

# Berberine induces apoptosis and arrests the cell cycle in multiple cancer cell lines

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## Abstract

**Introduction:** To examine the anti-cancer effects of berberine on multiple cancer cell lines, and to clarify the underlying molecular mechanisms.

**Material and methods:** The IC<sub>50</sub> values for the action of berberine on Tca8113 (oral squamous cell carcinoma), CNE2 (nasopharyngeal carcinoma cell), MCF-7 (breast cancer), Hela (cervical carcinoma), and HT29 (colon cancer) cells were determined by MTT cell viability assay. Early apoptosis and cell cycle arrest were examined by flow cytometry with annexin V and propidium iodide (PI) staining, respectively. For expression of BAX and BCL-2 genes and proteins were detected by real-time PCR and western blotting, respectively.

**Results:** Berberine displayed a cytotoxic effect on all the cell lines tested. The IC<sub>50</sub> values were determined (Tca8113, 218.52 ±18.71; CNE2, 249.18 ±18.14; MCF-7, 272.15 ±11.06; Hela, 245.18 ±17.33; and HT29, 52.37 ±3.45). PI staining revealed that berberine treatment resulted in cell cycle arrest at G2/M. The treatment also induced early apoptosis as shown by annexin V staining. In addition, berberine significantly elevated gene and protein expression of BAX, which was accompanied by substantial decreases in BCL-2 gene and protein levels. The effects of berberine on BAX and BCL-2 were time-dependent.

**Conclusions:** Berberine exhibited cytotoxic effects on multiple cancer cell lines by inducing apoptosis and cell cycle arrest. The BCL-2/BAX signaling pathway may be the common pathway underlying the anti-tumor effect of berberine. The findings support the notion that berberine is a dietary compound that can be further developed into a drug candidate for cancer treatment.

**Key words:** berberine, human epithelial cancer cells, MTT assay, apoptosis, BCL-2, BAX.

## Introduction

Cancer causes millions of deaths worldwide, and more unfortunately, its incidence in many countries is increasing. Nowadays, surgery, chemotherapy, radiation therapy, and immunotherapy are the four commonly used treatment modalities. For many cancers, surgical interventions are potentially curative; however, many tumors are at an advanced stage that cannot be managed by surgical resection at the time of diagnosis in many clinical scenarios. Chemotherapy, which inhibits multiplication

of cancer cells, is the major treatment option for most patients. The efficacy of chemotherapy has been widely documented [1, 2]; however, serious adverse effects associated with the use of chemotherapeutic drugs have also been reported [3–5]. Patients treated with chemotherapeutic drugs can develop drug resistance. For example, a significant number of patients with estrogen receptor-positive breast cancer are found to acquire resistance to tamoxifen [6, 7]. Treatment of patients with cancer by chemotherapy, radiotherapy, and surgery is also challenged by the post-treatment tumor recurrence. It therefore remains imperative to identify new modalities for cancer treatment.

Naturally occurring compounds are rich resources for novel anti-cancer drug candidates. Medicinal herbs containing a bioactive alkaloid called berberine have long been used by medical practitioners in China, Unani, and India to treat malignant diseases [8]. The compound berberine has been shown to display potent anti-cancer effects *in vitro* and *in vivo* [9–12], of which the underlying mechanisms are largely attributed to induction of the cell cycle and apoptosis in cancer cells [13–15]. More importantly, berberine is relatively non-toxic to humans compared to chemotherapeutic drugs [16]. All these studies collectively suggest that berberine is a promising drug candidate warranting further in-depth investigations. In the present study, we examined the cytotoxic effects of berberine on a panel of cancer cell lines including oral squamous cell carcinoma, nasopharyngeal carcinoma, breast cancer, cervical carcinoma, and colon cancer. We also deciphered the possible mechanism underlying the cytotoxicity of berberine towards cancer cells.

## Material and methods

### Cell cultures and reagents

Human oral squamous cell carcinoma Tca8113 cells were obtained from the Key Laboratory for Oral Biomedicine of Ministry of Education, Department of Endodontics, School and Hospital of Stomatology, Wuhan University, China. Human nasopharyngeal carcinoma CNE2 cells, human breast cancer MCF-7 cells, human cervical carcinoma Hela cells, and human colon cancer HT29 cells were purchased from the China Center for Type Culture Collection (CCTCC), Wuhan, China. Tca8113 and HT29 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific Inc., MA, US). Hela and MCF-7 cells were maintained in RPMI-1640 (Thermo Fisher Scientific Inc.), while CNE2 cells were maintained in Minimum Essential Medium with Earle's Balanced Salts (MEM/EBSS; Thermo Fisher Scientific Inc.). All the culture media were supplemented with 5% fetal bovine serum and 1% streptomycin/

penicillin. For the culture of MCF-7 cells, human insulin-like growth factor (1 µg/ml) was also added. All the cell cultures were maintained in a 5% CO<sub>2</sub> environment at 37°C.

Berberine hydrochloride and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). The stock solution of berberine was prepared by dissolving berberine hydrochloride in sterile water and filter-sterilized. 3-(4,5)-dimethylthiaziazolo (-z-y1)-3,5-di-phenyltetrazolium bromide (MTT) was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China).

### MTT cell viability assay

The effect of berberine on the viability of various cancer cell lines was examined using MTT assay. Cancer cells were seeded into 96-well plates at a density of  $1 \times 10^6$  cells/well, and on the next day, were treated with berberine of serial concentration (i.e. 12000, 6000, 3000, 1500, 750, 375, 188, 94, and 47 µM). Incubation was allowed for 48 h. After the incubation, 50 µl of MTT (2 mg/ml) was added. After 3 h, the optical density at a wavelength of 600 nm was detected. The IC<sub>50</sub> value of each cancer cell was determined from three independent determinations.

### Annexin V staining for apoptosis

Apoptosis of cancer cells after treatment with berberine was studied using annexin V staining (KeyGen Biotech, Nanjing, China). In brief, cancer cells were treated with berberine hydrochloride of concentration equivalent to the respective IC<sub>50</sub> for different time points (0, 6, 12, 24, and 36 h). The treated cells were then stained with annexin V-FITC and propidium iodide (PI, 10 µg/ml). The proportion of apoptotic cells (annexin V-positive/PI-negative) was determined using flow cytometry by FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

### Cell cycle analysis

The effect of berberine on the cell cycle of cancer cells was examined by PI staining. Briefly, cancer cells were treated with berberine hydrochloride of concentration equivalent to the respective IC<sub>50</sub> for different time points (0, 6, 12, 24, and 36 h). Cancer cells were fixed with ethanol for overnight, treated with RNase A (1 mg/ml), and finally stained with PI (100 µg/ml). PI-stained cells were quantified using flow cytometry by FACSCalibur (Becton Dickinson), with the cell cycle being analyzed by CellQuest and Modfit software.

### Quantitative real-time PCR

The expression of BAX and BCL-2 genes after berberine treatment was examined using quanti-

tative real-time PCR. Cancer cell lines were subjected to berberine hydrochloride treatment of a concentration equivalent to the respective  $IC_{50}$  for different time points (0, 6, 12, 24, and 36 h). After treatment, total RNA was isolated from the treated cells using TRIZOL (Thermo Fisher Scientific Inc.). First-strand cDNA was then synthesized from the isolated RNA using Superscript II reverse transcriptase (Thermo Fisher Scientific Inc.). Amplification of BAX and BCL-2 was performed using TOYOBO THUNDERBIRD SYBR qPCR Mix (Osaka, Japan) by the ABI 7500 Real-time System (Thermo Fisher Scientific Inc.) with the following oligonucleotides: H-BCL-2, 5'-CATTGGGAAGTTTCAAATCAGC-3' (sense) and 5'-CTTTGCATTCTTGGACGAGG-3' (antisense); H-BAX, 5'-TTGCTTCAGGGTTTCATCCA-3' (sense) and 5'-CAGCCTTGAGCACCAGTTTG-3' (antisense). Human actin (sense, 5'-GTCCACCGCAAATGCTCTA-3'; antisense, 5'-TGCTGTACCTTCACCGTTC-3') was also amplified as the internal control. Gene expression of both targets was determined as the expression relative to the control using the comparative cycle threshold method.

#### Western blotting

The expression of BAX and BCL-2 proteins after berberine treatment was studied by western blotting. Whole cell lysate (40 mg) from cancer cell lines, which were treated with berberine hydrochloride of concentration equivalent to the respective  $IC_{50}$  for different time points (0, 6, 12, 24, and 36 h), was resolved on 10% SDS-polyacrylamide gels and electroblotted on polyvinylidene fluoride membranes (Roche Applied Science, Germany). The blots were blocked overnight with 5% nonfat dry milk and probed with primary antibodies at dilutions recommended by the suppliers. Immunoblots were detected by horseradish peroxidase-conjugated secondary antibody (Pierce) using a chemiluminescence kit (Pierce) and photographed.

#### Statistical analysis

Data were analyzed using statistical software SPSS, and were presented as mean  $\pm$  standard deviation from at least three independent experiments.

**Table I.** The  $IC_{50}$  values for the killing of different cancer cells by berberine as determined by MTT assay

Cell lines	$IC_{50}$ [ $\mu$ M]
Tca8113	218.52 $\pm$ 18.71
CNE2	249.18 $\pm$ 18.14
MCF-7	272.15 $\pm$ 11.06
Hela	245.18 $\pm$ 17.33
HT29	52.37 $\pm$ 3.45

## Results

### Berberine suppressed cancer cell viability

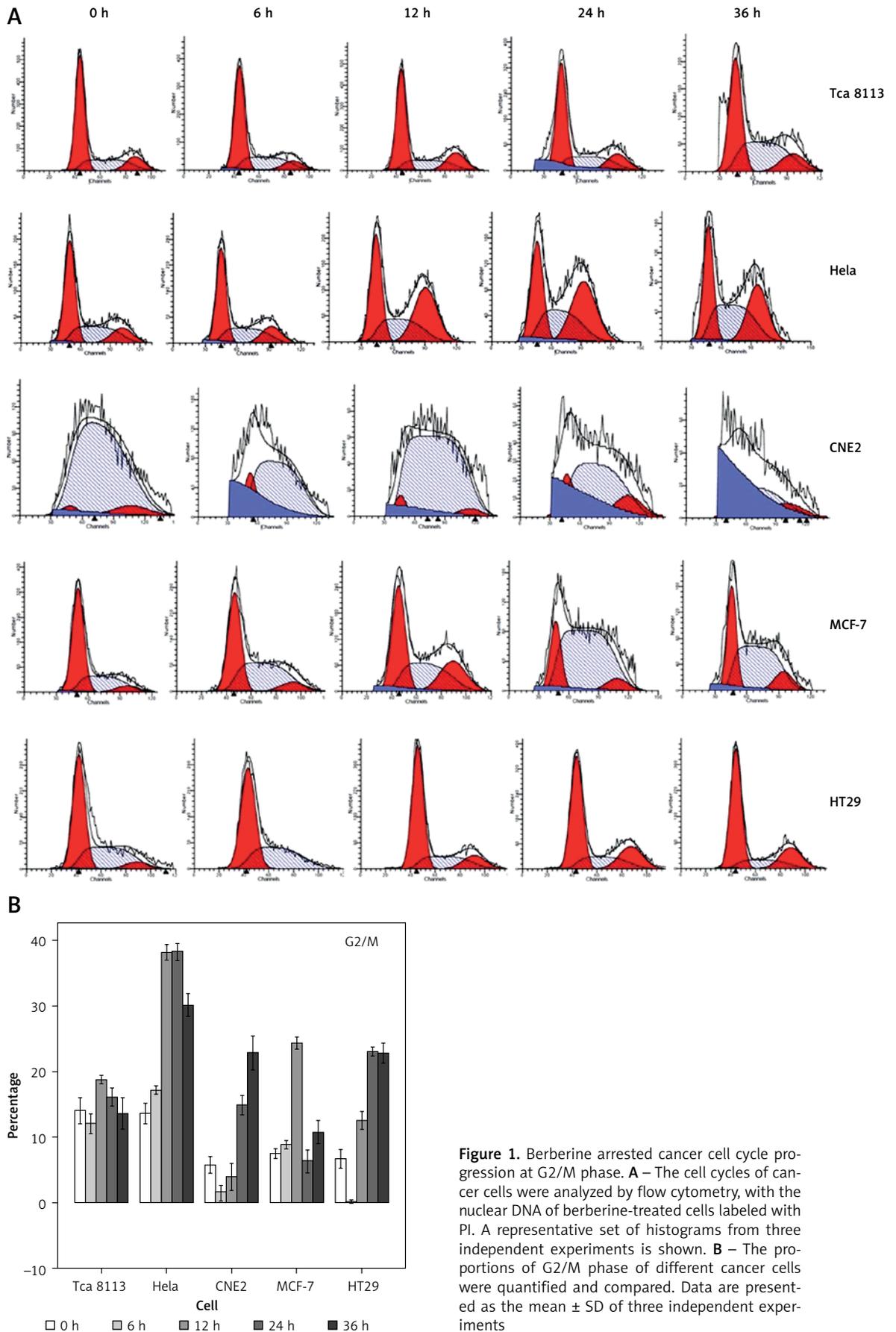
The suppression of cancer cell viability by berberine was studied using MTT viability assay after the cancer cells were incubated with berberine for 48 h. Berberine displayed an *in vitro* cytotoxic effect on all the tested cell lines. The  $IC_{50}$  of berberine treatment for each cell line was determined and compared (Table I). Colon cancer cell line HT29 was the most sensitive one among the five cell lines tested, with its  $IC_{50}$  measuring 52.37  $\pm$ 3.45  $\mu$ M. The  $IC_{50}$  values of the remaining four cell lines were comparable to each other.

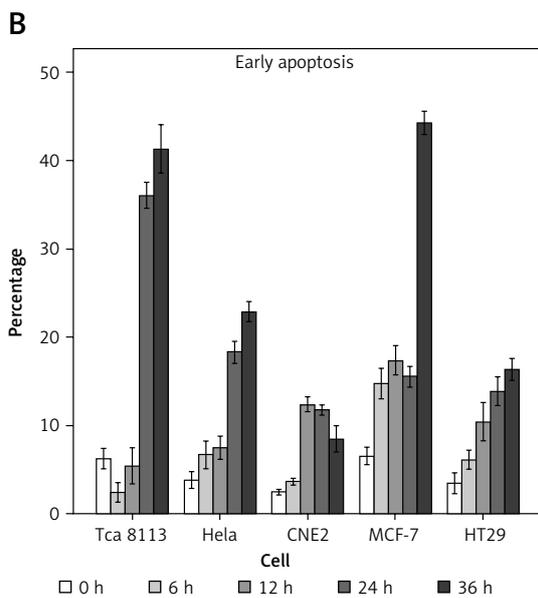
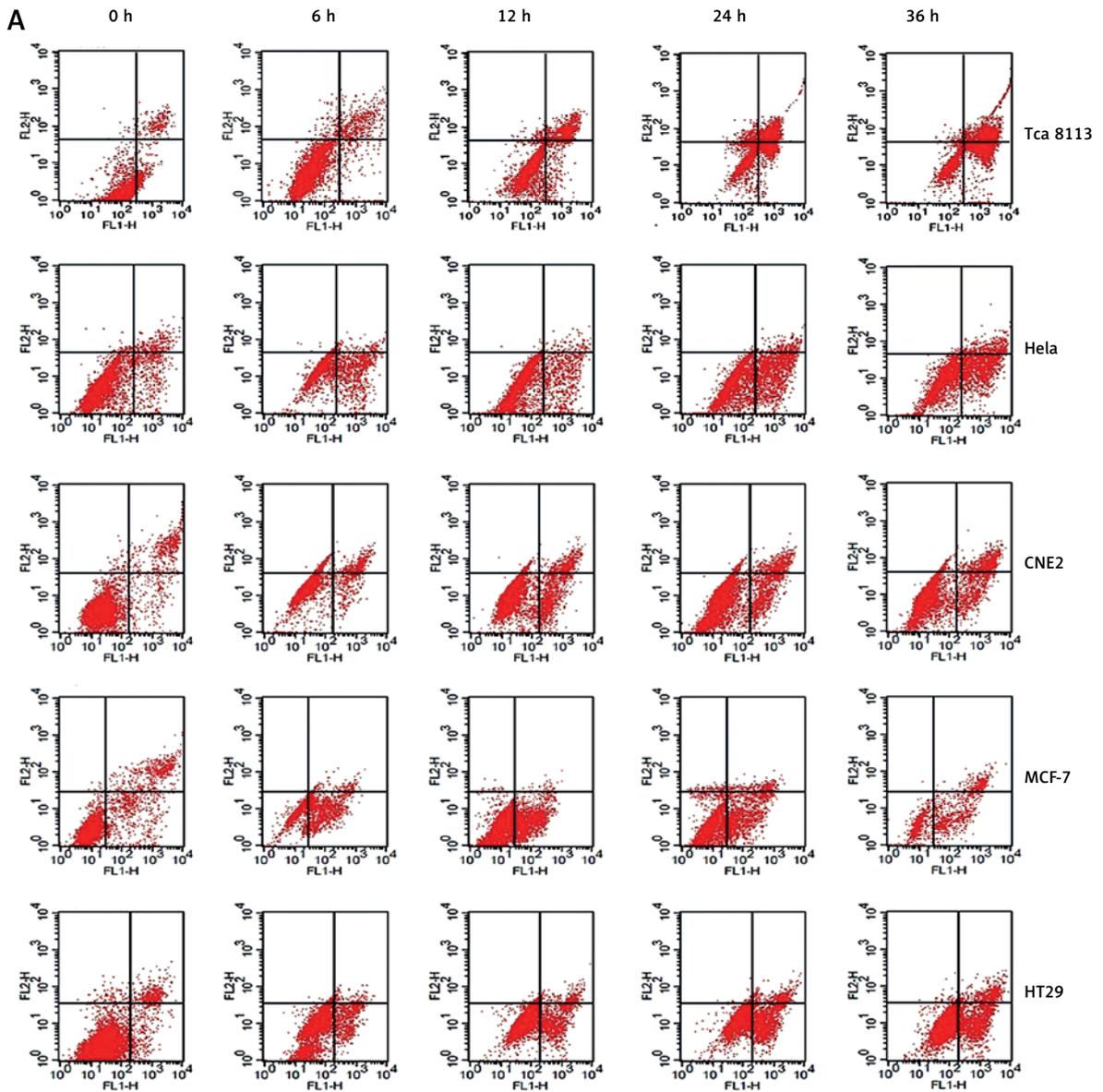
### Berberine induced G2/M-phase arrest

Since the treatment of berberine resulted in suppression in cancer cell viability, we examined whether berberine would affect the cell cycle of different cancer cells by PI staining (Figure 1 A). The proportion of cell cycle phases was determined, and results showed that berberine could induce cell arrest at the G2/M phase (Figure 1 B). For Tca8113 and MCF-7, the maximal arrests were seen after berberine treatment for 12 h. For Hela, CNE2, and HT29, the maxima arrest was observed at 24, 36, and 24 h, respectively.

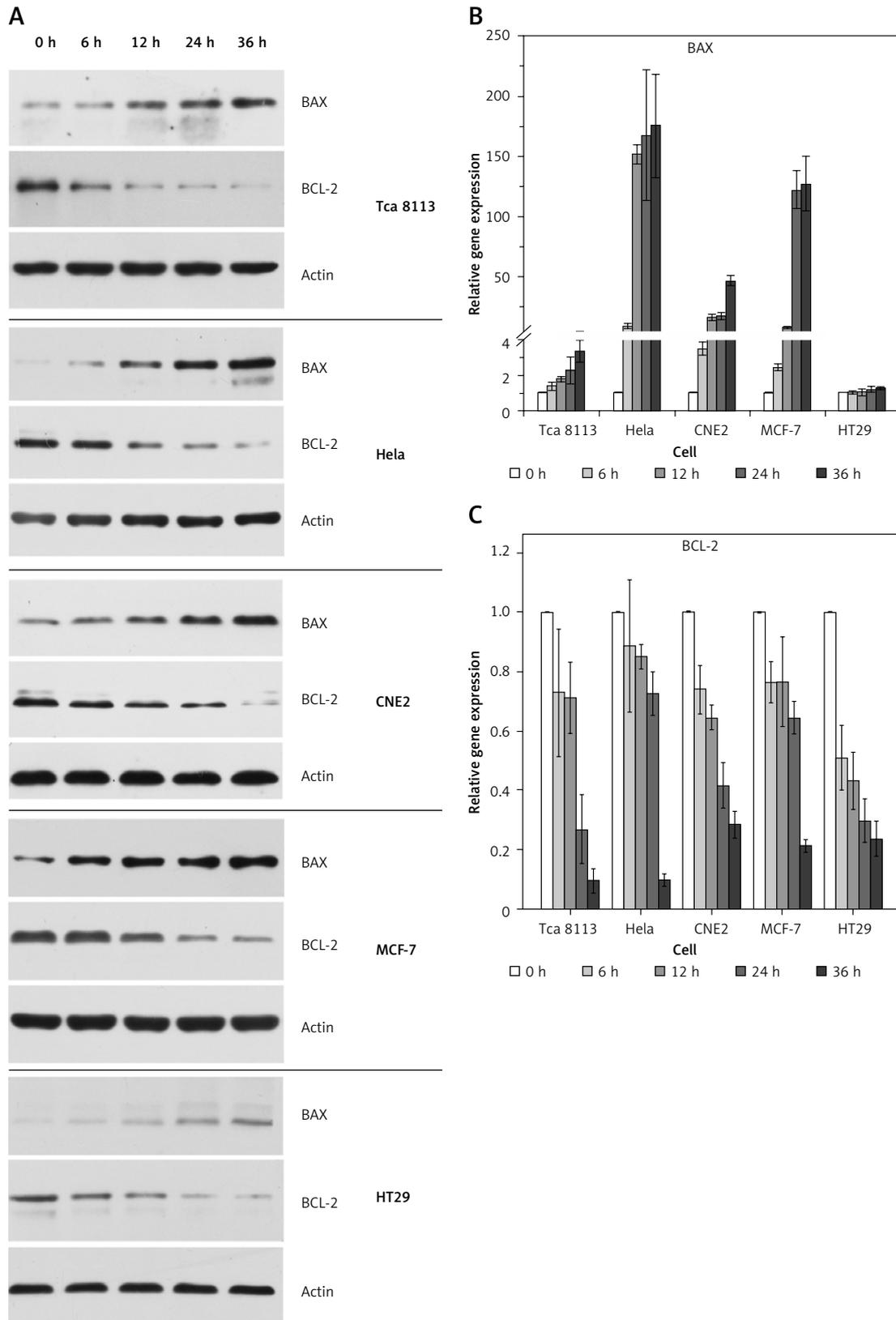
### Berberine triggered early apoptosis

We also performed annexin V staining to examine the induction of apoptosis in cancer cells by berberine treatment (Figure 2 A). The annexin V-positive cells were quantified and the results demonstrated that apoptosis was increased by berberine treatment in all the cell lines tested in a time-dependent manner (Figure 2 B). To delineate the molecular mechanism by which berberine induced apoptosis in cancer cells, we used western blotting to examine the protein expression of apoptotic regulators BAX and BCL-2 in berberine-treated cancer cell lines (Figure 3 A). It was clearly demonstrated that berberine treatment could increase BAX protein expression; the elevation was accompanied with significant suppression of BCL-2. The effect of berberine on the protein expression of BAX and BCL-2 was time-dependent. To investigate whether berberine would also regulate BAX and BCL-2 at the transcriptional level, real-time PCR was employed to determine the gene expression of BAX and BCL-2 after treatment of different cancer cells with berberine (Figure 3 B). For BAX, its gene expression was substantially elevated by berberine treatment in all cell lines tested except in HT29, while for BCL-2, its gene expression was significantly suppressed in all cell lines tested. Again, the effect of berberine





**Figure 2.** Berberine induced apoptosis in multiple cancer cells. **A** – Plots of sorted apoptotic cells. Early apoptotic cells (annexin V+ and PI-) are displayed in the lower right quadrant and late apoptotic cells (annexin V+ and PI+) are shown in the upper right quadrant. A representative set of sorted apoptotic cell plots from three independent experiments is shown. **B** – The early apoptotic cells were counted by flow cytometry. Data are presented as the mean  $\pm$  SD of three independent experiments



**Figure 3.** Berberine modulated the expression of BAX and BCL-2 in multiple cancer cells. **A** – Five cancer cells were exposed to berberine hydrochloride (respective  $IC_{50}$  for each cell line) for 0, 6, 12, 24 and 36 h. The protein levels of BAX and BCL-2 were determined by Western blotting assays. The representative set of western blotting from three independent experiments is shown. **B** – Relative mRNA expression of BAX at indicated time points. **C** – Relative mRNA expression of BCL-2 at indicated time points. Data are presented as the mean  $\pm$  SD of three independent experiments

on the gene expression of BAX and BCL-2 was time-dependent.

## Discussion

The present study demonstrated the anti-cancer property of berberine on multiple cancer cell lines including oral squamous cell carcinoma, nasopharyngeal carcinoma, breast cancer, cervical carcinoma, and colon cancer. Our in-depth mechanistic studies suggested that the anti-cancer property of berberine was apparently attributed to its ability to trigger apoptosis and to induce cell cycle arrest at G2/M phase. Treatment of cancer remains challenging, despite the recent advents in targeted therapy and immunotherapy. New therapeutic agents for cancer treatment are urgently needed. Over the last decades, in the search of new therapeutic agents, many naturally occurring dietary compounds have been shown to display profound anti-cancer properties with minimal toxicity in various *in vitro* and animal models [17–19].

Berberine, which is an alkaloid that exists as the major bioactive compound in many medicinal herbs, is one of the naturally occurring compounds that attract intense investigations. The anti-cancer effects of berberine have been characterized, suggesting that the compound could inhibit DNA and protein synthesis and arrested cell cycle progression [20–24]. In line with these studies, we further demonstrated the anti-cancer effect of berberine on different cancer cell lines including Tca8113, Hela, CNE2, MCF-7 and HT29 cells. In these cell lines, berberine prevented cell cycle progression at G2/M phase. The arrest of the cell cycle at G2/M by berberine was also reported in BALB/3T3 cells [25, 26], while in murine L1210 cells, berberine arrested the cell cycle at G0/G1 phase [27]. Our data together with the published findings collectively suggest that the regulation of the cell cycle by berberine would be cell type-dependent.

Induction of apoptosis represents one of the key mechanisms of action of chemotherapy, radiotherapy and immunotherapy [28–30]. Apoptosis is a tightly regulated process involving many anti- and pro-apoptotic proteins. Overexpression of anti-apoptotic members of the Bcl-2 family has been implicated in the chemoresistance of cancers [31–33], whereas high levels of pro-apoptotic proteins, e.g. BAX, promote apoptosis and sensitize tumor cells to various anticancer therapies [19, 20, 34]. In this context, whether cancer cells would be committed to apoptosis partly depends upon the balance between anti- and pro-apoptotic proteins. Our present study clearly showed that berberine treatment could induce apoptosis in multiple cancer cell lines by increasing BAX and decreasing BCL-2 expression. The consistent changes in expression of BAX and BCL-2 indicated

that the BAX/BCL-2 signaling pathway may represent a common mechanism of berberine to inhibit the growth of tumors, which is a promising direction for developing strategies in the treatment of various tumors. The increased BAX might override the protective action of BCL-2 on cancer cells; however, the elevation of BAX alone was found not sufficient to initiate apoptosis in the absence of additional stimuli [25]. The berberine-induced apoptosis likely involved other pro-apoptotic pathways or factors yet to be identified.

In conclusion, our results clearly illustrated the anti-cancer action of berberine on multiple cancer cell lines, covering oral squamous cell carcinoma, nasopharyngeal carcinoma, breast cancer, cervical carcinoma, and colon cancer. Advanced tumors of these cancers are difficult to treat, despite the recent advents in new treatment modalities such as immunotherapy. Berberine holds promise as a drug candidate that can be developed into new cancer drugs with minimal toxicity. Our study and findings from other research groups worldwide collectively suggest that berberine can trigger apoptosis and induce cell cycle arrest in cancer cells. Nevertheless, the pharmacologic actions of berberine will require further in-depth investigation in different animal models of cancers.

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## Conflict of interest

The authors declare no conflict of interest.

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