 h and permeabilized in 0.2% Triton X-100 (Sigma-Aldrich; Merck KGaA) at 37°C for 15 min. Thereafter, cells were blocked with 5% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) for 1 h at room temperature and incubated with γH2AX antibody (80312, dilution, 1:1,000, OBBio Technology, Corp., Ltd.) at room temperature for 1 h. Cells were incubated with an Alexa Fluor 594-conjugated secondary antibody (dilution, 1:5,000, Jackson ImmunoResearch) for 0.5 h at 37°C. Cells were treated with DAPI (Beyotime Institute of Biotechnology, Shanghai, China) for 30 min in the dark at 4°C and visualized using confocal fluorescence microscopy under three fields of view at magnification x40 (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Western blotting analysis. ECA-109 cells were cultured on 6-well plates at a density of 1x10\(^5\) cells/well and treated with liriodenine (1 μM) or 8 Gy X-rays. Radioimmunoprecipitation assay buffer (OBI Biotechnology, Corp., Ltd.) was used to lyse cells, and a Bicinchoninic Acid Protein Quantiﬁcation kit (Beyotime Institute of Biotechnology, Haimen, China) was used to quantify the amount of protein in the lysates. Equal amounts of 15 μg proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene ﬂuoride membranes (EMD Millipore, Billerica, MA, USA). Western blot analysis was performed using the aforementioned rabbit polyclonal primary antibodies to assess the protein levels of caspase-3, Bax, Bcl-2 and GAPDH, followed by horseradish peroxidase-conjugated secondary antibodies (dilution, 1:5,000; BioWorld Technology, Inc.) for 1 h at 37°C. Immunoblotted proteins were analyzed with the ChemiDoc XRS imaging system and QuantityOne software (Version 4.6.9, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. The mean ± standard deviation (SD) from triplicate assays was calculated, and differences between treatment groups were determined using one-way analysis of variance with post hoc Tukey's test. Statistical analysis was performed using SPSS 18 (SPSS, Inc., Chicago, IL, USA) and figures were generated using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.
Results

Liriodenine significantly promotes radiosensitivity of ECA-109 cells. Liriodenine decreased proliferation of ESCC ECA-109 cells in a dose-dependent manner (Fig. 1A; P<0.01). Liriodenine at a low cytotoxic concentration (0.1 and 1 µM) was selected to assess the sensitizing effects towards radiotherapy. Liriodenine plus radiotherapy exhibited a marked reduction in the colony formation of ESCC cells, as shown in Fig. 1B (P<0.01). The sensitization enhancement ratios of liriodenine concentrations at 0.1 and 1 µM were 1.11 and 1.69, and survival fraction (Gy) were 0.6 and 0.38 in ECA-109 cells according to methods in a clonogenic assay, respectively (Table I). The ability to form colonies was used as an indication of resistance to IR. The combination treatment of liriodenine and radiation significantly promoted the radiosensitivity of ECA-109 cells.

Treatment with radiation and liriodenine together increase apoptosis and modify cell cycle distribution. Whether liriodenine induced radiosensitization due to increased apoptosis and changes to the cell cycle was investigated. ECA-109 cells treated with liriodenine and IR together exhibited significantly increased apoptosis rates compared with either treatment alone (Fig. 2; P<0.01). To investigate the antitumor effects of liriodenine, changes in the cell cycle distribution were evaluated. The variation of the percentage of cells in each phase of the cell cycle is depicted in Fig. 3. Flow cytometry analysis indicated that treatment with liriodenine and IR resulted in a significant proportion of cells arrested in G2/M phase (60.02% for ECA-109) (P<0.01) as compared with single agent treatments. Exposure of the cells to liriodenine or IR alone caused a small degree of accumulation of the cells in G2/M phase, and a slight decrease in the proportion of cells in G0/G1 phase. Therefore, liriodenine appears to induce the arrest of cells in G2/M phase.

Liriodenine significantly decreases double-strand break (DSB) repair in ECA-109 cells. ECA-109 cells with or without pretreatment with liriodenine were harvested at 2, 12 and 24 h post-irradiation. The mean numbers of γ-H2AX foci were calculated to examine the amount of DSBs in cells. DNA repair following double-stranded DNA breaks requires a variant histone protein called H2A.X. Radiation induces phosphorylation of H2A.X at Ser139 following DNA breaks (15). Liriodenine primarily enhanced the radiosensitivity of cells by delaying the repair of DNA damage. As shown in Fig. 4, treatment with liriodenine plus irradiation significantly delayed DNA DSB repair compared with irradiation alone.

Liriodenine radiosensitizes ESCC cells by regulating protein expression of Bax, Bcl-2 and Caspase-3. ESCC was radiosensitized by liriodenine through the regulation of Bax, Bcl-2 and caspase-3 expression. Following combinatorial treatment with liriodenine and radiation, changes in the expression of Bax, Bcl-2 and caspase-3 were observed (Fig. 5). The expression of Bcl-2 protein decreased, whereas that of Bax and caspase-3 increased in cells that received combined treatment, compared with the cells treated with liriodenine or radiation alone. These results indicated that liriodenine induced changes in cell proliferation and apoptosis.

Discussion

The aim of the present study was to evaluate the radiosensitizing effect of liriodenine, an aporphine alkaloid from Enicosanellum pulchrum, Fissistigma glaucescens, Annona glabra, Liriodendron tulipifera, and to investigate the underlying molecular mechanisms through which this agent functions.

Table I. Radiosensitization effects of liriodenine on esophageal squamous cell carcinoma ECA-109 cells.

<table>
<thead>
<tr>
<th>ECA-109 treatment</th>
<th>D0, mean lethal dose</th>
<th>Dq, quasi-threshold dose</th>
<th>SF2, survival fraction (2 Gy)</th>
<th>SER, sensitization enhancement ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.11</td>
<td>3.13</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>Liriodenine 0.1 µM</td>
<td>1.91</td>
<td>2.17</td>
<td>0.60</td>
<td>1.11</td>
</tr>
<tr>
<td>Liriodenine 1 µM</td>
<td>1.25</td>
<td>1.44</td>
<td>0.38</td>
<td>1.69</td>
</tr>
</tbody>
</table>

D0, mean lethal dose; Dq, quasi-threshold dose; SF2, survival fraction (2 Gy); SER, sensitization enhancement ratio.

Figure 1. Liriodenine sensitizes ECA-109 cells to IR. (A) ECA-109 cells were treated with liriodenine (0.1, 0.5, 1, 2.5, 5, 7.5, 10 and 20 µM) or dimethyl sulfoxide as a control for 24 or 48 h. Liriodenine suppresses cell viability in a dose- and time-dependent manner in ECA-109 cells. (B) A clonogenic assay was performed to calculate the sensitization enhancement ratio of ECA-109 cells in response to different doses (0.1 and 1 µM) of liriodenine. IR, irradiation. *P<0.05 and **P<0.01.
Liriodenine was first reported to exhibit tumor suppressive properties in 1969 (6). In previous studies, liriodenine was shown to inhibit cell proliferation in hepatoma, ovarian, laryngocarcinoma and lung cancer (12-14). Similarly, liriodenine significantly inhibited the proliferation of melanoma cells in a cell viability assay (16). Results demonstrated that liriodenine, particularly when combined with radiation therapy, induced a significant decrease in the viability and proliferation of ESCC cells in a dose-dependent manner in vitro.

It has previously been reported that liriodenine affects cell cycle distribution. Li et al (16) revealed that liriodenine and cisplatin significantly induced cell cycle arrest at the G2/M phase and the S phase in human hepatoma BEL-7404 cells, while cell cycle arrest at G2/M was also observed in human lung adenocarcinoma A549 cells treated with liriodenine (13). In addition, combination treatment with liriodenine and glucocorticoid induced a moderate accumulation of cells in G2/M phase in the human melanoma A375.S2 cells (17). In the present study, it was revealed that IR and nanomolar concentration of liriodenine significantly induced cell cycle arrest at the G2/M phase. The present results are thus in agreement with those of previous studies.

Radiotherapy represents the primary modality of treatment for patients with unresectable ESCC. However, despite this treatment, 5-year survival rates remain poor, which is attributed to radiation resistance (18). Previous studies have reported that liriodenine may induce apoptosis in human lung, ovarian and laryngocarcinoma cancer cells (12-14). In the present study,
compared with liriodenine or IR alone, liriodenine and IR together strongly induced apoptosis in ESCC cells. The radiosensitizing effect of liriodenine may be mediated by numerous molecular pathways. The results of the present study indicated that cell cycle arrest and apoptosis represent the primary effects of liriodenine treatment.

Figure 3. Effects of liriodenine on the cell cycle distribution of ECA-109 cells. (A) The cell cycle distribution of ESCC cells was examined by flow cytometry. (B) ECA-109 cells were treated with liriodenine alone or in combination with IR for 24 h. Cell cycle distribution was calculated by flow cytometry. Liriodenine induced arrest in G2/M phase. Results were obtained from three independent experiments. Error bars represent the standard error of the mean. IR, irradiation.
High Bcl-2 expression levels have been shown to lead to radioresistance in cancer patients, and radiation-induced activity of Bcl-2 family members are known to cause radioresistance in human prostate cancer cell lines (19). Similarly, high expression of Bax may sensitize patients with head and neck cancer to radiotherapy (20). As such, several direct Bax activators have been used to overcome resistance to chemotherapy and radiotherapy (21). Caspases are central for the induction of apoptosis (22). Hypofractionated RT induces cell death only in caspase-3-proficient breast cancer cells (23). In the present study, increased expression of Bax and caspase-3 was observed following treatment.
with liriodenine and IR, whereas that of Bcl-2 was signifi-
cantly decreased. As such, these results indicated that
liriodenine may induce apoptosis of radioresistant ESCC
cells by regulating the level of the key apoptotic-associated
proteins.

In summary, the present study demonstrated that lirio-
denine affects ESCC cell viability and proliferation, which was
associated with changes in cell cycle distribution. Liriodenine
may also induce apoptosis of radioresistant ESCC cell lines by
increasing expression of Bax and caspase-3, and decreasing
that of Bcl-2, indicating that liriodenine may be a promising
anticancer agent in ESCC radiation therapy.

Acknowledgements
The authors would like to thank Professor Xinchun Sun
(The First Affiliated Hospital of Nanjing Medical University,
Nanjing, China) for his technical support.

Funding
The present study was supported by Key Academic
Discipline of Jiangsu Province ‘Medical Aspects of Specific
Environments’, A Project Funded by the Priority Academic
Program Development of Jiangsu Higher Education
Institutions (grant no. JX10231801), A Phase III Clinical
Trial Funded by Wuxi Hospital Management Center (grant
no. YGZXM14033).

Availability of data and materials
All data generated or analyzed during this study are included
in this published article.

Authors' contributions
GW and GC performed the experiments, analyzed the data
and wrote the manuscript. IZ participated in flow cytomtery
analysis. IZ and JC helped with western blot analysis and
data collection. HZ and FZ conceived the idea, designed the
study and helped draft the manuscript. All authors read and
wrote the manuscript. JZ participated in flow cytometry
and wrote the manuscript. JZ participated in flow cytometry
and wrote the manuscript.

Ethics approval and consent to publish
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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