Induction of Differentiation in Human Hepatocarcinoma Cells by Isoverbascoside

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Abstract

Isoverbascoside, a phenylethanoid glycoside, was isolated from Chinese folk medicine herb *Pedicularis striata* Pall. Here we report that isoverbascoside is capable of inducing differentiation in human hepatocellular carcinoma (HCC) cell line SMMC-7721. When treated with isoverbascoside, the proliferation of SMMC-7721 cells was markedly inhibited in a dose- and time-dependent manner, and the average cell population doubling time was delayed. Exposure of cells to 20 μmol/l isoverbascoside led to the decline of colony formation efficiency on soft agar, induced G_0/G_1 arresting, and resulted in the decrease of γ-glutamyltransferase (γ-GT) activity and the increase of tyrosine aminotransferase (TAT) activity, two marker enzymes, respectively, representing HCC malignance and differentiation stage. These results suggest that isoverbascoside possess the activity of inducing differentiation in SMMC-7721 cells.

Letter

Phenylpropanoid glycosides are a subset of natural phenolic compounds mainly extracted from medicinal herbs. Some of them have been reported possessing anti-tumor activity [1], [2]. Isoverbascoside (Fig. **1**), a phenylethanoid glycoside, was isolated from *Pedicularis striata* Pall, a Chinese folk medicine herb which is used as cardiac tonic for the treatment of collapse, exhaustion and senility by local inhabitants living in Northwestern China [3]. It has been reported that isoverbascoside has strong capabilities for scavenging reactive oxygen species and chelating iron [4]. The aim of this study is to investigate the effects of isoverbascoside on inducing the differentiation in the human hepatocellular carcinoma (HCC) cell line SMMC-7721, using all-*trans*-retinoic acid (ATRA) as positive control [5].

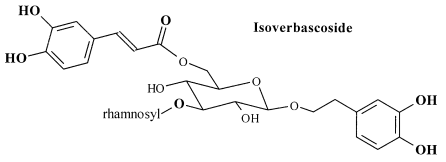

Fig. **1** Chemical structure of isoverbascoside.

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The purpose of induction of differentiation is to reverse the cancer malignant phenotypes, rather than to kill cancer cells [6]. In this regard, we performed the trypan blue dye exclusion test to measure the effects of isoverbascoside on both cell growth and cell death. Inhibition of cell growth by isoverbascoside was observed in a dose- and time-dependent manner in SMMC-7721 cells, as shown in Fig. **2A**. The half-maximal inhibition dose (IC_{50}) was observed at 19.0 ± 1.8 μmol/l. When treated with 10, 20 and 30 μmol/l isoverbascoside, the average cell population double times were respectively delayed by 6.3, 15.3 and 26.1 hours, and 11.1 hours in the 20 μmol/l ATRA-treated group at the sixth day. Compared to control cells, the maximum mitotic indexes were all declined after treating with either 20 μmol/l isoverbascoside or 20 μmol/l ATRA (Fig. **2B**), indicating a remarkable inhibitory effect of isoverbascoside on cell proliferation. Moreover, under the experimental concentration indicated, no significant cytotoxic or apoptotic effect was observed in either isoverbascoside or ATRA treated groups (data not shown).

Tumorigenicity is one of the important phenotypes of cancer [7]. In methodology, the tumorigenicity of cancer cells can be estimated with colony formation assay [8]. Therefore, we conducted the semi-soft agar colony assay for determining the tumorigenicity of the treated and untreated cells. Exposure of SMMC-7721 cells to 10 and 20 μmol/l isoverbascoside or 20 μmol/l ATRA resulted in a decline of the colony formation efficiency in a dose-dependent manner in isoverbascoside treated groups (Table **1**), which suggests that the treatment of isoverbascoside significantly suppresses the tumorigenicity of HCC cells. Moreover, our results revealed further that, similar to ATRA, the treatment of isoverbascoside resulted in the suppression of γ-glutamyl-

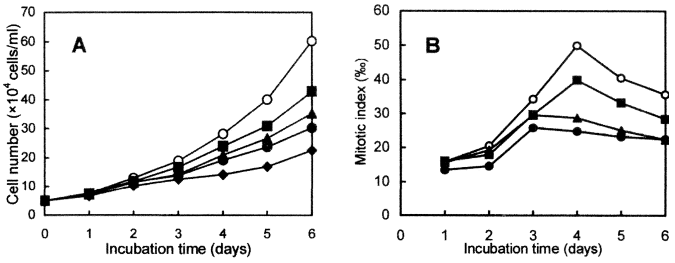

Fig. **2** Effects of isoverbascoside and ATRA on SMMC-7721 cell growth (**A**) and mitosis (**B**). (**A**) Cells were treated with final concentration of 0 (○), 10 (■), 20 (●) and 30 (◆) μmol/l isoverbascoside or 20 μmol/l ATRA (▲). (**B**) Cells were treated with final concentration of 0 (○), 10 (■) and 20 (●) μmol/l isoverbascoside or 20μmol/l ATRA (▲).

Table **1** Effects of isoverbascoside and ATRA on colony formation efficiency of SMMC-7721

		Number of colonies	
	Test group	*X ± SD*[a]	*Inhibition rate*
Control	0	37.5 ± 2.65	/
Isoverbascoside	10 μmol/l	33.3 ± 2.95	11.2 %[b]
	20 μmol/l	26.3 ± 2.63	30.7 %[c]
ATRA	20 μmol/l	27.5 ± 3.12	26.7 %[c]

[a] Mean values of three independent determinations.
[b] $p > 0.01$.
[c] $p < 0.005$.

transferase (γ-GT) activity and the elevation of tyrosine aminotransferase (TAT) activity in a time-dependent manner (Table **2**). Many studies have suggested that γ-GT is a marker enzyme of HCC [9] and contrarily, TAT is an indicator of differentiated hepatic cells [10]. Taken together, these results suggest that isoverbascoside possesses the activity of inducing SMMC-7721 cells differentiation.

The mechanism by which the isoverbascoside induces differentiation in HCC cells remains to be investigated. In this study, one effect observed in HCC cells exposed to isoverbascoside was the accumulation of cells in G_0/G_1 phase and the decrease in S phase in a time-dependent manner (Table **3**). G_0/G_1 arrest is a common phenomenon in the cells undergoing induction of differentiation [11]. Although we do not now know what factors lead to cell cycle arrest, such arrest, we consider, may be associated with the effect of isoverbascoside on inducing differentiation in HCC cells. Further investigation on regulation of cell cycle related genes by isoverbascoside might lead to revealing the molecular mechanism of induction of differentiation in HCC cells.

Our study presented here initially proved that isoverbascoside, a phenylethanoid glycoside [3], has the capacity of induction of differentiation in human HCC cells. Moreover, most inducers successfully induce differentiation restricted to leukemia cells, while our study succeeded in solid tumor cells.

Materials and Methods

Materials: ATRA was purchased from Sigma Chemical Co. (St. Louis, MO, America). Isoverbascoside was kindly provided by Dr. Li J (Department of Biology of Lanzhou University, Lanzhou, China) [3], [4]. Human HCC cell line SMMC-7721 was purchased from Cell Collection of Shanghai Cell Research Institute of Academia Sinica (Shanghai, China).

Assay of cell growth and mitotic index: Cells were plated in triplicate into wells of 24-well plates (Nunc, Roskilde, Denmark) in 1 ml aliquots at density of 5×10^4 cells/ml. Medium was replaced on the following day (day 0) with new complete medium containing indicated concentration of isoverbascoside or 20 μmol/l ATRA and renewed every 2 days. Viability of cells was examined by trypan blue dye exclusion test. Cell numbers were counted using a heamocytometer. For determining the mitotic index, cells were seeded in triplicate at density of 2.5×10^4 cells/ml in 24-well plate with cover slip strip. After treatment with isoverbascoside or ATRA, the cover slip strips were fixed and stained by HE staining procedure. The mitotic cells at each cover slip strip were counted in at least 1,000 cells under light microscopy.

Soft agar colony assay: Cells were cultured in liquid medium with isoverbascoside or ATRA for 6 days. After harvest, viable cells were enumerated and plated in triplicate into 35-mm Petri dishes using two-layer soft agar system with a total of 5×10^4 cells per dish, according to the reported method [8]. Colonies containing more than 30 cells were counted after 14 days of incubation.

Assay of enzyme activity: For detecting the enzymatic activity, the harvested cells were resuspended to a cell density of 10^7 cells/ml and sonicated in ice-cooled 100 mmol/l sodium phosphate (pH 7.2). The homogenates were centrifuged at 12,000 × g for 20 min at 4 °C and the supernatants were subjected to assay of enzymatic activity. Proteins were quantified with Bradford's method [12]. Activity of γ-GT was determined according to the method described by Huseby et al. [13] and the TAT activity was tested according to the reported method [14]. Enzymatic activity was expressed as units/mg protein.

Cell cycle analysis: Cells treated with 20 μmol/l isoverbascoside were harvested at the indicated day. After extensive wash with ice-cold D-Hank's solution, about 5×10^6 cells of each sample were fixed in 75% ethanol overnight and subjected to the cell cycle distribution analysis with FACS Calibur flow cytometry (Becton Dickinson, USA), according to the reported method [15].

Statistical evaluation: Results of cell proliferation, mitotic index, colony formation and enzymatic activity data are expressed as mean values of three independent determinations. Different between means were analyzed using Student's *t*-test, with $p < 0.05$ being considered statistically significant different.

Table **2** Effects of isoverbascoside and ATRA on γ-GT and TAT activities of SMMC-7721 cells

		Enzymatic activities[a]		
Enzyme	**Group**	**2**	**4**	**6[b]**
	Control	13.82	14.53	13.92
γ-GT	Isoverbascoside	9.56	6.3	6.4
	ATRA	11.05	8.22	7.25
	Control	7.57	7.67	6.86
TAT	Isoverbascoside	15.3	17.49	17.49
	ATRA	10.5	13.81	13.38

[a] Mean values of three independent determinations. Comparing with control, all groups have significant different in statistical analysis ($p < 0.005$).

[b] Days of treatment.

Table **3** Effect of isoverbascoside on cell cycles of SMMC-7721

	Treatment	Cycle distribution (%)		
Group	**(day)**	**G_0/G_1**	**S**	**G_2/M**
Control	0	48.5	41.1	10.5
Isoverbascoside	2	58.3	27.7	14.0
20 μmol/l	4	61.3	22.1	16.6
	6	62.9	19.2	18.0

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