The role of Nrf2 and apoptotic signaling pathways in oroxylin A-mediated responses in HCT-116 colorectal adenocarcinoma cells and xenograft tumors

Rong Hu, Nan Chen, Jing Yao, Qing Zhao, Fengyi Zhang, Zhi-Yu Li, Qi-Dong You and Qing-Long Guo

Oroxylin A is a flavonoid found in the roots of *Scutellaria baicalensis* Georgi, a herbal medicine commonly used as an antipyretic, analgesic, antitumor, and anti-inflammatory agent. It has recently been investigated for its anticancer activities in hepatoma, gastric, and breast tumors. Here, we investigated the antitumor effects of oroxylin A in human colon carcinoma HCT-116 cells *in vitro* and *in vivo*. We characterized the proapoptotic effect of oroxylin A using diamidino-phenyl-indole (DAPI) and annexin V/PI staining. We then found that both caspase-3 and caspase-9 were activated, the expression of Bcl-2 protein decreased, and the expression of Bax protein increased after treatment with oroxylin A. In addition, oroxylin A increased nuclear transcription factor erythroid-related factor 2 (Nrf2) expression and induced Nrf2 translocation into the nucleus. Furthermore, we found that oroxylin A treatment elevated intracellular reactive oxygen species levels and increased the protein expression level of two of the Nrf2 target genes heme oxygenase-1 and NADP(H):quinone oxidoreductase-1 in HCT-116 cells. Finally, our study demonstrated that oral administration of oroxylin A significantly decreased tumor volume and weight in immunodeficient mice that were inoculated with HCT-116 cells. The in-vivo chemopreventive efficacy of oroxylin A against HCT-116 human colon cancer was accompanied by its proapoptotic and Nrf2-inducing activities, which correlates with the in-vitro study. This is the first demonstration of oroxylin A-dependent chemoprevention in colon cancer and may offer a potential mechanism for its anticancer action *in vivo*. *Anti-Cancer Drugs* 23:651–658 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Despite the tremendous progress in the early detection and treatment of cancer, the overall mortality rates for most cancers of epithelial origin have not declined in the past three decades [1]. Thus, considerable attention has been paid to cancer prevention in recent years. Studies have proven that this strategy is indeed effective in reducing the incidence of cancer in well-defined high-risk groups [2,3]. Chemopreventive compounds offer great potential in the fight against cancer by inhibiting the carcinogenesis process through the regulation of cell-defensive and cell death machineries. To block the initiation of carcinogenesis, enhancement of the detoxifying and antioxidant enzyme system for efficient neutralization and elimination of endogenous or exogenous carcinogenic species represents a good starting point, particularly for high-risk populations exposed to environmental carcinogens. With regard to the latter stages of carcinogenesis, induction of apoptosis and cell cycle arrest in precarcinoma and carcinoma cells is an attractive goal for many dietary antiproliferation agents.

Nuclear transcription factor erythroid-related factor 2 (Nrf2) is an important protein for chemoprevention and is crucial for protection against xenobiotics that cause DNA damage and initiate carcinogenesis [4]. Blocking agents, acting by preventing carcinogens from reaching the target sites, from undergoing metabolic activation, or from subsequently interacting with crucial cellular macromolecules such as DNA, RNA, and proteins at initiation stages, increase Nrf2 activity and activate downstream phase II and antioxidant enzymes. These enzymes detoxify and eliminate harmful reactive intermediates that are formed by carcinogens [5]. Oltipraz, 3*H*-1,2,-dithiole-3-thione, sulforaphane, and curcumin are considered potential chemopreventive agents because these compounds increase Nrf2 activity [6–9].

Oroxylin A ($C_{16}H_{12}O_5$) is a flavonoid isolated from the root of *S. baicalensis* Georgi, a conventional herbal medicine commonly used as an antipyretic, analgesic, antitumor, and anti-inflammatory agent [10–12]. PC-SPES, a proprietary herbal blend containing *S. baicalensis* Georgi, which has been used since 1996 by thousands of men for 'prostate health,' has been demonstrated to have anticancer activities against colon cancer *in vitro* using colon cancer cell lines and *in vivo* using the Apc^{min} mouse

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model [13]. In addition, studies have shown that oroxylin A could be a promising candidate for the selective and effective management of inflammation and cancer. Chen *et al.* [14] reported that oroxylin A inhibited lipopolysaccharide-induced expression of inducible nitric oxide synthase and cyclooxygenase-2 in RAW264.7 macrophages that overexpress Bcl-2. Recently, our laboratory demonstrated that treatment with oroxylin A inhibited the growth of human gastric carcinoma BGC-823 cells by inducing G_2/M cell cycle arrest through the inhibition of Cdk7-mediated Cdc2/p34 expression [15].

In the current study, we showed that oroxylin A has chemopreventive activity against colon cancer; this may be due to its induction of apoptosis and Nrf2 activity. This compound was found to induce apoptosis and increase Nrf2 expression and its translocation to the nucleus in HCT-116 cells. Examination of the Nrf2 target genes heme oxygenase-1 (*HO-1*) and NADP(H):quinone oxidoreductase-1 (*NQO-1*) provided a mechanistic framework for the exploration of oroxylin A as a novel chemopreventive agent for human colon cancer. In addition, the chemopreventive activity of oroxylin A was demonstrated in an in-vivo xenograft immunodeficient mouse model.

Materials and methods

Reagents

Oroxylin A was isolated from the root of *S. baicalens* as previously described [16]. Samples containing oroxylin A at a minimum of 99% purity were used for the experiments unless otherwise indicated. Oroxylin A (200 mmol/l) was dissolved in dimethylsulfoxide (DMSO) and stored at −20°C. Concentrations of 50, 100, and 200 μmol/l were used in this study and were freshly diluted using the basal medium to a final DMSO concentration of 0.1%. Controls were treated with the same amount of DMSO as that used in the corresponding experiments. Antibodies against poly (ADP-ribose) polymerase (PARP), procaspases 3, 8, and 9, Bcl-2, Bax, Lamin A, Nrf2, HO-1, and NQO-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). Antibodies against β-actin (BM0627) were from Boster (Wuhan, China). IRDyeTM800-conjugated secondary antibodies were obtained from Rockland Inc. (Philadelphia, Pennsylvania, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) was purchased from Sigma (St Louis, Missouri, USA).

Cell culture

Human colon carcinoma HCT-116 cells were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Cells were cultured in 90% McCoy's 5A medium (Sigma) supplemented with 10% fetal bovine serum (Sijiqing, Zhejiang, China), 100 U/ml of benzyl penicillin, and 100 μg/ml of streptomycin. Cells were cultured in a humidified environment with 5% CO_2 at 37°C.

Cell viability assay

Cell viability was measured using the colorimetric MTT assay as described previously. Experiments were performed in triplicate and in a parallel manner for each concentration of compound tested, and the data are presented as the mean ± SE. Control cells were treated with culture media alone. After incubation for 24 h, absorbance (A) was measured at 570 nm. The survival ratio (%) was calculated using the following equation: survival ratio (%) = $(A_{\text{treatment}}/A_{\text{control}}) \times 100$. IC_{50} values were measured as the concentration that caused 50% inhibition in cell viability and were calculated using the Logit method.

Diamidino-phenyl-indole staining

HCT-116 cells were cultured in McCoy's 5A medium until they reached the mid-log phase. At that time, cells were treated with 50, 100, and 200 μmol/l of oroxylin A for 24 h. Cell morphology was monitored using an inverted light microscope. Floating and attached cells were harvested with 0.02% (w/v) EDTA and 0.25% (w/v) trypsinase. Cells were then fixed with ice-cold 4% paraformaldehyde for 20 min and washed with ice-cold pPBS before permeabilization with 0.3% Triton X-100. Finally, the cells were washed with ice-cold PBS and stained with fluorochrome dye diamidino-phenyl-indole (Santa Cruz). Fluorescence was observed using a fluorescence microscope (Olympus IX51, Tokyo, Japan) with a peak-excitation wavelength of 340 nm.

Annexin V/propidium iodide staining

Apoptosis was measured using the fluorescein isothiocyanate-labeled annexinV/propidium iodide (PI) Apoptosis Detection kit (Biovision, MoutainView, California, USA) according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after supravital staining. The data acquisition and analysis were performed in a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences, San Jose, California, USA) using CellQuest software (Becton Dickinson, San Jose, California, USA). Cells that were in the early stages of apoptosis were annexin V positive, whereas cells that were both annexin V and PI positive were in the late stage of apoptosis.

Western blot analysis

After treatment with 100 μmol/l of oroxylin A for 15 min, 30 min, 1 h, 2 h, and 4 h, HCT-116 cells were collected and lysed in 1 ml of cold lysis buffer (100 mmol/l of Tris-Cl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol, 200 mmol/l of β-mercaptoethanol, 1 mmol/l of phenylmethylsulfonyl fluoride, and 1 g/ml of laprotinin). Lysates were transferred to a 1.5-ml microcentrifuge tube and centrifuged at 12 000*g* for 15 min at 4°C. Protein concentration was determined using the Bradford method (Bio-Rad, Richmond, California, USA). Equal amounts of protein samples

(40 μg) were subjected to electrophoresis on a 12% SDS polyacrylamide gel. The separated proteins were transferred onto nitrocellulose membranes (Millipore, Billerica, Massachusetts, USA) that were blocked with 10% nonfat milk (w/v) in PBS for 1 h at 37°C and incubated with primary antibodies in PBS containing 0.1% Tween-20 buffer (PBST) for 1 h at 37°C or overnight at 4°C. The membranes were washed three times with PBST and incubated with the IRDye800-conjugated secondary antibody for 1 h at 37°C, followed by washing three times with PBST. Signal detection was achieved using the two-color infrared imaging system (Odyssey, LI-COR Biosciences, Lincoln, Nebraska, USA). All blots were stripped and reprobed with polyclonal anti-β-actin antibody to ascertain equal loading of protein.

Nuclear and cytoplasmic extraction

HCT-116 cells were passaged 24 h before treatment with 100 μmol/l of oroxylin A. After treatment, cells were harvested by centrifugation and washed twice with PBS. Cells were then lysed on ice in three volumes of cytoplasmic extraction buffer. Nuclear and cytosol lysates were isolated separately using a Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, California, USA) according to the manufacturer's instructions. The protein concentration of the nuclear and cytoplasmic extracts was determined by a Bio-Rad protein assay dye using the Bradford method. Extracts were stored at −70°C until further experimentation.

Measurement of intracellular reactive oxygen species

Reactive oxygen species (ROS) production was monitored by flow cytometry using 2′,7′-dichlorofluorescein-diacetate (DCFH-DA). Samples of 1×10^6 cells were collected and washed with cold PBS, resuspended in PBS, and incubated for 30 min at 37°C with 10 μmol/l of DCFH-DA. Fluorescence was measured at 488 nm (excitation) and 525 nm (emission) using the FACSC alibur flow cytometer.

HCT-116 xenograft tumor study

Six-week-old male BALB/c immunodeficient mice were obtained from the Shanghai Slac Laboratory Animal Limited Company. The animals were housed in sterile filter-capped microisolator cages and provided with a sterilized diet and water. HCT-116 cells (2×10^6/0.2 ml/mouse) were suspended in 90% McCoy's 5A medium and injected subcutaneously into the right flank of each mouse to initiate tumor growth. Oroxylin A was dissolved in a solution (vehicle) containing polyethylene glycol, benzyl alcohol, ethanol, and water (40 : 0.1 : 10 : 49.1). Twenty-four mice were randomly separated into four groups (six mice/group). Oroxylin A was administered by an oral gavage at 50, 100, and 200 mg/kg every day beginning a week before injection of the tumor cells. The mice were killed 3 weeks after injection of the tumor cells. Body weight and diet consumption were recorded twice weekly throughout the study. After xenografts started growing, their sizes were measured every other day. Tumor volume was calculated according to the equation: '$0.5236\,L_1(L_2)^2$, where L_1 is the long axis and L_2 is the short axis of the tumor'. At euthanasia, tumors were excised, weighed, and stored at −80°C until further analysis. Animal care was in accordance with the approved protocol and institutional guidelines.

For western blot analysis, tumor samples were crushed into a powder with a mortar and pestle. After evaporation of most of the nitrogen, powdered tissues were lysed with lysis buffer (10 mmol/l of Tris–HCl, pH 7.4, 50 mmol/l of sodium chloride, 30 mmol/l of sodium pyrophosphate, 50 mmol/l of sodium fluoride, 100 mmol/l of sodium orthovanadate, 2 mmol/l of iodoacetic acid, 5 mmol/l of $ZnCl_2$, 1 mmol/l of phenylmethylsulfonyl fluoride, and 0.5% Triton X-100). The lysate was homogenized by passing through a 23-gauge needle three times or sonicating for 10 s, and kept in ice for 30 min The homogenate was centrifuged at 13 000 rpm for 15 min at 4°C. The supernatant was transferred to a clean tube and stored at −80°C until further analysis.

Statistical analysis

All results were presented as means ± SD from duplicate or triplicate experiments performed in a parallel manner. Statistical analyses were performed using analysis of variance. All comparisons were made relative to untreated controls and significant differences were indicated as $^*P < 0.05$ and $^{**}P < 0.01$.

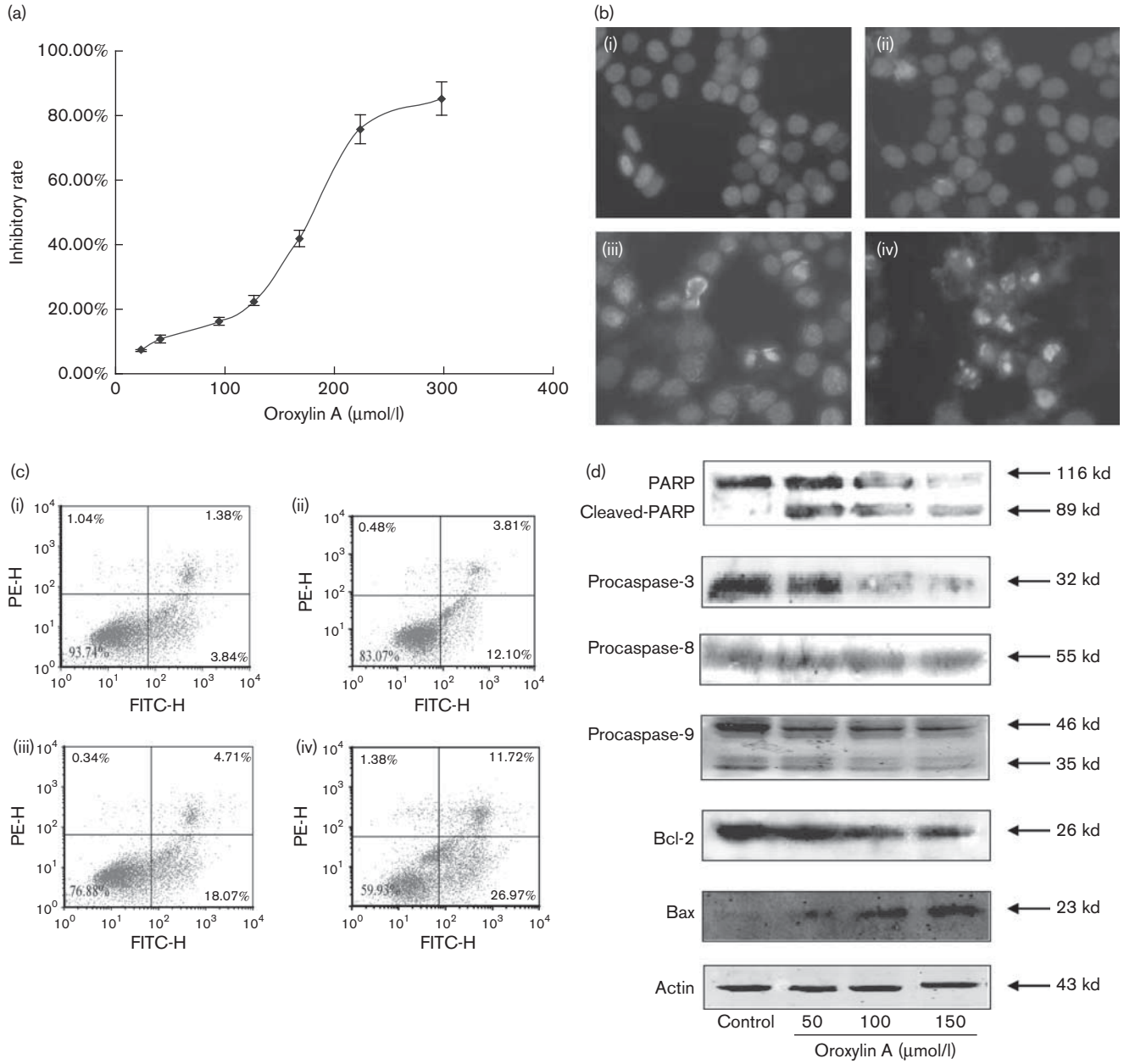
Results

Oroxylin A induced apoptosis in Human colon carcinoma HCT-116 cells

The effects of oroxylin A on HCT-116 cell viability were measured by an MTT assay. As shown in Fig. 1a, cell viability decreased dose dependently in cultures exposed to oroxylin A for 24 h. Cell death was further assayed by diamidino-phenyl-indole staining, which detects nuclear morphology (Fig. 1b). The number of condensed and fragmented nuclei increased with the exposed concentrations of oroxylin A. The annexinV/PI staining assay showed that the percentage of apoptotic cells increased with an increase in concentration, and when cells were treated with 200 μmol/l of oroxylin A for 24 h, the apoptosis rate reached 38.7% (both early and late apoptosis combined, Fig. 1c). These results demonstrated that oroxylin A induced apoptosis in HCT-116 cells in a dose-dependent manner.

Caspase-mediated PARP cleavage likewise showed that oroxylin A-induced apoptosis of HCT-116 cells was a concentration-dependent process (Fig. 1d). We then explored the involvement of caspases in oroxylin A-mediated apoptosis. Immunoblot analysis showed that, compared with the control, both caspase-3 and caspase-9 were activated after oroxylin A treatment for 24 h, whereas caspase-8 remained unchanged. Furthermore,

Fig. 1



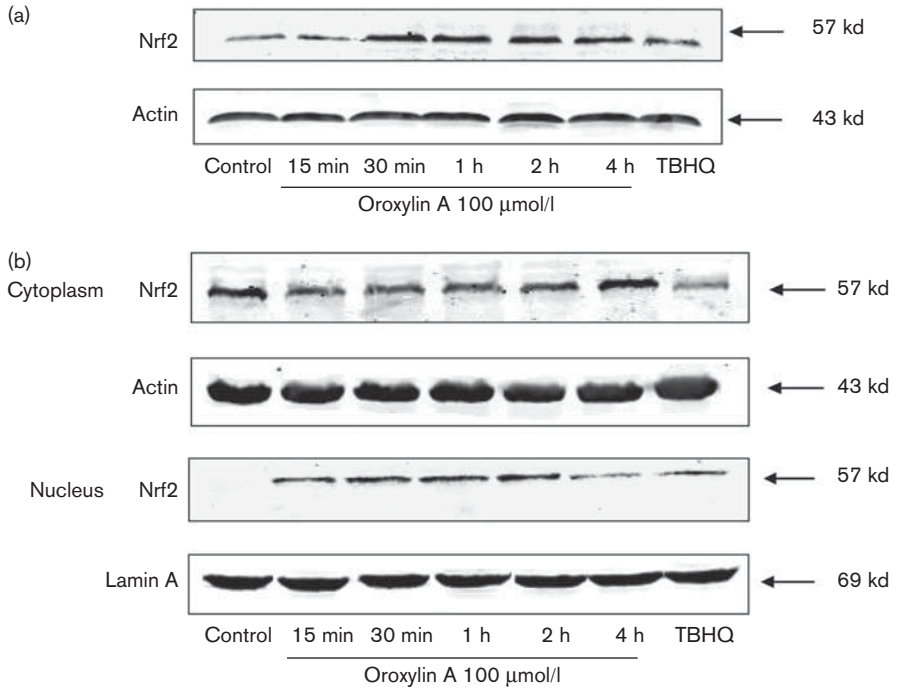
Oroxylin A induces apoptosis in HCT-116 cells. (a) Inhibitory effect of oroxylin A on the proliferation of HCT-116 cells. Data are shown as the mean±SD ($n=3$). (b) Diamidino-phenyl-indole staining of HCT-116 cells treated for 24 h with dimethylsulfoxide (DMSO; i), oroxylin A (50 μmol/l; ii), oroxylin A (100 μmol/l; iii), and oroxylin A (200 μmol/l; iv). (c) Fluorescence-activated cell sorter analysis of annexin-V and PI staining of control (i), oroxylin A (50 μmol/l; ii), oroxylin A (100 μmol/l; iii), and oroxylin A (200 μmol/l) after treatment for 24 h (iv). The results are given as the mean±SEM of three independent experiments. (d) Effects of oroxylin A on the expression of poly (ADP-ribose) polymerase (PARP), procaspase-3, 8, 9, Bcl-2, and Bax proteins. FITC, fluorescein isothiocyanate.

the expression of Bcl-2 protein decreased and the expression of Bax protein in HCT-116 cells increased after treatment with oroxylin A.

Oroxylin A-dependent Nrf2 activation and translocation to the nucleus

Compounds that increase Nrf2 activity are potential chemopreventive agents. In this study, we determined whether oroxylin A treatment increases the expression of Nrf2, and 100 μmol/l of tBHQ was used as a positive control. As shown in Fig. 2a, Nrf2 protein expression was increased 30 min after 100 μmol/l of oroxylin A treatment. Under homeostatic conditions, Nrf2 is suppressed by association with Keap1, but is stimulated upon exposure to oxidative or electrophilic stress. Once activated, Nrf2 translocates into nuclei and upregulates a group of genes that act in concert to

Fig. 2



Oroxylin A increases Nrf2 expression and its nuclear translocation. (a) Cells were incubated with 100 μmol/l of oroxylin A for the indicated times. Whole-cell extracts were prepared and analyzed by western blot using an anti-Nrf2 antibody. (b) The nucleus and cytoplasm extracts were separated and analyzed by western blot using an anti-Nrf2 antibody.

combat oxidative stress. In this study, we demonstrated that the protein level of Nrf2 in the nucleus was increased after 100 μmol/l of oroxylin A treatment for 15 min, 30 min, 1 h, and 2 h and decreased in the cytoplasm in comparison with the control (Fig. 2b). Thus, oroxylin A treatment induced the translocation of Nrf2 to the nucleus.

Effects of oroxylin A on reactive oxygen species levels and Nrf2 target gene expression

As shown in Fig. 3a, oroxylin A treatment for 15 min, 30 min, 1 h, 2 h, and 4 h increased the intracellular level of ROS in a time-dependent manner, and a 1-h pretreatment with *N*-acetyl-L-cysteine effectively blocked the induction of ROS. In addition, oroxylin A treatment increased the protein expression of two of the Nrf2 target genes *HO-1* and *NQO-1* in a time-dependent manner (Fig. 3b). These data demonstrated that oroxylin A treatment increased the intracellular level of ROS, which in turn might cause the activation of Nrf2 and induction of its downstream phase II enzymes HO-1 and NQO-1.

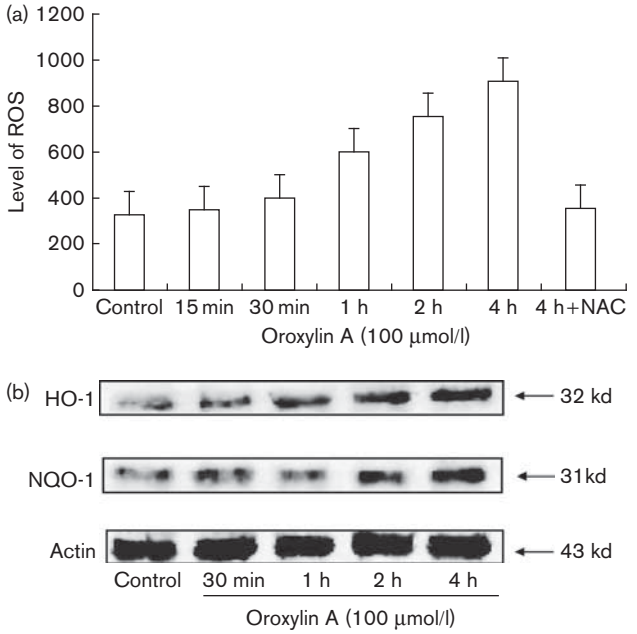
Effects of oroxylin A oral administration on the growth of HCT-116 exnograft tumors in immunodeficient mice

Male immunodeficient mice were treated with vehicle or oroxylin A (50, 100, 200 mg/kg) by an oral gavage once a day for 28 days beginning a week before the injection of HCT-116 colon cancer cells. Tumor growth was measured every other day, and tumor volume (*V*) was calculated (Fig. 4a). At the end of the experiment, the tumor volume was significantly different between the treatment and the control groups. The mean ± SEM tumor volume was $1868 \pm 624\,mm^3$ for the control group and 560 ± 251, 390 ± 83, and $195 \pm 112\,mm^3$ for mice treated with 50, 100, and 200 mg/kg of oroxylin A, respectively. In addition, tumor weight was also significantly different between the treatment and the control groups, with the 200 mg/kg of oroxylin A treatment group being the lowest (Fig. 4b). Oral oroxylin A administration did not show any gross sign of toxicity as monitored by body weight and diet consumption, as there was no considerable change in body weight gain and diet intake profiles between control and oroxylin A-fed groups (data not shown). These results suggested the in-vivo chemopreventive efficacy of oroxylin A when mice were exposed to it before colon tumor xenograft implantation, without any toxicity.

Oroxylin A induced apoptosis in HCT-116 xenograft tumors

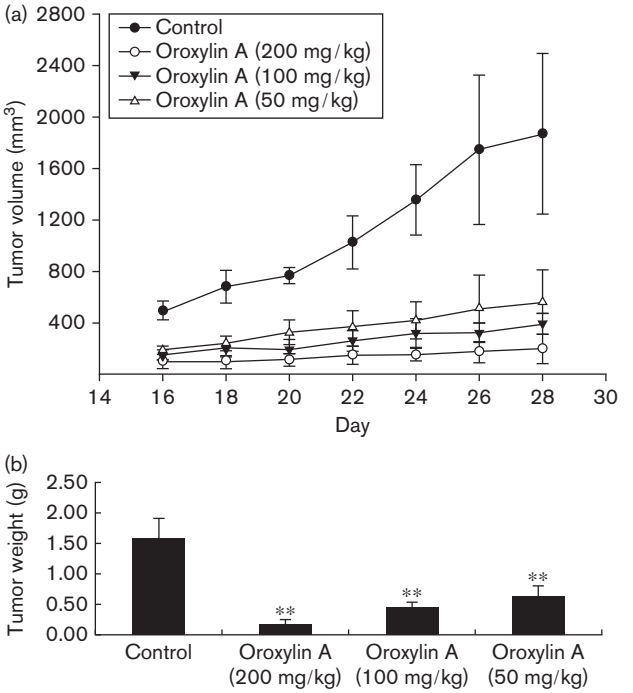
Resistance to apoptosis is a peculiar feature of almost every type of cancer, including colon cancer. Therefore, we analyzed HCT-116 tumor xenografts for the potential proapoptotic effects of oroxylin A that may have played a role in its overall antitumor efficacy. Caspase-mediated PARP cleavage showed that oral administration of oroxylin A induced apoptosis in HCT-116 xenograft tumors (Fig. 5). In addition, both caspase-3 and caspase-9 were activated

Fig. 3



Effects of oroxylin A on intracellular reactive oxygen species (ROS) and expression of the Nrf2 target genes. ROS production was monitored by flow cytometry using 10 μmol/l of DCFH-DA after cells were treated with oroxylin A for the indicated time (A). The protein expressions of heme oxygenase-1 (HO-1) and NADP(H) : quinone oxidoreductase-1 (NQO-1) were determined by western Blot using anti-HO-1 and anti-NQO-1 antibodies. NAC, *N*-acetyl-L-cysteine.

Fig. 4



Effects of dietary feeding of oroxylin A on growth in athymic male nude mice. Mice were given oroxylin A (50, 100, and 2000 mg/kg; $n=6$ mice per group) or vehicle orally once per day 1 week before xenograft implantation, and the mice were killed 3 weeks after injection of the tumor cells. Tumor growth was monitored and is presented as the tumor volume (a) and tumor weight (b) as a function of time. Data are presented as the mean±SEM. All comparisons were made relative to untreated controls and significant differences were indicated as $^{**}P<0.01$.

after oroxylin A treatment for 24 h, whereas caspase-8 remained unchanged, which agreed with the in-vitro cell culture study. Furthermore, the expression of Bcl-2 protein decreased and the expression of Bax protein increased in HCT-116 xenograft tumors after treatment with oroxylin A. These findings suggested that the antitumor efficacy of oroxylin A against in-vivo colon tumor growth involved induction of apoptosis, and the effects of oroxylin A on caspases, Bcl-2, and Bax levels may, in part, account for its proapoptotic and chemopreventive effects in HCT-116 tumors.

Oroxylin A activated Nrf2 in xenograft tumors

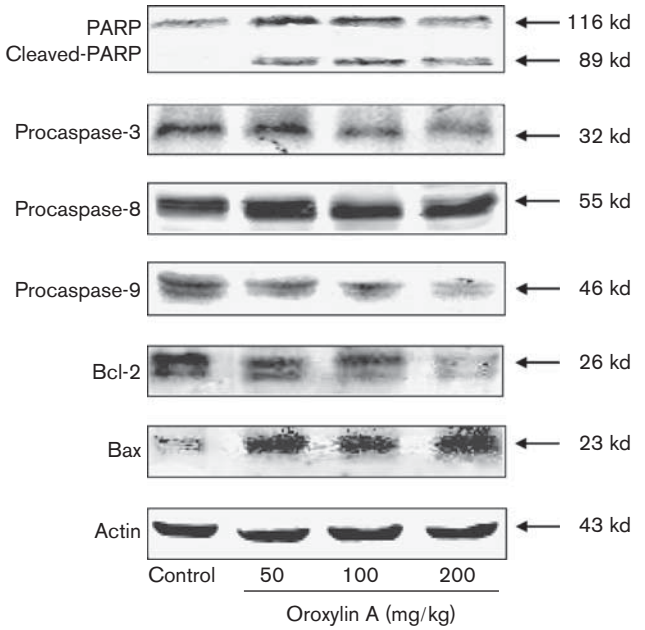
To determine whether oroxylin A could activate Nrf2 *in vivo* compared with the in-vitro cell culture study, we used western blot to measure Nrf2 protein levels in HCT-116 xenograft tumors. Indeed, it was found that the Nrf2 level decreased in the cytoplasm and increased in the nucleus in a dose-dependent manner, indicating that Nrf2 was activated after oral administration of oroxylin A in the xenograft tumor tissues (Fig. 6), which may also account for the chemopreventive effects of oroxylin A in HCT-116 tumors.

Discussion

In recent years, interest in the pharmacological effects of flavonoids in cancer chemoprevention has increased markedly. Oroxylin A, a naturally occurring monoflavonoid extracted from *S. baicalensis* Georgi, has been shown to be a promising candidate for selective and effective management of inflammation. Clinical studies have determined the effects of compounds isolated from *S. baicalensis* on the prevention and treatment of prostate cancer [17]. Studies in our lab and others have demonstrated that oroxylin A induced apoptosis in various cancer cells [18,19]. However, the chemopreventive effect of this compound as well as its potential mechanism have not been determined.

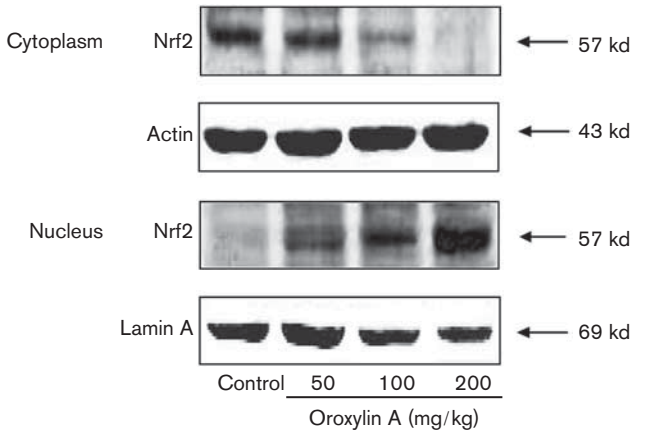
Oroxylin A-dependent apoptosis of preinitiated and/or neoplastic cells may indicate a protective mechanism against neoplastic transformation and tumor development. This may result from the elimination of genetically damaged cells or cells that divide inappropriately due to mitogenic and/or proliferative stimuli. Many chemopreventive agents induce apoptosis. These agents include curcumin, phenethyl isothiocyanate, sulforaphane, silibinin, silymarin, and resveratrol [6–9,20–22]. Studies (including ours) have found that the induction of apoptosis by some of these agents is at least partially responsible for their chemopreventive activities [7,9,20–23]. Therefore, we hypothesized that oroxylin A

Fig. 5



Oroxylin A induces apoptosis in the HCT-116 tumor xenograft. Xenograft tumor samples were lysed and homogenized. The homogenate was centrifuged and the supernatant was analyzed by western blot to show the effects of oroxylin A on the expression of poly (ADP-ribose) polymerase (PARP), procaspase-3, 8, 9, Bcl-2, and Bax proteins.

Fig. 6



The effect of oroxylin A on the activation of Nrf2 in xenograft mouse tissues. Xenograft tumor samples were lysed and homogenized. The homogenate was centrifuged and the nucleus and cytoplasm extracts were separated and analyzed by western blot using an anti-Nrf2 antibody.

may exert its chemopreventive activity through the induction of apoptosis. We examined the effects of oroxylin A treatment on HCT-116 cell proliferation, and our results demonstrated that oroxylin A inhibited HCT-116 cell growth and induced apoptosis through induction of the caspase cascade and affecting the expression of the Bcl-2 family proteins.

Chemical insults by environmental toxicants and endogenous oxidants can cause degenerative diseases such as cancer. Phase II and antioxidative enzymes provide a cellular defense against chemical stress and act in concert to detoxify and eliminate harmful reactive carcinogenic intermediates [1]. Under physiological conditions, these enzymes are expressed at a relatively low level. However, their expression is greatly induced in response to exposure to a variety of chemical compounds including isothiocyanates (e.g. sulforaphane) and dithiolthiones (e.g. 1,2-dithiole-3-thione, oltipraz). In addition, it has been established that these compounds exert their protective response partly through the activation of the Nrf2 signaling pathway. Therefore, a plausible mechanism by which agents may impart their chemopreventive activity is by the induction of detoxification and antioxidant enzymes through the activation of Nrf2. Upon release from Keap1, Nrf2 translocates into the nucleus. After translocation, Nrf2 dimerizes with Maf and binds the antioxidant response elements present in the promoter of Nrf2-target genes, which results in the transcriptional activation of these genes [24,25]. Indeed, in our present study, we found that oroxylin A induced the translocation of Nrf2, which increased the expression of its downstream target genes *HO-1* and *NQO-1*.

It has been shown that exposure to the chemopreventive agents produces a certain level of ROS or electrophiles, and causes mild oxidative/electrophilic stresses in cells [26–28]. Such mild oxidative stresses are sufficient to initiate the signaling pathways that, in turn, activate a variety of cellular events, such as induction of phase II and antioxidant enzymes, expression of tumor-suppressor genes, and inhibition of cell proliferation [29]. Here, we found that oroxylin A treatment increased the intracellular level of ROS in a time-dependent manner, which in turn might cause the activation of Nrf2 and induction of its downstream phase II enzymes HO-1 and NQO-1.

Animal models that are currently used to assess the efficacy of potential chemopreventive agents, including carcinogen induced with carcinogens specific for particular organ sites, transgenic/mutant animals with insertions, deletions, or mutations at targeted gene sites known to enhance cancers in a specific organ. For colon cancer, rats induced with the carcinogen azoxymethane, and genetically engineered models including Min/+, APC 1638, and the MSH2 mismatch repair-deficient mouse, have been used as chemopreventive models [30]. In addition, the xenograft nude mice model has also been used to validate the chemopreventive activities of natural products against head and neck, prostate, lung, and colon cancers [31–36], by administering the compounds before the implantation of the tumor cells. Therefore, here, we used a xenograft nude mice model to study the chemopreventive activity of oroxylin A.

Our study showed that continuous oral administration of oroxylin A (starting 1 week before implantation of HCT-116 colon xenografts) to nude mice significantly retarded the growth of tumors in comparison with mice treated with vehicle. In addition, this compound induced apoptosis and also activated Nrf2 in the xenograft tumor tissues. Future in-vivo and in-vitro correlation studies are necessary to better understand the pharmacological efficacy of oroxylin A as a potential chemopreventive agent.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

References

1 Hong WK, Sporn MB. Recent advances in chemoprevention of cancer. *Science* 1997; **278**:1073–1077.
2 Kelloff GJ, Lieberman R, Steele VE, Boone CW, Lubet RA, Kopelovich L, *et al*. Agents, biomarkers, and cohorts for chemopreventive agent development in prostate cancer. *Urology* 2001; **57**:46–51.
3 Kakizoe T. Chemoprevention of cancer: focusing on clinical trials. *Jpn J Clin Oncol* 2003; **33**:421–442.
4 Giudice A, Montella M. Activation of the Nrf2-ARE signaling pathway: a promising strategy in cancer prevention. *Bioessays* 2006; **28**:169–181.
5 Shen G, Hebbar V, Nair S, Xu C, Li W, Lin W, *et al*. Regulation of Nrf2 transactivation domain activity: the differential effects of mitogen-activated protein kinase cascades and synergistic stimulatory effect of Raf and CREB-binding protein. *J Biol Chem* 2004; **279**:23052–23060.
6 Balogun E, Hoque M, Gong P, Killeen E, Green CJ, Foresti R, *et al*. Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. *Biochem J* 2003; **371**:887–895.
7 Kang KW, Cho IJ, Lee CH, Kim SG. Essential role of phosphatidylinositol 3-kinase-dependent CCAAT/enhancer binding protein beta activation in the induction of glutathione S-transferase by oltipraz. *J Natl Cancer Inst* 2003; **95**:53–66.
8 Kensler TW, Qian GS, Chen JG, Groopman JD. Translational strategies for cancer prevention in liver. *Nat Rev Cancer* 2003; **3**:321–329.
9 Kwak MK, Wakabayashi N, Itoh K, Motohashi H, Yamamoto M, Kensler TW. Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 pathway: identification of novel gene clusters for cell survival. *J Biol Chem* 2003; **278**:8135–8145.
10 Horinaka M, Yoshida T, Shiraishi T, Nakata S, Wakada M, Sakai T. The dietary flavonoid apigenin sensitizes malignant tumor cells to tumor necrosis factor-related apoptosis-inducing ligand. *Mol Cancer Ther* 2006; **5**:945–951.
11 Yoon SB, Lee YJ, Park SK, Kim HC, Bae H, Kim HM, *et al*. Anti-inflammatory effects of *Scutellaria baicalensis* water extract on LPS-activated RAW 264.7 macrophages. *J Ethnopharmacol* 2009; **125**:286–290.
12 Sonoda M, Nishiyama T, Matsukawa Y, Moriyasu M. Cytotoxic activities of flavonoids from two Scutellaria plants in Chinese medicine. *J Ethnopharmacol* 2004; **91**:65–68.
13 Huerta S, Arteaga JR, Irwin RW, Ikezoe T, Heber D, Koeffler HP. PC-SPES inhibits colon cancer growth *in vitro* and *in vivo*. *Cancer Res* 2002; **62**:5204–5209.
14 Chen Y, Yang L, Lee TJ. Oroxylin A inhibition of lipopolysaccharide-induced iNOS and COX-2 gene expression via suppression of nuclear factor-kappaB activation. *Biochem Pharmacol* 2000; **59**:1445–1457.
15 Yang Y, Hu Y, Gu HY, Lu N, Liu W, Qi Q, *et al*. Oroxylin A induces G2/M phase cell-cycle arrest via inhibiting Cdk7-mediated expression of Cdc2/p34 in human gastric carcinoma BGC-823 cells. *J Pharm Pharmacol* 2008; **60**:1459–1463.
16 Li HB, Chen F. Isolation and purification of baicalein, wogonin and oroxylin A from the medicinal plant *Scutellaria baicalensis* by high-speed counter-current chromatography. *J Chromatogr A* 2005; **1074**:107–110.
17 Bonham M, Posakony J, Coleman I, Montgomery B, Simon J, Nelson PS. Characterization of chemical constituents in *Scutellaria baicalensis* with antiandrogenic and growth-inhibitory activities toward prostate carcinoma. *Clin Cancer Res* 2005; **11**:3905–3914.
18 Fas SC, Baumann S, Zhu JY, Giaisi M, Treiber MK, Mahlknecht U, *et al*. Wogonin sensitizes resistant malignant cells to TNFalpha- and TRAIL-induced apoptosis. *Blood* 2006; **108**:3700–3706.
19 Liu W, Mu R, Nie FF, Yang Y, Wang J, Dai QS, *et al*. MAC-related mitochondrial pathway in oroxylin-A-induced apoptosis in human hepatocellular carcinoma HepG2 cells. *Cancer Lett* 2009; **284**:198–207.
20 Huang C, Ma WY, Goranson A, Dong Z. Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. *Carcinogenesis* 1999; **20**:237–242.
21 Yu R, Mandlekar S, Harvey KJ, Ucker DS, Kong AN. Chemopreventive isothiocyanates induce apoptosis and caspase-3-like protease activity. *Cancer Res* 1998; **58**:402–408.
22 Hu R, Kim BR, Chen C, Hebbar V, Kong AN. The roles of JNK and apoptotic signaling pathways in PEITC-mediated responses in human HT-29 colon adenocarcinoma cells. *Carcinogenesis* 2003; **24**:1361–1367.
23 Kim BR, Hu R, Keum YS, Hebbar V, Shen G, Nair SS, *et al*. Effects of glutathione on antioxidant response element-mediated gene expression and apoptosis elicited by sulforaphane. *Cancer Res* 2003; **63**:7520–7525.
24 Hu R, Saw CL, Yu R, Kong AN. Regulation of NF-E2-related factor 2 signaling for cancer chemoprevention: antioxidant coupled with antiinflammatory. *Antioxid Redox Signal* 2010; **13**:1679–1698.
25 Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, *et al*. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* 1997; **236**:313–322.
26 Chen C, Shen G, Hebbar V, Hu R, Owuor ED, Kong AN. Epigallocatechin-3-gallate-induced stress signals in HT-29 human colon adenocarcinoma cells. *Carcinogenesis* 2003; **24**:1369–1378.
27 Kim SG, Cho MK, Choi SH, Kim HJ, Kwak MK, Kim ND. Molecular basis for hepatic detoxifying enzyme induction by 2-(allylthio)pyrazine in rats in comparison with oltipraz: effects on prooxidant production and DNA degradation. *Drug Metab Dispos* 1999; **27**:667–673.
28 Paolini M, Perocco P, Canistro D, Valgimigli L, Pedulli GF, Iori R, *et al*. Induction of cytochrome P450, generation of oxidative stress and in vitro cell-transforming and DNA-damaging activities by glucoraphanin, the bioprecursor of the chemopreventive agent sulforaphane found in broccoli. *Carcinogenesis* 2004; **25**:61–67.
29 Hu R, Kong AN. Activation of MAP kinases, apoptosis and nutrigenomics of gene expression elicited by dietary cancer-prevention compounds. *Nutrition* 2004; **20**:83–88.
30 Steele VE, Lubet RA. The use of animal models for cancer chemoprevention drug development. *Semin Oncol* 2010; **37**:327–338.
31 Khor TO, Keum YS, Lin W, Kim JH, Hu R, Shen G, *et al*. Combined inhibitory effects of curcumin and phenethyl isothiocyanate on the growth of human PC-3 prostate xenografts in immunodeficient mice. *Cancer Res* 2006; **66**:613–621.
32 Amin AR, Wang D, Zhang H, Peng S, Shin HJ, Brandes JC, *et al*. Enhanced anti-tumor activity by the combination of the natural compounds (–)-epigallocatechin-3-gallate and luteolin: potential role of p53. *J Biol Chem* 2010; **285**:34557–34565.
33 Hu H, Lee HJ, Jiang C, Zhang J, Wang L, Zhao Y, *et al*. Penta-1,2,3,4,6-O-galloyl-beta-D-glucose induces p53 and inhibits STAT3 in prostate cancer cells *in vitro* and suppresses prostate xenograft tumor growth *in vivo*. *Mol Cancer Ther* 2008; **7**:2681–2691.
34 Howells LM, Britton RG, Mazzoletti M, Greaves P, Broggini M, Brown K, *et al*. Preclinical colorectal cancer chemopreventive efficacy and p53-modulating activity of 3′,4′,5′-trimethoxyflavonol, a quercetin analogue. *Cancer Prev Res (Phila)* 2010; **3**:929–939.
35 Singh RP, Deep G, Blouin MJ, Pollak MN, Agarwal R. Silibinin suppresses in vivo growth of human prostate carcinoma PC-3 tumor xenograft. *Carcinogenesis* 2007; **28**:2567–2574.
36 Zhang X, Zhang H, Tighiouart M, Lee JE, Shin HJ, Khuri FR, *et al*. Synergistic inhibition of head and neck tumor growth by green tea (–)-epigallocatechin-3-gallate and EGFR tyrosine kinase inhibitor. *Int J Cancer* 2008; **123**:1005–1014.