Oroxylin A induces G_2/M phase cell-cycle arrest via inhibiting Cdk7-mediated expression of Cdc2/p34 in human gastric carcinoma BGC-823 cells

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Abstract

We reported previously that oroxylin A, a natural product isolated from Scutellariae Radix, was a potent apoptosis inducer of human hepatoma HepG2 cells. In this study, cell-cycle arrest of BGC-823 human gastric carcinoma cells caused by oroxylin A has been investigated. Based on our 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay and flow cytometric analysis, treatment of BGC-823 cells with growth suppressive concentrations of oroxylin A caused an irreversible arrest in the G_2/M phase of the cell cycle. Western blot analysis demonstrated that oroxylin A-induced cell-cycle arrest in BGC-823 cells was associated with a significant decrease in cdc2/p34, cyclin B1 and cyclin A expression. In addition, oroxylin A-treated cells decreased the expression of Cdk7, which was responsible for the low expression of M phase promoting factor (cyclin B1/Cdc2). The results suggested that oroxylin A induced G2/M phase cell-cycle arrest via inhibiting Cdk7-mediated expression of Cdc2/p34 in human gastric carcinoma BGC-823 cells.

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Introduction

Flavonoids are one type of polyphenols including flavones, isoflavones and flavanones (Lee et al 2002). *Scutellaria* plants are known to contain large amounts of flavonoids. Two flavonoid-rich *Scutellaria* species, *S. baicalensis* Georgi and *S. rivularis* Wall, are important medicinal plants in the traditional medicine of China and Japan. Scutellariae Radix, the root of *S. baicalensis*, is a conventional herbal medicine widely used in traditional herbal preparations in these two countries. In Chinese medicine it is prescribed as an antipyretic, analgesic, anti-inflammatory and antitumour agent (Horinaka et al 2006). Flavonoids, the chief ingredients found in Scutellariae Radix, are well-known for their physiological anti-inflammatory, anti-allergy activity. Furthermore, some flavonoids found in this plant inhibit the promotion of skin tumours, and baicalin, which is the main ingredient in *S. baicalensis*, inhibited the proliferation of several human prostate cancer cells by inducing apoptosis (Chen et al 2001; Russo et al 2003; Sonoda et al 2004; Yoshimizu et al 2004).

Oroxylin A, a naturally occurring monoflavonoid extracted from Scutellariae Radix, has been shown to be a promising candidate for selective and effective management of inflammation (Sonoda et al 2004; Kim et al 2006). Chen et al (2000) reported that oroxylin A inhibited lipopolysaccharide (LPS)-induced expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in a concentration-dependent manner. The inhibitory effects of oroxylin A on LPS-induced iNOS and COX-2 gene expression were also demonstrated in Bcl-2-overexpressing RAW264.7 macrophages. Furthermore, oroxylin A blocked nuclear factor-κB (NF-κB) binding and transcriptional activation associated with decreased p65 proteins in the nucleus induced by LPS. Hu et al (2006) demonstrated for the first time that oroxylin A could exert a pro-apoptotic effect on HepG2 cells. Oroxylin A treatment significantly reduced the expression of Bcl-2 and pro-caspase-3 protein of HepG2 cell in a concentration-dependent manner. It was also found that the proportion of G_2/M phase arrest cells increased dramatically after treatment with oroxylin A. However, the exact mechanism remains unknown. In this study, we have focused on the G_2/M cell-cycle arrest of human gastric carcinoma cells BGC-823. Our results suggested that the inhibition of

cell-cycle progression by oroxylin A was likely attributed to the modulation of the expression of Cdc2 and Cdk7 protein, which was critical to G_2 progression and G_2/M transposition (Yu et al 2007).

Materials and Methods

Reagents

Oroxylin A was isolated from Scutellariae Radix according to the protocols reported by Li & Chen (2005), with slight modifications. Samples containing 99% or higher oroxylin A were used in all experiments unless otherwise indicated. Oroxylin A was applied in dimethyl sulfoxide (DMSO) to a final concentration of 100 mM and stored at $-20°C$. The maximum concentration used here was 200 μM and freshly diluted to the basal medium with a final DMSO concentration at 0.2%. Controls were always treated with the same amount of DMSO as used in the corresponding experiments. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was purchased from Fluka (US) and was dissolved in 0.01 M phosphate-buffered saline (PBS). Primary antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and IRDye800 conjugated anti-goat and anti-rabbit second antibodies were obtained from Rockland Inc. (Philadelphia, PA, USA). CycleTest Plus DNA reagent kit was obtained from Becton Dickinson (Mountain View, CA, USA).

Cell culture

BGC-823 cells were purchased from the Cell Bank of Shanghai Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were grown in 90% RMPI-1640 (lot no. 1313804; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Sijiqing, Hangzhou, China), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Exponentially growing cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Colorimetric MTT assay

BGC-823 was cultured in RMPI-1640 according to the protocols described by Guo et al (2006). Oroxylin A (0.2, 0.4, 0.82, 1.18, 1.68, 2.40, 3.43, 4.90, 7.00 or 10.0×10^{-4} M) was added, respectively. After incubating for 24, 48 or 72 h, the culture medium was removed and 20 μL 5 mg mL^{-1} MTT was added. The supernatant was discarded 4 h later and 100 μL DMSO was added to each well. The mixture was shaken and measured at 570 nm using a Universal Microplate Reader (EL800, Bio-Tek Instruments Inc.). Cell inhibition ratio (I%) was calculated by the following equation:

$$I\% = ((A_{control} - A_{treated})/A_{control}) \times 100$$

where $A_{treated}$ and $A_{control}$ were the average absorbance of three parallel experiments from treated and control groups, respectively. The IC50 value was taken as the concentration that caused 50% inhibition of cell proliferation and was calculated by SAS statistical software.

Cell morphological assessment

Oroxylin A (2×10^{-4} M) was added and incubated for an additional 24, 36 or 48 h. The morphology of the cells was monitored under an inverted light microscope.

DNA content and cell cycle analysed by flow cytometry

Cells were treated with oroxylin A (2×10^{-4} M) for 24, 36 or 48 h. The cells were washed with cold PBS and resuspended in hypotonic propidium iodide (PI) staining solution (0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100, 0.05 mg mL^{-1} PI, 0.01 mg mL^{-1} RNase), and kept from light for 30 min at 4°C. Cells were analysed on a FACScan flow cytometer (Becton Dickinson) and the percentage of cells in each phase of the cell cycle was quantitated using ModFit software (Becton Dickinson). Aggregates were excluded from the analysis by use of the doublet discrimination module and subsequent gating on the linear red fluorescence area and width parameters.

Western blot analysis for cell-cycle regulatory proteins

After incubation with 2×10^{-4} M oroxylin A for 24, 36 or 48 h, proteins were isolated by lysis buffer (100 mM Tris-HCl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol, 200 mM β-mercaptoethanol, 1 mM PMSF, and 1 g mL^{-1} aprotinin) and measured using the Bradford assay with BioPhotometer (BioPhotometer 6131 GB/HK, Eppendorf) at 595 nm. Protein samples were separated with 12% SDS-polyacrylamide gel (SDS-PAGE) and transferred onto the PVDF membranes (Millipore). Immune complexes were formed by incubation of the proteins with primary antibodies, rabbit anti-cyclin B1, mouse anti-Cdc2, rabbit anti-cyclin A, rabbit anti-Cdk7 and mouse anti-actin overnight at 4°C. Blots were washed and incubated for 1 h with IRDye800 conjugated anti-mouse and anti-rabbit second antibodies. All antibodies were obtained from Santa Cruz Biotechnology. Immunoreactive protein bands were detected with an Odyssey Scanning System (LI-COR Inc., Nebraska, USA).

Statistical evaluation

All results shown represent the means ± s.d. from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical analyses were performed using a Kruskal–Wallis test. All comparisons were made relative to untreated controls and a significance of difference was indicated as $*P < 0.05$ and $**P < 0.01$.

Results

Cell growth inhibition assessed by colorimetric MTT-assay

The cytotoxicity of oroxylin A on BGC-823 cells was assessed by the MTT assay. Oroxylin A treatment exhibited a marked inhibition on the survival of HepG2 BGC-823 cells time- and dose-dependently. The IC50 values of oroxylin A were 3.21 ± 0.36, 1.05 ± 0.15 and $0.51 \pm 0.10 \times 10^{-4}$ M obtained for 24, 48 and 72-h treatment, respectively (Figure 1).
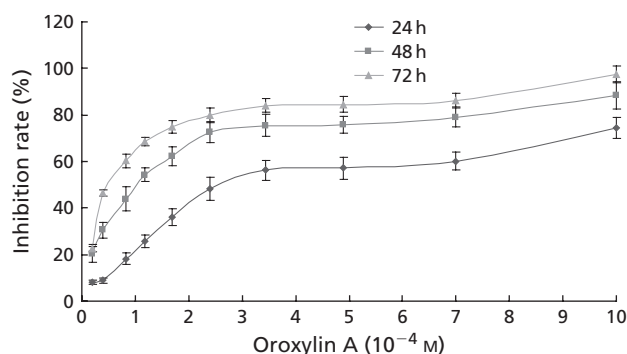
Figure 1 Inhibitory effects of oroxylin A on the variability of BGC-823 cells. Data shown are mean ± s.d., n = 3.

Cell morphological assessment

Inverted-microscopy demonstrated that the majority of BGC-823 cells were severely distorted (elongated), forming long filaments, while some cells turned round in shape after treatment with oroxylin A 2×10^{-4} M for 24, 36 or 48 h. The untreated cells displayed a normal healthy shape, demonstrated by the clear skeletons observed by the inverted-microscope (Figure 2).

DNA content and cell cycle analysed by flow cytometry

To test whether oroxylin A could affect the cell cycle of BGC-823 cells, synchronized cells treated with DMSO or oroxylin A 2×10^{-4} M for 24, 36 or 48 h were subjected to flow cytometric analysis after DNA staining. The effects of oroxylin A on BGC-823 cell-cycle distribution are summarized in Table 1. BGC-823 cells treated with oroxylin A 2×10^{-4} M for 24, 36 or 48 h resulted in the proportion of G₂/M phase cells significantly increasing in a time-dependent manner.

Table 1 Cell cycle analysis of BGC-823 cells treated with oroxylin A

Groups		Cell cycle		
		G_0/G_1 (%)	S (%)	G_2/M (%)
Control		68.55 ± 4.52	22.82 ± 2.65	8.63 ± 2.97
Oroxylin A	24 h	46.63 ± 4.98	40.44 ± 3.52	12.92 ± 3.44
	36 h	50.45 ± 6.55	30.38 ± 4.85	19.17 ± 2.15*
	48 h	41.45 ± 4.24	26.70 ± 3.87	31.78 ± 2.98**

Cell cycle analysis was performed after incubation with oroxylin A 2×10^{-4} M for 24, 36 or 48 h as described in Materials and Methods. The experimental data presented were at least three independent experiments. Data were presented as means ± s.d., *P < 0.05, **P < 0.01.

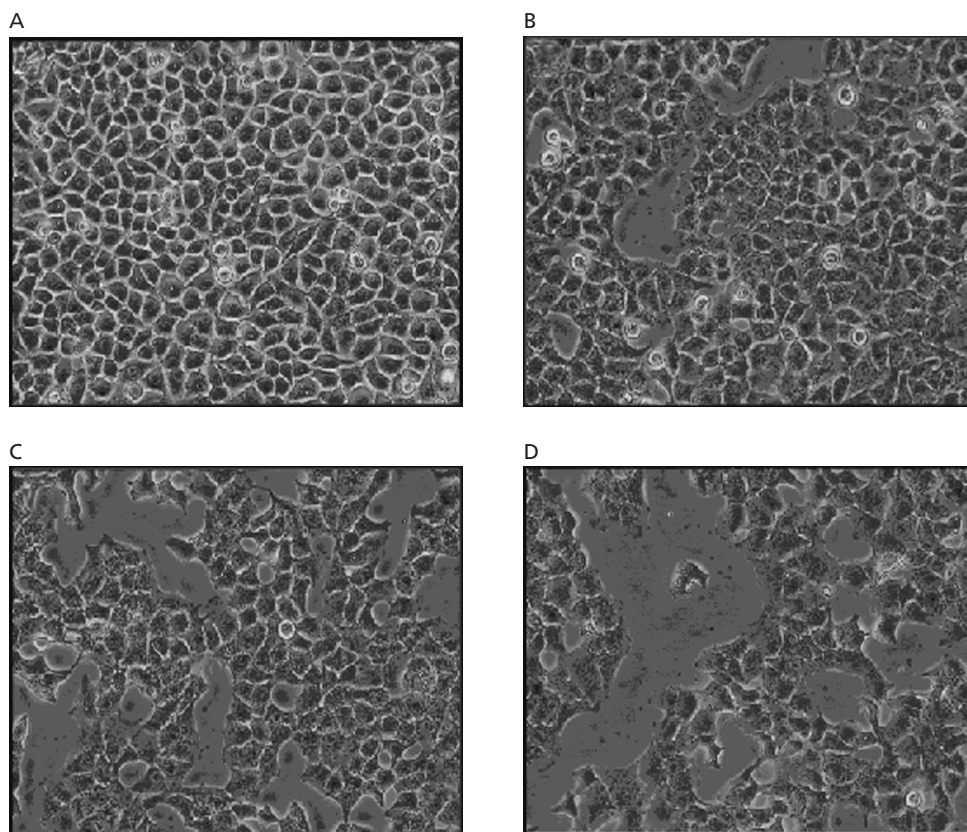**Figure 2** BGC-823 cell morphological changes under inverted-microscope. A. Control. B. BGC-823 cells treated with oroxylin A 2×10^{-4} M for 24 h. C. BGC-823 cells treated with oroxylin A 2×10^{-4} M for 36 h. D. BGC-823 cells treated with oroxylin A 2×10^{-4} M for 48 h (× 100).
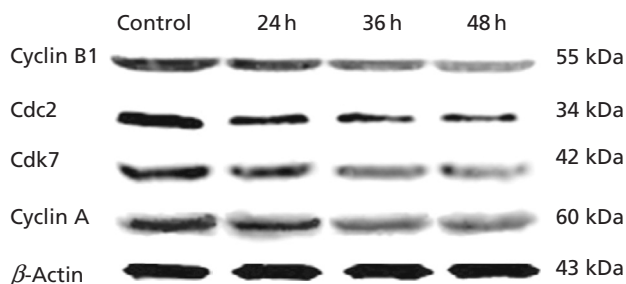
Figure 3 Effect of oroxylin A on the expression of cyclin B1, Cdc2, Cdk7 and cyclin A proteins in BGC-823 cells. BGC-823 cells were treated with oroxylin A 2×10^{-4} M for 24, 36 or 48 h.

Expressions of cell-cycle regulatory proteins

Cyclins are regulatory subunits which associate with kinases to form complexes that control many of the important steps in cell-cycle progression (King et al 1994). The best characterized of the cyclin-containing complexes is the association of cyclin B with Cdc2/p34 kinase. Western blot analysis was used to detect further the expression of cell-cycle regulatory proteins. After incubation with 2×10^{-4} M oroxylin A for 24, 36 or 48 h, the expression of cyclin B1, cyclin A, Cdc2 and Cdk7 protein decreased dramatically in a time-depended manner (Figure 3).

Discussion

In recent years, more and more researchers have focused on the effects of flavonoids in cancer treatment. We reported previously that wogonin, a flavonoid compound, was a potent apoptosis inducer of human hepatoma SMMC-7721 cells and murine sarcoma S_{180} cells (Wang et al 2006a, b). We demonstrated that oroxylin A may have inhibited Bcl-2 to trigger apoptosis signalling and G_2/M phase cell cycle that mediated the growth inhibition of HepG2 (Hu et al 2006). In this study, we have focused on illustrating the regulatory effect and mechanism of oroxylin A on the cell cycle of human gastric carcinoma BGC-823 cells.

Our results suggested that BGC-823 cells treated with three dose of oroxylin A (50-150 μM) for 48 h caused a dramatic increase in the G_2/M phase from 13.39% to 24.10%, while 100 and 150 μM had no distinguished variance (data not shown). When the dose of oroxylin A was increased to 200 μM, the proportion of G_2/M phase cells increased from 8.63% to 31.78% in a time-dependent manner. Research on the mechanism of G_2/M phase arrest via Western blot revealed that expression of cyclin B1, Cdc2/p34 and Cdk7 proteins decreased after incubation with oroxylin A, consistent with the results of flow cytometric analysis. Moreover content of cyclin A, which also plays an important role in the G_2 phase, presented a low level after incubation with oroxylin A.

Cyclins are regulatory subunits which associate with kinases to form complexes that control many of the important

steps in cell-cycle progression (King et al 1994). The best characterized of the cyclin-containing complexes is the association of cyclin B with Cdc2/p34 kinase. The human Cdc2 protein kinase, Cdc2/p34, represents the homologue of the $Cdc2^+$/Cdc28 yeast protein kinase. This 34 kDa polypeptide exhibits protein kinase activity in-vitro and exists in a complex with both cyclin B and a 13 kDa protein that is homologous to p13Suc1. Cdc2 kinase is the active subunit of the M phase promoting factor (MPF) and the M phase-specific histone H1 kinase. The p34Cdc2/cyclin B complex is required for the regulation of G_2 progression and G_2/M transition in all eukaryotic cells. Cyclin A accumulates before cyclin B in the cell-cycle, appears to be involved in control of S phase and has been shown to associate with cyclin-dependent kinase-2 (Nurse 1990). In addition, cyclin A has been implicated in cell transformation and is found in complexes with E1A, transcription factors DRTF1, E2F and retinoblastoma protein, p110. A second form of cyclin A, named cyclin A1 because of its high sequence homology to *Xenopus* cyclin A1, is most highly expressed in germ cells. It has been proposed that cyclin A1 can associate with Cdk2, p39 and Cdc2/p34 (Margolis et al 2006).

Progression through the cell cycle requires activation of a series of enzymes designated cyclin dependent kinases (Cdks) (Morgan 1995). The monomeric catalytic subunit Cdk2, a critical enzyme for initiation of cell-cycle progression, is completely inactive. Partial activation is achieved by the binding of regulatory cyclins such as cyclin D1, while full activation requires additional phosphorylation at Thr 160. The enzyme responsible for the phosphorylation of Cdk2 on Thr 160 and also of Cdc2/p34 on Thr 161, designated Cdk-activating kinase (CAK), has been partially purified and shown to be comprised of a 42 kDa catalytic subunit and a 37 kDa regulatory subunit. The catalytic subunit, designated Cdk7, has been identified as the mammalian homologue of MO15, a protein kinase demonstrated in starfish and *Xenopus*. The regulatory subunit is a novel cyclin (cyclin H) and is required for activation of Cdk7. Like other Cdks, Cdk7 contains a conserved threonine residue for full activity; mutation of this residue severely reduces CAK activity (Shiekhattar et al 1995).

In conclusion, the G_2/M phase arrest of oroxylin A-treated BGC-823 cells was associated with the decreased production of Cdk7 protein, which was responsible for the low level of Cdc2/p34 expression and cell-cycle arrest. The kinase activity of Cdk7 and Cdc2 should be investigated further, as should p53, phosphorylation of Thr^{14}/Tyr^{15} Cdc2/p34 (inactive), dephosphorylation of Thr^{16} Cdc2/p34 (active) and DNA damage checkpoint chk1/2.

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