Research Note

Journal of INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
2014, Vol. 42(4) 984–992

DOI: 10.1177/0300060513507389
imr.sagepub.com
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Preliminary study on the mechanism of oridonin-induced apoptosis in human squamous cell oesophageal carcinoma cell line EC9706

Jun-bao Liu[1] and Jing-yu Yue[2,3]

Abstract

Objective: To study the apoptosis-inducing effect of the Chinese medicine oridonin in the human oesophageal squamous cell carcinoma cell line EC9706, in vitro.

Methods: The effect of oridonin on cell proliferation was studied using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Effects on the cell cycle and apoptosis were studied by flow cytometry; effects on intracellular Ca^{2+} concentration were studied by measuring the fluorescence intensity of a fluorescent Ca^{2+} probe by laser scanning confocal microscopy.

Results: The EC9706 cell-proliferation rate decreased with time and oridonin concentration (10–40 μmol/l). The number of cells in G_0 and G_1 phases increased significantly following exposure to oridonin for 48 and 72 h respectively, and oridonin was shown to be most effective at inducing apoptosis in EC9706 cells at 40 μmol/l. Compared with the control group, all concentrations of oridonin tested (10–40 μmol/l) significantly increased the Ca^{2+} fluorescence intensity of EC9706 cells.

Conclusions: Oridonin was shown to inhibit proliferation and induce apoptosis in the human oesophageal squamous cell carcinoma cell line EC9706, in vitro. These data provide preliminary experimental evidence for the anticancer effects of oridonin, which is as a traditional Chinese medicine used to treat various cancers, including oesophageal squamous cell carcinoma. Further studies are required to elucidate the mode of action.

[1]Department of Integrated Chinese and Western Medicine, First Clinical Hospital of Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China
[2]Department of Integrated Chinese and Western Medicine, People's Hospital of Zhengzhou University, Zhengzhou, Henan, China
[3]Experimental Centre, First Teaching Hospital of Henan, Chinese Medicine University, Zhengzhou, Henan, China

Corresponding author:
Jing-yu Yue, Department of Internal Medicine, People's Hospital of Henan Province, Zhengzhou 225000, Henan, China.
Email: zhongyi450003@163.com

Keywords
Apoptosis, EC9706 cells, oesophageal carcinoma, oridonin

Date received: 21 August 2013; accepted: 10 September 2013

Introduction

Oesophageal carcinoma is a common malignant tumour of the digestive system; worldwide there are 450 000 new cases annually and the fatality rate ranks sixth among malignant neoplasms.[1] In Henan Province, China, the areas with highest oesophageal carcinoma incidence and mortality rates are Linzhou, Anyang and Huixian.[2] Traditional Chinese medicine has unique advantages in the treatment of oesophageal carcinoma, such as inhibition of carcinoma growth, slowing of progression through the clinical stages, prolonging patient survival and improving health-related quality-of-life.[3] Oridonin is a tetracyclic diterpenoid compound based on the kaurene skeleton, extracted from the herb *Rabdosia rubescens*[4] and developed for medical use by Chinese researchers.

The present study aimed to provide an experimental basis for the clinical treatment of oesophageal carcinoma with oridonin, by investigating its apoptosis-inducing effect on the oesophageal squamous cell carcinoma cell line EC9706 in vitro, and exploring the mechanism of this effect.

Materials and methods

Drug and reagents

The present study included the following: EC9706 cells (Biological Engineering Technology Research Centre of Henan Province; Zhenghou City, Henan, China); oridonin with 98% purity (Boxing Biotechnology, Zhengzhou City, Henan, China); trypsin/ethylenediaminetetra-acetic acid (EDTA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Dulbecco's modified Eagle's medium (DMEM) (Beijing Solarbio, Beijing, China); RPMI 1640 medium (Beijing Tianyi Science company, Beijing, China); mycoplasma-free newborn bovine serum (Hangzhou Tianhang Biotechnology, Hangzhou, China); 5-bromo-2′-deoxyuridine (BrdU) Cell Proliferation Assay Kit for flow cytometry (Nanjing KeyGen Biotechnology Co. Ltd., Nanjing, China); annexin-V fluorescein isothiocyanate (FITC) apoptosis detection kit (KGA108; Biosynthesis Biotechnology, Beijing, China); calcium fluorescence probe fluo-3/acetoxymethyl (AM) (Biotium, Hayward, CA, USA); chaotropic agent F-127 (Biotium).

To prepare calcium-free Tyrode's solution, 4.0 g NaCl, 0.1 g KCl, 0.5 g $NaHCO_3$, 0.07 g $Na_2HPO_4{\cdot}12H_2O$, 0.105 g $MgCl_2{\cdot}6H_2O$ and 0.5 g glucose were dissolved in 400 ml ultrapure water and the pH was adjusted to 7.4 at a constant volume of 500 ml; the solution was sterilized by filtration with a 0.22-μm porosity membrane, and stored at 4°C until use.

Experimental groups

Each well in the oridonin groups contained EC9706 cells, culture medium (DMEM with 10% newborn bovine serum) and oridonin at final concentrations of 10, 20 or 40 μmol/l. Blank control wells contained EC9706 cells and culture medium (DMEM with 10% newborn bovine serum).

Cell culture

The EC9706 cells (provided by Henan Biology Engineering Technology Research Centre) were routinely cultured in DMEM with 10% mycoplasma-free newborn bovine serum, and incubated at 37°C/5% CO_2 with

95% relative humidity. Subconfluent cells were passaged every 2–3 days by incubating with 0.2% trypsin/EDTA for 12 min at 37°C. Cells were routinely seeded at 5×10^4 cells/ml (16 ml into a 75 cm^2 culture flask) and the culture medium was changed on alternate days.

MTT proliferation assay

The EC9706 cells in logarithmic phase were routinely digested using 0.2% trypsin/EDTA (12 min at 37°C) and prepared as single-cell suspensions containing 50 000 cells/ml. Each well of a 96-well plate was seeded with 200 µl of the cell suspension (1×10^4 cells/well), and the cells were incubated at 37°C/5% CO_2 for 24 h. The culture medium was then replaced with 200 µl of culture medium containing oridonin at 10, 20 or 40 µmol/l, or blank control medium, with five replicates for each condition. All 96-well plates were incubated at 37°C/5% CO_2 for 24, 48, 72 or 96 h after which the culture medium was discarded and replaced with 180 µl of RPMI 1640 medium and 20 µl of MTT solution. Following incubation at 37°C/5% CO_2 for a further 4 h, the supernatant was discarded, dimethyl sulphoxide (DMSO) was added at 150 µl/well, and the plates were shaken for 10 min on a table concentrator. The optical density (OD) of each well was determined with a microplate reader (Lifecare Medical Equipments., Ningbo, Zhejiang, China) set at 490 nm wavelength. The rate of inhibition of proliferation was calculated using the following formula: inhibition rate (%) = (OD of blank control group − OD of drug test group)/OD of blank control group × 100.[5] Tests were repeated three times for each group.

Morphological analyses

Suspensions of EC9706 cells in logarithmic phase were seeded into 25 cm^2 culture flasks (5 ml, 1×10^8 cells/flask) in DMEM with 10% newborn bovine serum and incubated for 24 h at 37°C/5% CO_2, to allow cells to adhere. The medium was then replaced with 5 ml DMEM with 10% newborn bovine serum (blank control) or complete medium (DMEM with 10% newborn bovine serum) containing oridonin at 10, 20 or 40 µmol/l final concentration and the cells were incubated for 48 h at 37°C/5% CO_2. Cells in logarithmic growth were observed for morphological characteristics using an inverted optical microscope (Olympus 11389-210; Olympus Corporation, Tokyo, Japan).

Flow cytometry measurements of cell cycle and apoptosis

Suspensions of EC9706 cells in logarithmic phase were seeded into 25 cm^2 culture flasks (5 ml, 5×10^4 cells/ml) in DMEM with 10% newborn bovine serum and incubated for 24 h at 37°C/5% CO_2, to allow cells to adhere. The medium was then replaced with 5 ml DMEM with 10% newborn bovine serum (blank control) or medium (DMEM with 10% newborn bovine serum) containing oridonin at 10, 20 or 40 µmol/l final concentration. After incubation for 48 or 72 h at 37°C/5% CO_2, the cells were digested with 0.2% trypsin/EDTA (12 min at 37°C), washed twice with 0.2 mol/l phosphate-buffered saline (PBS, pH 7.4), centrifuged for 5 min at 372 ***g***, and resuspended to a concentration of 1×10^6 cells/ml. Within 1 h of resuspending, the cell-cycle stage and presence of apoptosis were evaluated by flow cytometry (EPICS® Altra™ flow cytometer; Beckman Coulter, Brea, CA, USA) according to the instructions provided with the BrdU cell proliferation assay kit and annexin V-FITC apoptosis detection kit, respectively.

Confocal laser scanning microscopy for detection of intracellular free calcium

The calcium fluorescence probe fluo-3/AM was prepared as a 5-mmol/l solution in

DMSO, and the chaotropic agent F-127 (Biotium) was dissolved in DMSO to a final mass-to-volume ratio of 20%. The two prepared solutions (200 µl of each) were mixed with 12.5 ml RPMI 1640 and stored in the dark at −20°C prior to use. Suspensions of EC9706 cells in logarithmic phase were seeded into 96-well plates (5×10^3 cells/well) and incubated for 24 h at 37°C/5% CO_2 to allow cells to adhere. The medium was then replaced with 200 µl DMEM containing 10% newborn bovine serum (blank control) or medium (DMEM with 10% newborn bovine serum) containing oridonin at 10, 20 or 40 µmol/l final concentration, and the cells were incubated for 2 h at 37°C/5% CO_2. At the end of the incubation period, cells were washed three times with 1 ml of calcium-free Tyrode's solution. The fluo-3/AM and F-127 solution (200 µl) was then added to the central circular groove of the 96-well plate and the solution was incubated for 30 min in darkness at 37°C to allow absorption of the loading solution.

Following three washes with calcium-free Tyrode's solution and adding 200 µl calcium-free Tyrode's solution to the groove of each well, the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined by confocal laser scanning microscopy (Zeiss Optics Instruments [Shanghai] International Trade Co., Shanghai, China). EC9706 cells loaded with fluo-3/AM were placed on the microscope's loading platform and the focus was adjusted to make the image as clear as possible. Cells with a structured rather than irregular shape were selected for observation. The specimen was first pre-scanned with a low-speed argon ion laser, then properly scanned when a clear view had been obtained. The same parameters were used for all scans. Following the technique described previously,[6] six to eight views in all experimental groups were selected, each view including five cells. The cells were subjected to fluorescence intensity analysis and curves showing the change in fluorescence intensity of the Ca^{2+} indicator over time were generated. FV10-ASW Viewer software, version 1.6 (Zeiss Optics Instruments [Shanghai] International Trade Co.) was used to calculate the average fluorescence intensity value (arbitrary units); the greater the fluorescence intensity, the higher the $[Ca^{2+}]_i$.

Statistical analyses

All experimental data were analysed with SPSS® software, version 18.0 (SPSS®, Chicago, IL, USA). Data were expressed as mean ± SD, and analysis of variance was used to compare intra- and intergroup differences. A P-value <0.05 was considered statistically significant.

Results

Cell proliferation and morphology

Oridonin inhibited the proliferation of EC9706 cells. Observation of the cells under an inverted microscope showed that cells exposed to oridonin for 48 h had changed from a shuttle-like to an irregular form, and that cell density was low with increased intercellular spaces. Some cells had become rounded, detached floating cells were observed, and some cells were vacuolated. Part of the cell membrane had contracted and had a foamy appearance, a small number of cells showed a pyknotic state with a translucent round or oval shape and individual cells were small (Figure 1). Incubation with oridonin for 24 h was shown to inhibit the rate of EC9706 cell proliferation by 19.45 ± 2.34%, 41.38 ± 3.32% and 78.83 ± 4.99% (10 µmol/l, 20 µmol/l and 40 µmol/l oridonin), respectively. Differences in rates of inhibition were statistically significant compared with the control group at all oridonin concentrations ($P = 0.0001$). In terms of antiproliferation effect, the IC_{50} for oridonin incubated with

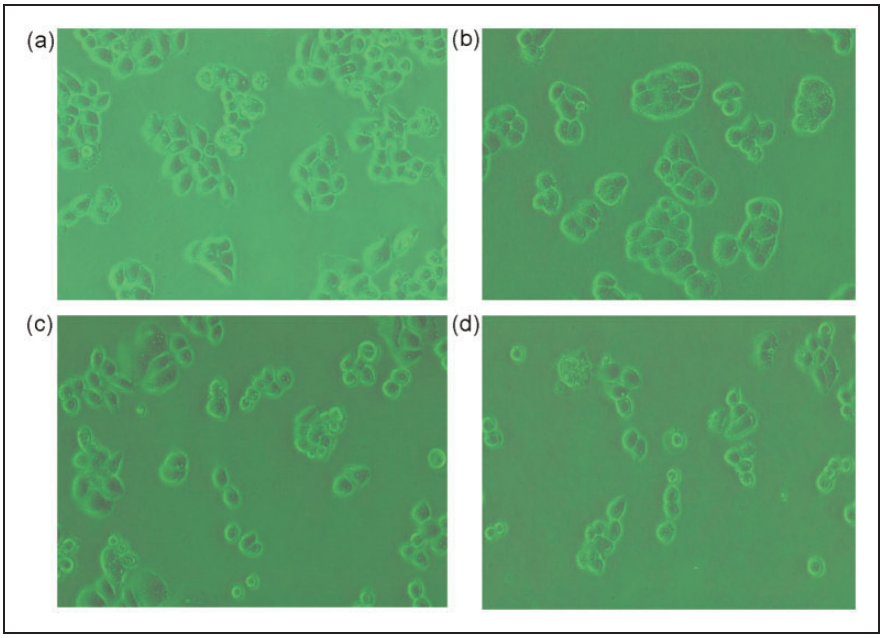

Figure 1. Representative photomicrographs showing the effects of oridonin on the morphology and proliferation of human oesophageal squamous cell carcinoma EC9706 cells following 48 h incubation at 37°C in: (a) blank control Dulbecco's modified Eagle's medium with 10% newborn bovine serum; (b) oridonin at 10 μmol/l; (c) oridonin at 20 μmol/l; (d) oridonin at 40 μmol/l. Original magnification, ×200. The colour version of this figure is available at: http://imr.sagepub.com.

EC9706 cells for 48 h was 21.48 ± 1.43 μmol/l. Inhibition of proliferation was observed in EC9706 cells incubated with oridonin for 24, 48, 72 and 96 h at 10, 20 and 40 μmol/l (Table 1).

Cell-cycle phases and apoptosis

Following 48 h of exposure to oridonin, the percentage of cells in G_1/G_0 phase was significantly higher than in the control group for all oridonin concentrations ($P < 0.05$; Table 2), and the percentage of cells in G_1/G_0 phase was significantly higher in the 40 μmol/l oridonin group than in the 20 and 10 μmol/l oridonin groups ($P < 0.05$). The percentage of cells in S phase was significantly lower in the 40 μmol/l oridonin group than in the control group and 10 and 20 μmol/l oridonin groups ($P < 0.05$; Figure 2 and Table 2). The percentage of cells in G_2/M phase was significantly lower in the 40 μmol/l oridonin group than in the control group ($P < 0.05$; Table 2).

Following 72 h of exposure to oridonin, the percentage of cells in G_1/G_0 phase was significantly higher, and the percentage of cells in the S and G_2 phases were significantly lower, in the 40 and 20 μmol/l oridonin groups than in the control group ($P < 0.001$); the difference was greatest in the 40 μmol/l oridonin group.

The apoptosis rate in all oridonin groups was significantly higher than in the control group at both 48 h (Figure 3) and 72 h (data not shown) ($P < 0.001$), and the apoptosis rate in the 40 μmol/l oridonin group was significantly higher than in the 10 and 20 μmol/l oridonin groups ($P < 0.05$).

Table 1. Inhibition of proliferation of human oesophageal squamous cell carcinoma EC9706 cells incubated with oridonin at 10, 20 or 40 μmol/l concentrations for 24, 48, 72 and 96 h at 37°C, measured using the MTT assay.

Oridonin concentration, μmol/l	Incubation time, h			
	24	48	72	96
0 (Control)	0	0	0	0
10	19.45 ± 2.34*	22.14 ± 8.65*	18.52 ± 5.11*	31.26 ± 6.21*
20	41.38 ± 3.32*	32.37 ± 5.67*	36.01 ± 4.70*	48.39 ± 2.29*
40	78.83 ± 4.99*†	51.17 ± 3.66*	67.41 ± 2.13*†	82.04 ± 0.92*†

Data presented as % inhibition of proliferation ± SD.
*$P < 0.05$ compared with control (analysis of variance).
†$P < 0.05$ compared with 10 μmol/l or 20 μmol/l oridonin (analysis of variance).
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Table 2. Effect of oridonin on the cell-cycle phases (%) of human oesophageal squamous cell carcinoma EC9706 cells following 48 h incubation at 37°C.

Oridonin concentration, μmol/l	Cell-cycle phase, %		
	G_1/G_0	S	G_2/M
0 (Control)	51.62 ± 0.41	37.86 ± 0.61	10.76 ± 0.76
10	61.77 ± 4.19*†	32.73 ± 2.21†	5.51 ± 3.04
20	61.90 ± 2.93*†	32.21 ± 1.34†	5.89 ± 1.99†
40	71.40 ± 3.65*†	24.16 ± 1.49*	4.44 ± 1.48*

Data presented as mean ± SD.
*$P < 0.05$ compared with control.
†$P < 0.05$ compared with 40 μmol/l oridonin.

Intracellular free calcium

From the beginning of the scanning period, the fluorescence intensity of the cultures incubated with oridonin increased at a significantly higher rate than the control group ($P < 0.05$; Table 3). Intracellular free calcium fluorescence intensities were significantly higher in all groups exposed to oridonin for 2 h compared with the control group ($P < 0.05$; Table 3).

Discussion

The aetiology and underlying molecular mechanisms of oesophageal carcinoma onset and progression remain unknown. Principle treatment strategies include surgery, radiotherapy, chemotherapy and biotherapy, and there are currently no effective preventive measures and no specific diagnostic index or methods. The morbidity and fatality rates, therefore, cannot be effectively controlled. Traditional Chinese medicine has few toxic and adverse effects in the treatment of oesophageal carcinoma, whether in single use or combined with radiotherapy or chemotherapy.[7] In the treatment of oesophageal carcinoma, certain forms of traditional Chinese medicine can improve dysphagia, relieve cancer pain, enhance immunity, reduce tumour volume, improve the general state of the body,

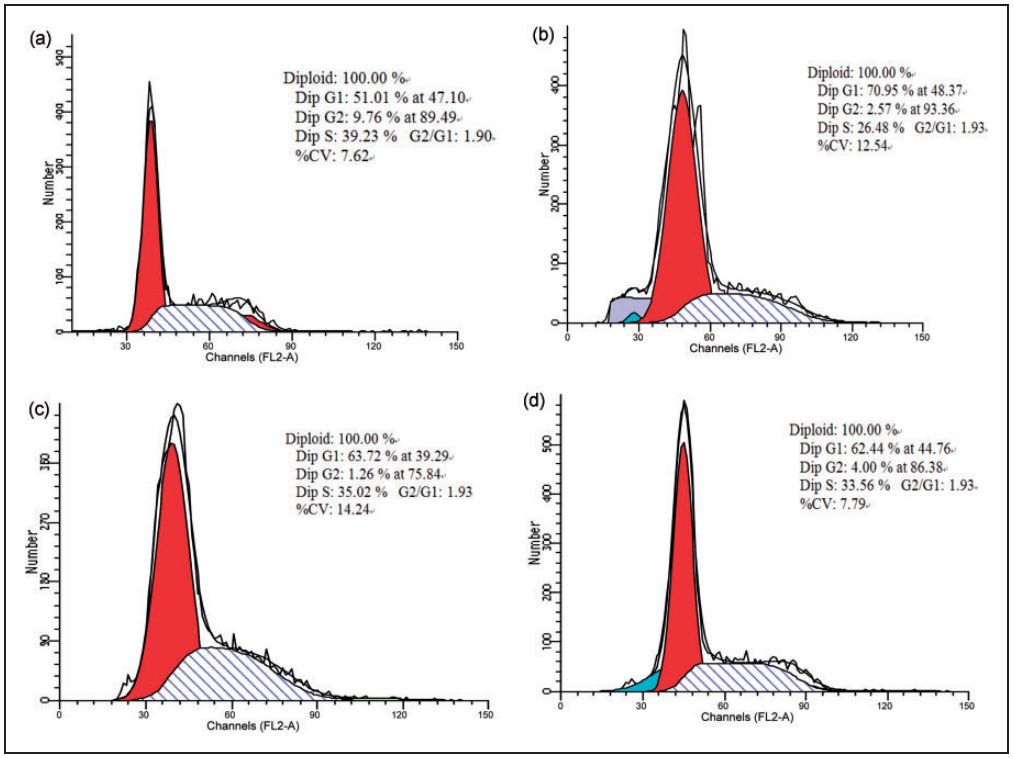

Figure 2. Representative flow cytometry plots showing the effect of oridonin on the human oesophageal squamous cell carcinoma EC9706 cell cycle following . 48 h incubation at 37°C. (a) Control medium; oridonin at (b) 40 μmol/l, (c) 20 μmol/l and (d) and 10 μmol/l concentrations. The colour version of this figure is available at: http://imr.sagepub.com.

decrease relapses and metastases, increase the health-related quality-of-life and 5-year survival rate, reduce toxins and enhance the effects of radiotherapy and chemotherapy.[7,8] Oridonin has inhibitory effects on various malignant tumour cells,[9–11] and its therapeutic effect on solid tumours has been widely used clinically, especially in the treatment of oesophageal carcinoma[7] and gastric tumours (author's personal communication), with notable clinical success. The in vitro antitumour effects of oridonin have been shown against cell lines derived from cancers such as those of the lung, prostate and breast.[10] Oridonin has been shown to induce human cancer-cell apoptosis, with mitochondrial hyperplasia and swelling observed in the early stage of apoptosis.[10,12] The herb *Rabdosia rubescens*, from which oridonin is extracted, has been shown to inhibit the synthesis of DNA and RNA,[13,14] its target being DNA polymerase.[15] The mechanism of antitumour activity may involve an effect on apoptosis through actions such as decreasing the expression level of B-cell chronic lymphocytic leukaemia/lymphoma 2 (*BCL-2*) and activation of the caspase pathway,[16,17] in which telomerase is closely involved,[18] but also by increasing the phagocytosis of apoptotic cells.[13] Oridonin is also thought to affect intracellular signal transduction.[19]

Oridonin at concentrations of 10–40 μmol/l induced apoptosis of EC9706

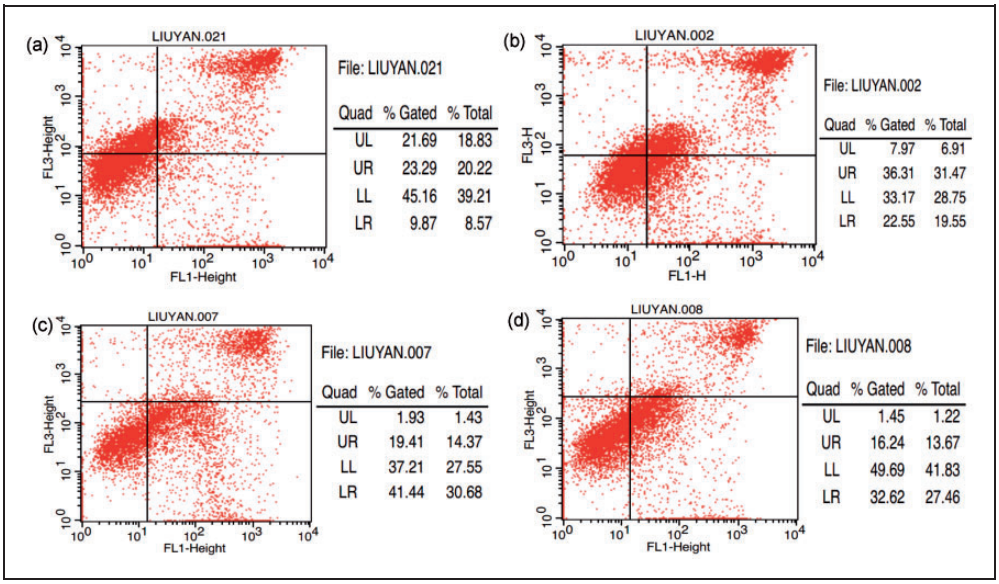

Figure 3. Representative flow cytometry plots showing the effect of oridonin on apoptosis of human oesophageal squamous cell carcinoma EC9706 cells following 48 h incubation at 37°C. (a) Control medium; oridonin at (b) 40 μmol/l, (c), 20 μmol/l and (d) 40 μmol/l concentrations. The colour version of this figure is available at: http://imr.sagepub.com.

Table 3. Effect of oridonin on intracellular Ca^{2+} fluorescence intensity in human oesophageal squamous cell carcinoma EC9706 cells following 2 h incubation at 37°C.

Oridonin concentration, μmol/l	Cell population, wells analysed, *n*	Fluorescence intensity, arbitrary units
0 (Control)	30	553.83 ± 133.67
10	30	2090.241 ± 296.24*
20	30	2354.60 ± 511.78*
40	30	2680.22 ± 276.59*

*$P < 0.05$ compared with control (analysis of variance).

cells in vitro, with peak hypodiploid detection observed following 48 h of exposure, and a concentration-dependent proapoptotic effect. These results are consistent with the apoptosis-inducing effect of oridonin shown in other tumour cells.[9,16]

In the present study, oridonin inhibited the proliferation of oesophageal squamous cell carcinoma EC9706 cells in vitro. Furthermore, increases in cytoplasmic intracellular free calcium (measured by laser scanning confocal microscopy) were seen to correspond with EC9706 cells blocked at the G_1 or G_2/M phases of the cell cycle (measured by flow cytometry), in response to incubation with oridonin. It may be that oridonin inhibits EC9706 cell proliferation through interaction with membrane calcium transport systems or activation of the apoptosis signal transduction pathway. Further studies are required to understand the mechanism by which oridonin inhibits proliferation and induces apoptosis in oesophageal cancer cells.

Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

Funding

This research was funded under Project 5451 of Health Science and Technology of Henan Province (Hospital research No. 2011077).

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