ISSN 1671-4083
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
http://www.ChinaPhar.com

Effect of isoverbascoside, a phenylpropanoid glycoside antioxidant, on proliferation and differentiation of human gastric cancer cell[1]

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KEY WORDS isoverbascoside; phenylpropanoid glycoside; antioxidants; cell division; cell cycle; cell differentiation; cultured tumor cells; stomach neoplasms

ABSTRACT

AIM: To investigate the effects of phenylpropanoid glycoside antioxidant isoverbascoside on cell proliferation and differentiation of human gastric cancer cell line MGC 803. **METHODS:** MGC 803 cells were treated with isoverbascoside. Its effects on cell proliferation, tumorigenicity, enzymatic activities, cell cycles, and gene expression were respectively evaluated with cell counting, tumor formation assay, enzymatic assay, flow cytometer analysis, and Western blotting, with Me_2SO as positive control. **RESULTS:** Isoverbascoside could markedly inhibit cell proliferation in dose- and time-dependent manner. Isoverbascoside 20 μmol/L strikingly suppressed cell tumorigenicity, activities of alkaline phosphatase (ALP), and lactate dehydrogenase (LDH), and caused G_0/G_1 arrest. The expression of $G_1 \rightarrow S$ checkpoint related proteins, p53, p21/WAF1, and p16/INK4, were up-regulated after MGC 803 cells were treated with isoverbascoside 20 μmol/L for 4-8 h. Contrarily, the expression of C-myc protein was suppressed after 8 h treatment. **CONCLUSION:** Isoverbascoside inhibited cell proliferation, reversed cell malignant phenotypic characteristics, and consequently caused differentiation in MGC 803 cells. These effects might be associated with its activities of causing G_0/G_1 arrest and regulating the expression of cell cycle related proteins.

INTRODUCTION

Phenylpropanoid glycosides are a subset of natural phenolic compounds mainly extracted from herbal medicine. Some of them have been reported possessing multi-pharmacological activities of antioxidation[1] and antitumor[2]. Isoverbascoside, one of phenylpropanoid glycoside compounds, was isolated from *Pedicularis striata* Pall, a Chinese folk herbal medicine which is used as cardiac tonic for treatment of collapse, exhaustion, and senility by local inhabitants living in northwestern China[3]. We have previously reported that isoverbascoside possessed the activities of scavenging reactive oxygen species[1], inhibiting cell multiplication, and inducing morphological changes in HL-60 and MGC 803 cells[4,5], and inducing differentiation in HL-60 cells[6]. Here we examined the effects of isoverbascoside on cell proliferation and differentiation of human gas-

[1]Project partially supported by Natural Science Foundation of Fujian Province, № C97108.
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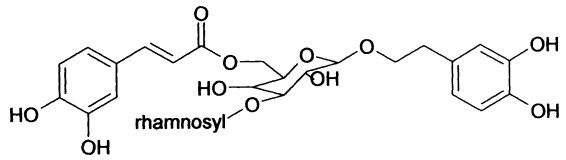
Received 2001-09-12 Accepted 2002-07-03

tric cancer cell line MGC 803, and Me_2SO, a well known differentiation inducer[7], used as positive control.



Structure of isoverbascoside

MATERIALS AND METHODS

Reagents Dimethyl sulfoxide (Me_2SO) was purchased from Sigma Chemical Co (St Louis, USA). Antibodies for Western blotting analysis were purchased from Santa Cruz Biotechnol, Inc (Santa Cruz, USA). Alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) detection kits were purchased from Shanghai Lianyang Biotech Co (Shanghai, China). Isoverbascoside was kindly provided by Department of Biology of Lanzhou University, and its solution was prepared as previous report[6].

Cell culture and treatment Human gastric cancer cell line MGC 803 was maintained in RPMI medium 1640 (GIBCO, Grand Island, USA) supplemented with 10 % fetal calf serum, penicillin 1×10^5 U/L, and streptomycin 100 mg/L at 37 ℃ in 5 % CO_2. Cells were always seeded at a starting density of 5×10^7 cells/L, and the medium containing indicated concentration of isoverbascoside or 1.5 % Me_2SO was renewed every 2 d. For morphological assessments and mitotic index determination, cells were grown on glass cover slides and stained with hematoxylin-eosin (HE). Mitotic cells were counted in at least 1000 cells per slide under light microscopy. For ultrastructural observation, cells samples were prepared according to the routine method and examined with a JEM-100CX II transmission electron microscope (MJ, USA).

Assay of cell growth Cells were plated in 24-well plates. Treated and control cells were harvested each day with trypsin/ethylene diamine tetraacetic acid procedure. Viable cells were counted using a heamocytometer after staining with trypan blue dye.

Assay of tumorigenicity Cells were cultured in medium containing isoverbascoside 0 or 20 μmol/L for 6 d and harvested by trypsinization. The amount of 5×10^6 viable cells in treated or control group were injected subcutaneously at a different side of the buttock in the same BALB/C nude mouse (from Experimental Animal Laboratory of Cancer Research Center, Xiamen University, Certificate No 23-007, SPF grade). After 4 weeks, tumor tissues were taken out from nude mice (n=5) and weighed.

Cell cycle analysis To evaluate the effect of isoverbascoside on cell cycle, MGC 803 cells were treated with isoverbascoside 20 μmol/L and harvested at the d 0, 2, 4, and 6, respectively. About 5×10^6 cells per sample were subjected to the cell cycle distribution analysis with FACS calibur flow cytometry (Becton Dickinson, USA), according to the reported method[8].

Assay of enzymatic activity For detecting the enzymatic activity, the harvested cells were suspended and sonicated in ice-cold sodium phosphate buffer (100 mmol/L, pH 7.2). The homogenates were centrifuged at 12 000×g for 30 min at 4 ºC. The supernatants were subjected to quantification of protein with Bradford assay[9], and assay of ALP and LDH activities with detection kits. Enzymatic activities were expressed as kU/g (protein).

Western blotting Harvested cells were lysed with lysis buffer 100 μL containing 1 % Nonidet P-40 (NP-40), 1 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), edetic acid 1 mmol/L, phenylmethyl-sulfonyl fluoride (PMSF) 1 mmol/L, and leupetin 0.5 mg/L. Total cell lysates were centrifuged at 20 000×g for 20 min at 4 ºC. Aliquots of 75 μg protein were resolved in SDS-polyacrylamide gel, transferred to nitrocellulose filter membranes, and detected with appropriate antibodies as the reported method[10].

Statistics Data were expressed as mean±SD. Difference between groups was analyzed using Student's t-test. $P<0.05$ was considered statistically significant.

RESULTS

Effects of isoverbascoside on MGC 803 cell proliferation and tumorigenicity Inhibition of cell growth by isoverbascoside was observed in a concentration- and time-dependent manner (Fig 1A). Comparing to control, the mitotic peak of either 1.5 % Me_2SO or isoverbascoside 20 μmol/L treated cells was moved from d 4 to d 3 (Fig 1B). The maximum mitotic index was declined by 1.46 % in isoverbascoside treated group ($P<0.05$ *vs* control group). Treatment with 20 μmol/L isoverbascoside also caused cells striking morphological and ultrastructural changes: predominantly spread and flatted epithelioid cells with regularly shaped nuclear,

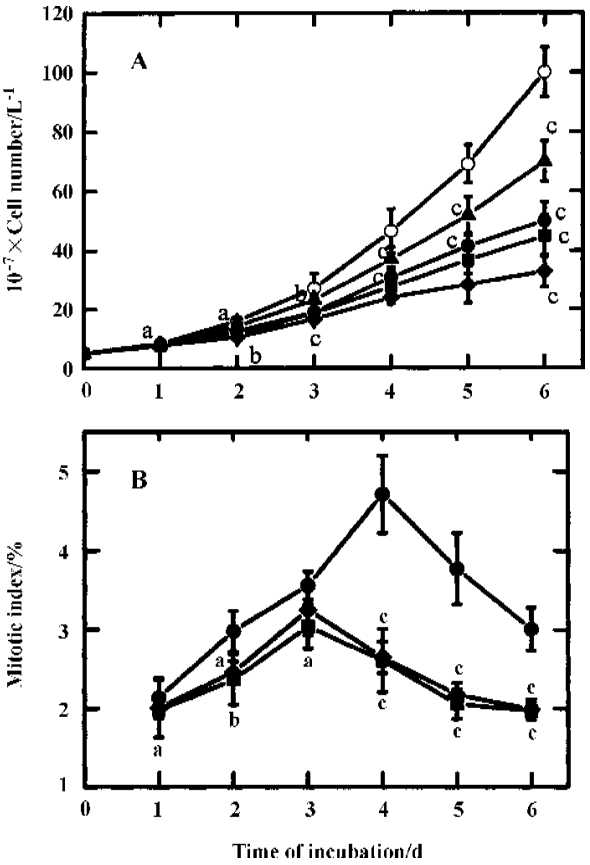


Fig 1. Effect of isoverbascoside and Me_2SO on MGC 803 cell growth (A) and mitotic index (B). (○) control; (▲) isoverbascoside. 10 μmol/L; (●) isoverbascoside 20 μmol/L; (◆) isoverbascoside 30 μmol/L; (■) 1.5 % Me_2SO. *n*=4 experiments. Mean±SD. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control.

declined nucleo-cytoplasmic ratio and less nucleoli in nuclear, markedly decreased polyribosome, and increased rough endoplasmic reticulum (data not shown). In 5 nude mice for tumorigenicity assay, both control and isoverbascoside treated groups developed tumors in each mouse. However, comparing with control group (average tumor weight 0.89 g±0.19 g), the average tumor weight of isoverbascoside treated group (0.40 g± 0.08 g) was markedly declined by 55.1 % ($P<0.05$ *vs* control group, Fig 2).

Effects of isoverbascoside on enzymatic activities Untreated MGC 803 cells displayed relative high ALP and LDH activities. While treated with isoverbascoside 20 μmol/L or 1.5 % Me_2SO, the activities of ALP and LDH were both inhibited in a time-dependent manner (Fig 3A, 3B). Compared to control, the ALP and LDH activities of isoverbascoside treated cells were respectively declined by 40.7 % and 60.2 % at d 6

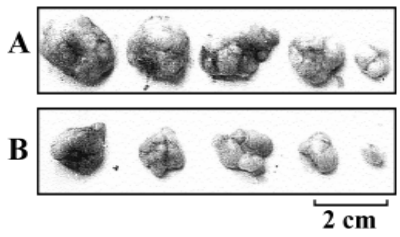


Fig 2. Effect of isoverbascoside on MGC 803 cell tumorigenicity. A: control group; B: isoverbascoside 20 μmol/L.

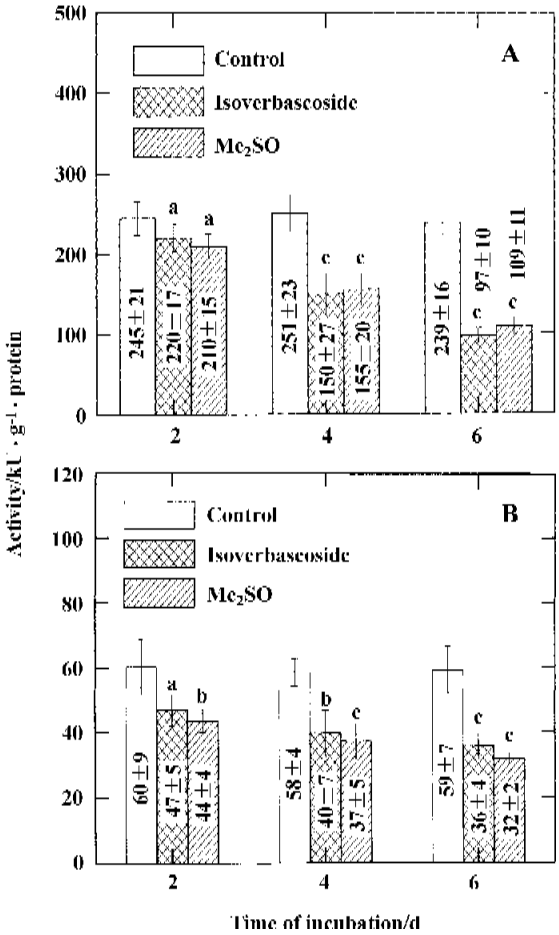


Fig 3. Effects of isoverbascoside and Me_2SO on MGC 803 cell ALP (A) and LDH (B) activities. *n*=3. mean±SD. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control.

($P<0.05$, $P<0.01$ *vs* control).

Effects of isoverbascoside on cell cycle and gene expression The analysis of flow cytometer showed that the cells had a dramatic increase in the percentage of G_0/G_1 phase and decrease in S phase in time-dependent manner (Tab 1), indicating that isoverbascoside mainly caused G_0/G_1 arrest. Immunoblotting results showed that the expressions of p53, p21/WAF1, and p16/INK4 proteins were up-regulated after

Tab 1. Effects of isoverbascoside on MGC 803 cell cycles.

Group	Treated time/d	Cell cycle/%		
		G_0/G_1	S	G_2/M
Control	0	45.5	49.2	5.4
Isoverbascoside	2	51.2	43.2	5.7
20 μmol/L	4	60.6	30.0	9.3
	6	66.0	27.4	6.6

treating MGC 803 cell with isoverbascoside 20 μmol/L for 4-8 h, and the expression of C-myc protein was markedly down-regulated after 8 h (Fig 4).

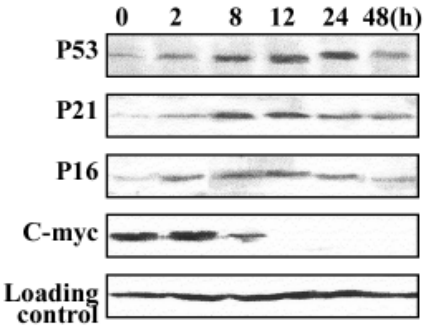


Fig 4. Effects of isoverbascoside on cell cycle related proteins expression in MGC 803 cells.

DISCUSSION

The determination results have proved that, similar to Me_2SO, isoverbascoside could inhibit both cell growth and multiplication, and induce cell morphological and ultrastructural changes. Moreover, under the experimental concentration, no significant cytotoxic or apoptotic effect was observed in either isoverbascoside or Me_2SO treated groups (data not shown). The results of tumorigenicity assay showed an expected significant inhibition of isoverbascoside on tumor formation in nude mice. Thus, the treatment of isoverbascoside has significantly suppressed the tumorigenical phenotype of MGC 803 cells. Our results further revealed that, similar to Me_2SO, the treatment of isoverbascoside also significantly inhibited both ALP and LDH activities, suggesting that isoverbascoside possess the activity of impelling MGC 803 cells reversion against the malignant phenotypes.

The mechanism of isoverbascoside reversing the malignant phenotypes of MGC 803 cell remains to be investigated. In the cells exposed to isoverbascoside, the accumulation of cells in G_0/G_1 phase and the up-regulation of protein expression of p53, p21/WAF1, and p16/INK4 as well as the suppression of C-myc expression were observed in this study. G_0/G_1 arrest is a common phenomenon in the cells undergoing induction of differentiation[11]. The cyclin-dependent kinase inhibitors such as p21/WAF1 and p16/INK4 proteins play central roles in this process[12,13]. These proteins exert their functions by combining with cyclins/CDKs complexes, therefore inhibiting the complexes kinase activity, and finally blocking the cell cycle at the $G_1 \rightarrow$ S checkpoint[12]. The results suggested that the p21/WAF1 and p16/INK4 proteins were associated with the G_0/G_1 arrest after the MGC 803 cells were treated with isoverbascoside. As a tumor suppression gene, *p53* can exert its cell cycle arresting function by up-regulating the expression of p21/WAF1 protein, and thus play an important role in $G_1 \rightarrow$ S checkpoint[14,15]. C-myc onco-protein, a well-known nuclear transcription factor, exerts its regulation function in cell cycle by promoting the transcription of DNA synthesis related genes and therefore impelling cell cycle into S phase[13]. Suppression of its expression will block the cell cycle at $G_1 \rightarrow$S checkpoint.

In conclusion, our study has proved that, in addition to induce HL-60 differentiation, isoverbascoside has the activities of inhibiting cell proliferation and causing differentiation in human gastric cancer cell line. Moreover, our study succeeded in solid tumor cells while most differentiation inducers successfully induce differentiation restricted to leukemia cells.

ACKNOWLEDGEMENT We thank academician and Prof XU Xun for kind helps in conducing experiments and Prof Bill AHO for the key review of this article.

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苯丙素苷类抗氧化剂异毛蕊花苷对人胃癌细胞生长和分化的影响[1]

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关键词 异毛蕊花苷；苯丙素苷；抗氧化剂；细胞分裂；细胞周期；细胞分化；培养的肿瘤细胞；胃肿瘤

目的：探讨苯丙素苷类抗氧化剂异毛蕊花苷对人胃癌MGC 803细胞生长和分化的影响. **方法**：以1.5 %二甲基亚砜为阳性对照，采用细胞计数、肿瘤形成率实验、酶活性测试、流式细胞仪分析和Western blot等方法分别评价异毛蕊花苷对MGC 803细胞生长、致瘤性、碱性磷酸酶(ALP)及乳酸脱氢酶(LDH)活性、细胞周期和周期相关基因表达的影响. **结果**：MGC 803细胞经异毛蕊花苷处理后，细胞生长和增殖呈浓度和时间依赖性抑制；异毛蕊花苷20 μmol/L可使细胞致瘤性明显受抑，胃癌细胞分化标志酶ALP和LDH活性均呈时间依赖性下降，细胞周期表现为G_0/G_1期阻滞，$G_1 \rightarrow S$期调控点相关蛋白p53、p21/WAF1和p16/INK4表达上调，C-myc蛋白表达受抑. **结论**：异毛蕊花苷可明显抑制MGC 803细胞生长及逆转胃癌细胞恶性表型进而诱导分化，这可能与细胞周期G_0/G_1期阻滞及其对周期相关基因表达的调控作用有关.

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