Biochemical Pharmacology 81 (2011) 1192–1200

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Cudratricusxanthone G inhibits human colorectal carcinoma cell invasion by MMP-2 down-regulation through suppressing activator protein-1 activity

Lisha Kuang [a,1], Lei Wang [a,1], Qian Wang [a], Qufei Zhao [a], Bing Du [a], Dali Li [a], Jian Luo [a], Mingyao Liu [a], Aijun Hou [b,*], Min Qian [a,**]

[a] The Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, 500 Dongchuan Road, Shanghai 200241, China
[b] Department of Pharmacognosy, School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai 201203, China

ARTICLE INFO

Article history:
Received 9 January 2011
Accepted 23 February 2011
Available online 4 March 2011

Keywords:
AP-1
Cudratricusxanthone G (CTXG)
Invasion
MMP-2
Rac1

ABSTRACT

Cudratricusxanthone G (CTXG), a natural bioactive cudratricusxanthone extracted from *C. tricuspidata*, has shown anti-cancer properties. However, the function and mechanism of CTXG in tumor invasion have not been elucidated to date. In this study, we investigated the inhibitory effect of CTXG on the proliferation, migration and invasion of SW620 cells. We found that MMP-2, a pivotal factor in tumor invasion, was suppressed in both expression and activation by CTXG in a dose-dependent manner. The suppression of MMP-2 expression by CTXG led to an inhibition of SW620 cells invasion and migration by inactivating Rac1 and Cdc42 but not RhoA GTPase. Furthermore, CTXG also inhibited the transcriptional activity of AP-1 (activator protein-1). In conclusion, our data demonstrate that CTXG exerted anti-invasion action in SW620 cells by targeting MMP-2 though regulating the activities of Rac1, Cdc42 and their downstream transcriptional factor AP-1. These results are the first to reveal the novel functions of CTXG in cancer cell invasion and its molecular basis for the anti-cancer action.

1. Introduction

CTXG (cudratricusxanthone G) is a natural cudratricusxanthone extracted from the roots of *C. tricuspidata*, which is widely distributed in China, Korea, and Japan. The cortex and root bark of *C. tricuspidata* have been widely applied in clinic as a traditional medicine for the treatment of alimentary system cancer, especially gastric carcinoma [1,2]. It has been documented that cudratricusxanthones have multiple functions such as inhibitory activity against human digestive apparatus cancer cells [3], hepatoprotective effect on nitrofurantoin-induced cytotoxicity [4], anti-proliferation effect of vascular smooth muscle cells [5], protective activity on neuronal cells from glutamate-induced oxidative stress [6], and anti-inflammatory effect in macrophages [7]. However, whether cudratricusxanthones have any effect on tumor invasion has not been identified yet.

Colorectal cancer, the third most common form of cancer occurring worldwide, is one of the leading causes of cancer-related deaths globally [8–10]. Tumor metastasis, which is the main reason of colon cancer patient death, becomes a major challenge and a critical target for successful cancer therapy and leads to many novel treatments [11–14]. Degradation of the extracellular matrix (ECM) is one of the key mechanisms involved in cancer cell metastasis. MMPs (matrix metalloproteinases), especially MMP-2 (gelatinase A) and MMP-9 (gelatinase B), are the most vital proteases for the degradation of type IV collagen, which is the main constituent of ECM [15]. Rac1, an important member of small GTPase family, is also well known to play a key role in malignant tumor progression, such as proliferation, survival [16], and cytoskeleton organization. Thus, it has become one of the important targets of cancer chemotherapy [17]. Previous study has identified AP-1 as one of the major downstream transcriptional factors of Rac1 [18]. It was also reported that AP-1 could induce malignant phenotypic changes by MMP-2 up-regulation through Rac1 signaling. Therefore, agents targeting MMPs and its upstream signaling pathway factors are potential to be applied to treatment for colorectal cancer metastasis.

In this study, the anti-invasion effect of CTXG on colorectal cancer cell SW620 and its molecular mechanism have been identified for the first time. CTXG, one of the bioactive cudratricusxanthones, exerted great inhibition on colon cancer cell proliferation, migration and invasion by suppressing the activities of Rac1 and its downstream transcriptional factor AP-1.

2. Materials and methods

2.1. Materials

CTXG was purified in our previous study [3]. A 50 mM stock solution of CTXG was prepared in dimethyl sulfoxide (DMSO)

* Corresponding author. Tel.: +86 21 5198 0005; fax: +86 21 5198 0005.
** Corresponding author. Tel.: +86 21 6223 3569; fax: +86 21 5434 4922.
E-mail addresses: ajhou@shmu.edu.cn (A. Hou), mqian@bio.ecnu.edu.cn (M. Qian).
[1] These authors contributed equally to this work.

0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.02.017

(Sigma–Aldrich, St. Louis, MO), stored at −20 °C, and then diluted as needed concentrations in cell culture medium. Bacteria-derived recombinant human EGF (epidermal growth factor) was obtained from Jingmei Biotech (Shanghai, China). Matrigel was purchased from BD Biosciences (Bedford, MA). Antibodies against total and phosphorylated MAPK/ERK1/2 (EERK1/2), p38 MAPK, SAPK/c-Jun N-terminal Kinase (JNK), and MMP-2, MMP-9, Cdc42, RhoA were purchased from Cell Signaling Technology (Bedford, MA), while anti-CXCR4, anti-Rac and anti-β-actin antibodies were from Abcam (Bedford, MA), Upstate Biotechnology Inc (New York, NY) and Sigma, respectively.

2.2. Cell lines and cell culture

Human colorectal carcinoma SW620 cells, HCT-116 cells and LoVo cells were purchased from American Type Culture Collection (ATCC), and cultured in Leibovitz's L-15 medium (L-15, Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Shanghai, China) at 37 °C in a 1% CO_2 atmosphere. Human kidney epithelial cell 293 T cells (purchased from ATCC) were maintained in DMEM (Gibco) supplemented with 10% FBS at 37 °C under a humidified 95%:5% (v/v) mixture of air and CO_2. Cell lines were routinely analyzed for mycoplasma contamination and cell line authenticity.

2.3. Cytotoxicity assay

Cytotoxicity assay was performed to detect cytotoxicity of CTXG as previously described [19]. Briefly, SW620 cells (1×10^5/well in 96-well plate) were incubated with different concentrations of CTXG for 48 h, then stained with cytotoxicity reagent (5 μM ethidium homodimer and 5 μM calcein-AM) and incubated at 37 °C for 30 min. Cells were analyzed under a fluorescence microscope (DM 4000 B; Leica).

2.4. MTS assay

Following the manual of CellTiter 96 Aqueous One Solution Cell Proliferation assay (Promega, Madison, WI), MTS assay was performed to determine the anti-proliferative effect of CTXG. SW620 cells (5×10^3 cells/well) were seeded into 96-well plate and incubated for 24 h. Cells were then treated with different concentrations of CTXG for 48 h while control cells were treated with 0.1% DMSO. 20 μL MTS solution I was added to each well following the treatment. After 3 h incubation at 37 °C, absorption at 460 nm of each well was measured with VERSAmax microplate reader (Molecular Devices).

2.5. Wound healing migration assay

Cell motility was measured by wound healing assay as described previously [20]. Initially, SW620 cells were allowed to grow into full confluence in 0.1% gelatin (Sigma) pre-coated 12-well plates and then starved overnight with serum free L-15 medium to inactivate cell proliferation. Cells were then scraped with pipette tips and washed with PBS. L-15 containing 10% FBS and different concentrations of CTXG was added into wells with or without 20 ng/mL EGF. After treated for 24 h, cells were imaged and migrated cells were quantified by manual counting.

2.6. Boyden chamber invasion assay

Invasion assay was carried out using modified Boyden chambers consisting of 24-well Millicell (Millipore, Shanghai, China) membrane filter (8 μM pore size). Briefly, the upper surface of Millicells was coated with 100 μL Matrigel (100 μg/mL) while the bottom chambers were filled with 500 μL L-15 (10% FBS) supplemented with 20 ng/mL EGF. The top chambers were seeded with 1×10^5 cells/well in 100 μL L-15 (serum free) containing indicated concentrations of CTXG. After 24 h incubation, the cells on the top surface of the filter were scraped using a cotton swab while cells spreading on the bottom sides (invaded cells) were fixed with cold 4% paraformaldehyde (Sigma) and stained with 5% hematoxylin (Sigma). Then, invaded cells were imaged under inverted microscope (OLYMPUS) and quantified by manual counting.

2.7. Gelatin zymography assay

The activity of MMP-2 in cell culture medium was measured by gelatin zymography protease assay as described by Johansson and Smedsrod [21]. SW620 cells were incubated in a serum-free L-15 medium with or without CTXG for 24 h. Phorbol 12-myristate 13-acetate (PMA, Sigma) was used to stimulate MMP-2 expression. Collected cell culture medium of appropriate volumes were prepared in sample buffer without boiling or reduction and

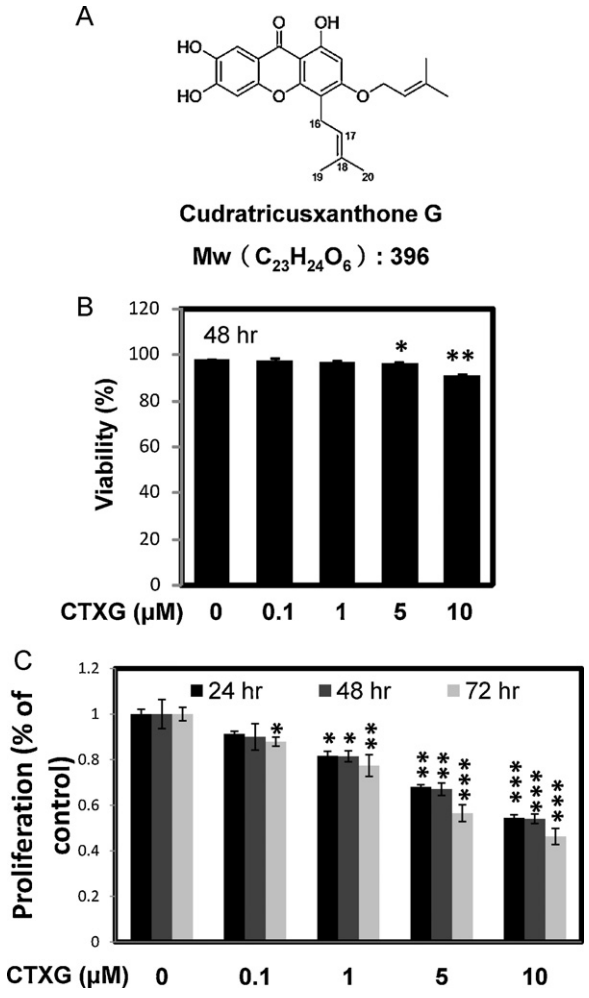

Fig. 1. CTXG (Cudratricusxanthone G) exhibits low cytotoxicity and inhibits cell proliferation. (A) The chemical structure of CTXG with a molecular weight 396.0 g/mol. (B) CTXG exhibits low cytotoxicity on SW620 cells. SW620 cells were treated with different concentrations of CTXG for 48 h. Viability was detected by cytotoxic assay and cells were quantified by manual counting. (C) CTXG inhibits cell proliferation in a dose-dependent manner. SW620 cells were treated with different concentrations of CTXG for 24, 48 and 72 h, respectively. Proliferation was quantified by MTS assay. *Columns*, mean from three different experiments with 3 duplicates; *bars*, SE (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus DMSO alone).

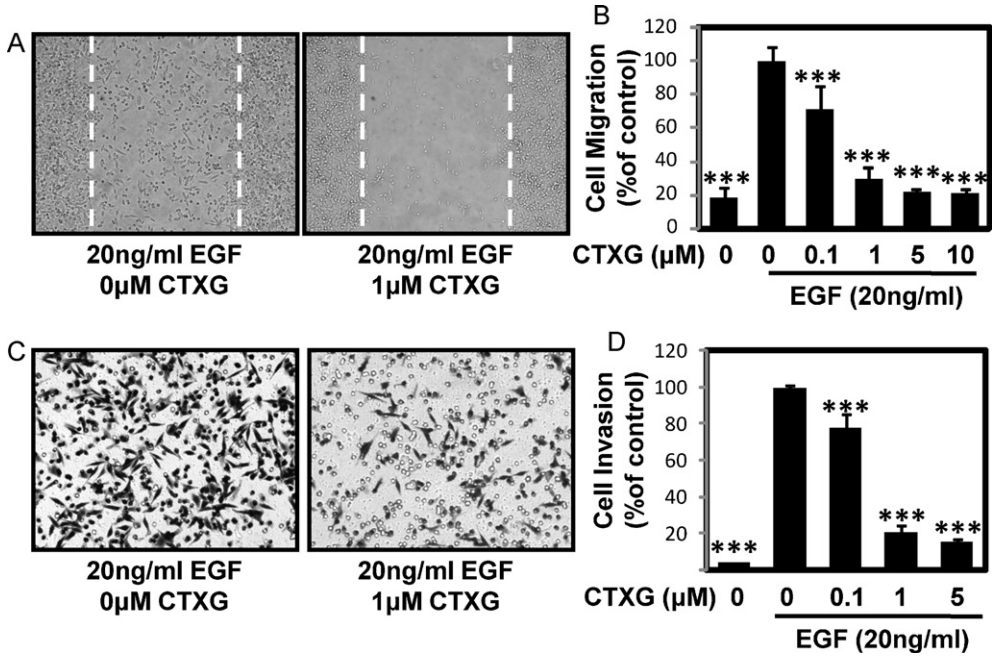

Fig. 2. CTXG inhibits cell migration and invasion. (A) SW620 cells were serum starved overnight, then treated with 20 ng/mL EGF and various concentrations of CTXG. Cells were photographed under phase-contrast microscopy (magnification, 200×). (B) Densitometric analysis of migrated cell numbers as in (A). Migrated cells were quantified by manual counting. (C) Cells penetrating through the Matrigel to the lower surface of the filter were stained with 5% hematoxylin and photographed under a light microscope at 400× magnification. (D) Densitometric analysis of invasive cells as in (C). Invasive SW620 cells were quantified by manual counting. *Columns*, mean from three different experiments with 3 duplicates; *bars*, SE (*$P < 0.05$; **$P < 0.01$; **$P < 0.001$ versus EGF alone).

subjected to 0.1% gelatin-8% SDS–PAGE electrophoresis. After electrophoresis, gel was washed with 2.5% Triton X-100 and incubated in zymogram developing buffer [50 mM Tris–HCl (pH 8.0), 5 mM $CaCl_2$, and 0.2 mol/L NaCl] at 37 °C for 12 h, and then stained with Coomassie brilliant blue R-250. Densitometric analysis of protein bands was conducted with software Image-Pro Plus.

2.8. Rac/Cdc42/RhoA activation assay

Activated GTPase in cells was detected by GST-PBD or GST-RBP pull-down as described previously [22]. SW620 cells were serum-starved overnight followed by incubation with different concentrations of CTXG for 12 h. Afterwards, treatment with 20 ng/mL platelet-derived growth factor (PDGF, Sigma) (for Rac, Cdc42) or 20 μmol/L lysophosphatidic acid (LPA, Sigma) (for RhoA) was applied for 10 min. Cells were then washed with PBS and lysed with RIPA buffer (20 mM Tris, 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mM NaF, 10 mM $Na_4P_2O_7$, and 1 mM PMSF). 30 mg of GST-PBD (Rac/Cdc42-binding domain) or GST-RBD (RhoA-binding domain) attached to beads were added to the cell lysate. After incubation at 4 °C for 60 min, the beads were washed three times with RIPA buffer. Active Rac1, Cdc42, and RhoA were detected by western blot.

2.9. Western blot analysis

To assess protein expression level, overnight serum-starved SW620 cells were treated with different concentrations of CTXG for 48 h. The whole-cell extracts were prepared in RIPA buffer. 40 μg cellular protein of each sample was proceeded to immunoblot after 8–12% SDS–PAGE electrophoresis and probed with specific antibodies, followed by a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibody (Cell Signaling Technology), as described previously [20].

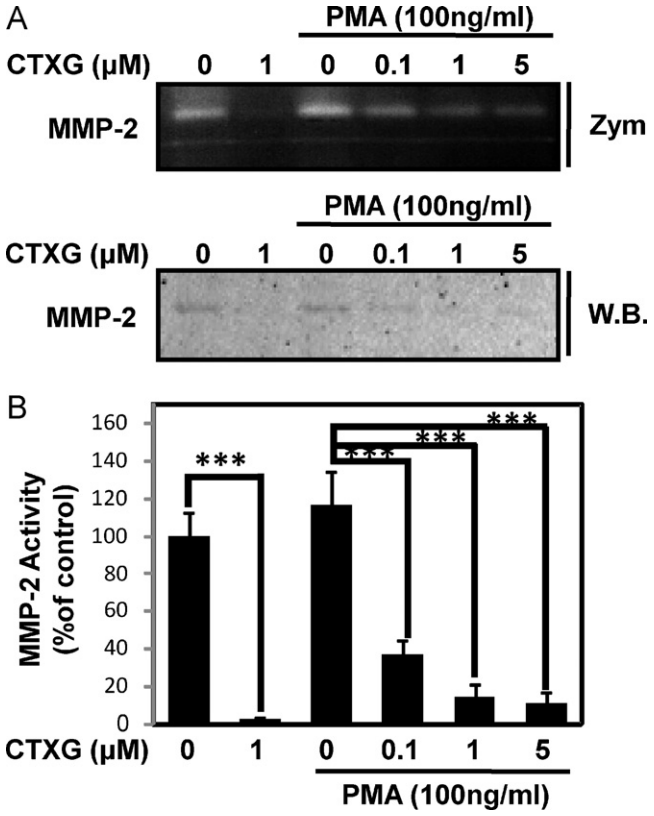

Fig. 3. CTXG inhibits MMP-2 activity and expression. (A) After treatment with CTXG for 24 h, MMP-2 enzyme activity was analyzed by gelatin zymography (Zym). Protein level in conditional culture medium from same amount of cells with different treatment was detected by western blotting (W.B.). PMA was used to stimulate expression of MMP-2. (B) Densitometric analysis of bands from gel zymogram. *Columns*, mean from three different experiments with 3 duplicates; *bars*, SE (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus with or without PMA alone).

2.10. Luciferase reporter assay

Luciferase reporter assay was carried out as described by Mitchell et al. [23]. 293T cells (1×10^6 cells/dish) were seeded into 10 cm cell culture dishes. When cells achieve 60% confluence, reportor gene constructs were transfected using the calcium phosphate method, with 8 μg pAP1-luciferase vector per each plate. After transfection, cells were reseeded in 24-well plates, and consequently pretreated with the indicated concentrations of CTXG for 24 h before being washed and lysed with luciferase lysis buffer (Promega). Rellina luciferase reporter was used as a transfection efficiency control and luciferase activity of protein lysate was measured following the manufacturer's protocol (Luciferase Assay System, Promega) with a Victor 3 microplate reader (PerkinElmer).

2.11. Electrophoretic mobility shift assay (EMSA)

SW620 cells were treated with various concentration of CTXG, and nuclear extracts were prepared as described [24,25]. Nuclear proteins (5 μg) were incubated with ^{32}P labeled AP-1 (activator protien-1) probe on ice for 30 min, and the DNA-protein complex was separated on a 5% acrylamide gel. The oligonucleotide sequences of AP-1 probes were 5′-CTGACCCCTGAGTCAG-CACTTGC-3′ (sense) and 5′-CAAGTGCTGACTCAGGGGTCAGG-3′ (antisense). The oligomers were annealed and double strand products were isolated with PAGE gel and used for labeling.

2.12. Statistical analysis

All arrays were performed at least three independent experiments. The data were presented as mean ± SD, and statistical comparisons between groups were performed using one-way ANOVA followed Dunnet test. P value ≤ 0.05 was considered statistically significant.

3. Results

3.1. CTXG exerts anti-proliferative effect but low cytotoxicity in SW620 cells

CTXG is a bioactive cudratricusxanthone with a molecular weight of 396.0 g/mol (Fig. 1A). To investigate the cytotoxicity of CTXG, cytotoxic assay was performed at first. Results show that the viability of SW620 cells was 91.8 ± 6.61% at 10 μM CTXG treatment for 48 h, indicating CTXG has low cytotoxicity on SW620 cells (Fig. 1B). The anti-proliferation function of CTXG on SW620 cells was determined using MTS assay. The cell proliferative rate compared with control is reduced along with rising concentrations of CTXG

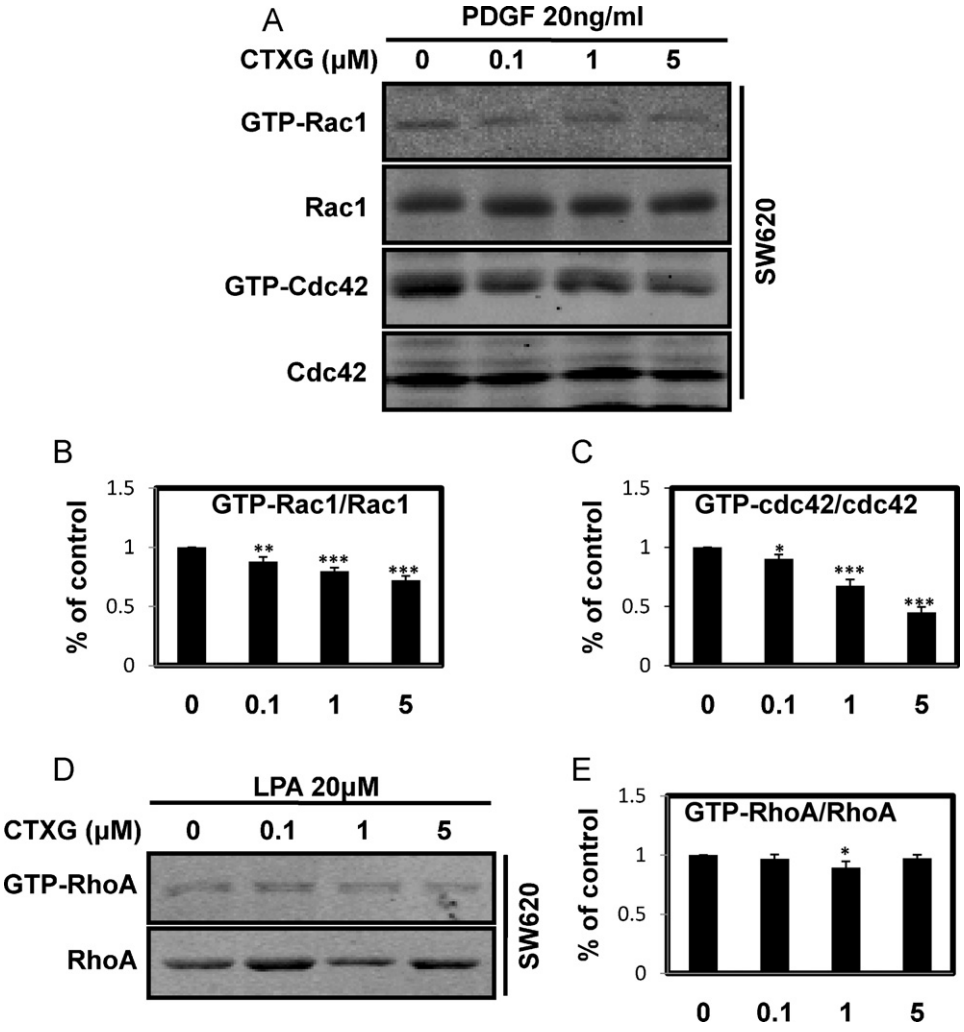

Fig. 4. CTXG inhibits activation of Rac1 and Cdc42 in SW620 cells. Cells were treated with CTXG for 12 h as described in Section 2. Samples were subjected to SDS–PAGE followed by western blot. (A) Effect of CTXG on the activity of Rac1 and Cdc42 stimulated by PDGF. (B) Densitometric analysis of bands of Rac1. (C) Densitometric analysis of bands of cdc42. (D) Effect of CTXG on the activity of GTP-RhoA stimulated by LPA. (E) Densitometric analysis of bands of RhoA. *Columns*, mean from three different experiments with 3 duplicates; *bars*, SE (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus PDGF alone).

treatment for 24, 48 and 72 h (Fig. 1C), suggesting that CTXG significantly inhibited proliferation of SW620 cells at 1, 5 and 10 μM in a concentration-dependent manner, with half-maximal inhibition between 5 and 10 μM.

3.2. CTXG inhibits SW620 cells migration and invasion in vitro

The effect of CTXG on the chemotactic motility of SW620 cells was measured by wound healing migration assay. After treatment with CTXG at various concentrations for 24 h, migratory cells were counted. As shown in Fig. 2A and B, CTXG significantly inhibited EGF-induced SW620 cells migration at concentrations over 0.1 μM in a dose-dependent manner. These results suggest that CTXG is highly effective on inhibiting SW620 cells migration. The effect of CTXG on invasion of SW620 cells was analyzed by Boyden chamber invasion assay. Result indicates that CTXG also markedly suppresses the EGF-induced cell invasion through Matrigel in a dose-dependent manner (Fig. 2C and D).

3.3. CTXG inhibits MMP-2 activity and expression in SW620 cells

The pivotal role of MMP-2 in tumor metastasis was previously demonstrated [26]. To detect effects of CTXG on the activity of MMP-2, we applied gelatin zymography assay to conditioned media from CTXG treated SW620 cells. In our results, MMP-2 expression was observed in SW620 cells and the activity of MMP-2 was decreased significantly ($P < 0.001$) after treatment with 1 μM CTXG. To amplify basal protein level, PMA was used to stimulate MMP-2 expression. As Fig. 3 shows, PMA increased MMP-2 activity and CTXG inhibited PMA induced MMP-2 activity in a dose dependent manner. Western blot confirmed that MMP-2 expression was inhibited with or without PMA stimulation. These results indicate MMP-2 is an important target of CTXG.

3.4. CTXG inhibits Rac1 and Cdc42 activation

To investigate whether CTXG could suppress the activation of small GTPases in tumor metastasis, we examined the effect of CTXG on Rac1, Cdc42 and RhoA. Since 10 μM CTXG treatment shows detectable cytotoxic effect in cells, to conduct this assay on cells under lower cytotoxic effect, 5 μM was used as the highest dose in this assay. In SW620 cells, CTXG was observed to suppress the activation of GTP-Rac1 and GTP-Cdc42 at 0.1 μM and above concentrations (Fig. 4A–C). However, the inhibitory effect of CTXG on RhoA was hardly detectable at 0.1–5 μM (Fig. 4D and E), suggesting that CTXG mainly affects activation of Rac1 and Cdc42 but not RhoA. Besides, the inhibitory effect of CTXG on Rac1 and Cdc42 were also confirmed in two other colon cancer cell lines, HCT-116 and LoVo cells (Fig. 5A and B). Densitometric analysis shows phosphory-Erk1 was also significantly inhibited by CTXG in SW620 cells at 0.1–10 μM (Fig. 6A and B), while phosphory-JNK only was inhibited at high dose treated (5–10 μM) (Fig. 6A, E and F). Despite the significant inhibitory effect on Erk1, CTXG was observed to exhibit subtle effect on the phosphorylation of other MAPKs such as ERK2, JNK and p38 MAPK (Fig. 6).

3.5. CTXG inhibits AP-1 transcriptional activity

To reveal the mechanism of how CTXG inhibited MMP-2 activity and expression, we examined the effect of CTXG on the activation of AP-1, which is an upstream regulator of MMP-2. Our results demonstrated that 1 μM CTXG could strongly inhibit the transcriptional activity of AP-1 in AP-1 luciferase reporter assay (Fig. 7A). Additionally, the effect of CTXG on the DNA binding activity of AP-1 in SW620 cells was investigated by EMSA. As shown in Fig. 7B, the DNA-binding activities of AP-1 were strongly

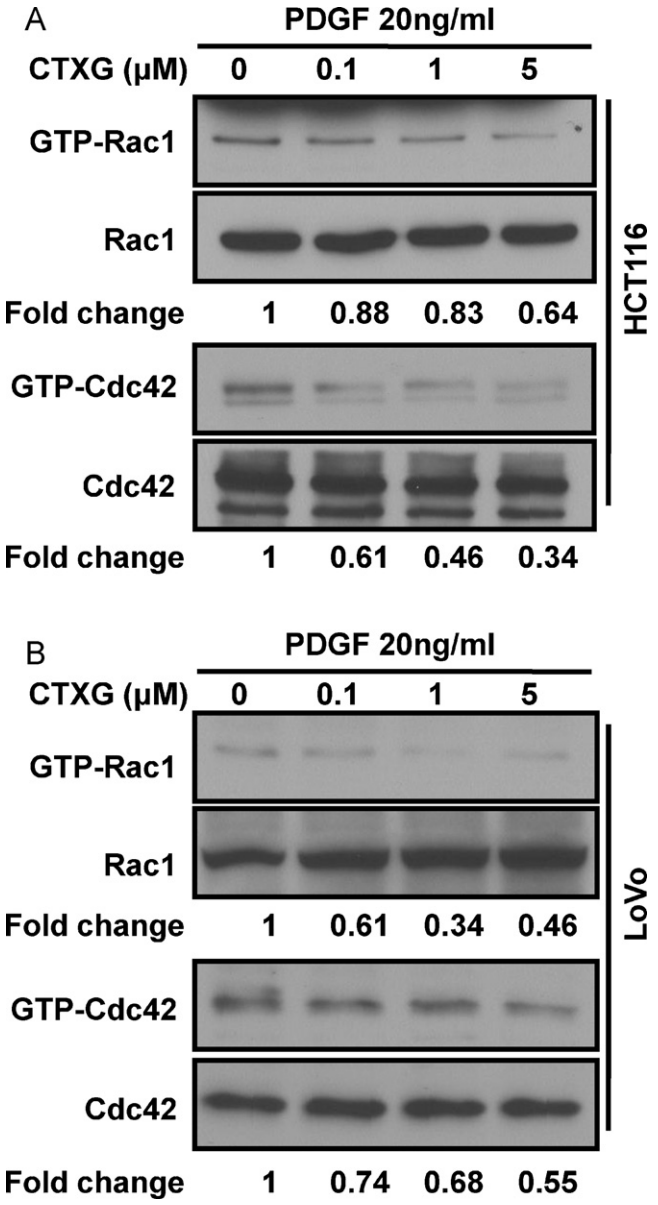

Fig. 5. CTXG inhibits activation of Rac1 and Cdc42 in HCT-116 and LoVo cells. Cells were treated with CTXG for 12 h as described in Section 2. Samples were subjected to SDS–PAGE followed by western blot. (A) Effect of CTXG on the activity of Rac1 and Cdc42 stimulated by PDGF in HCT-116 cells. (B) Effect of CTXG on the activity of Rac1 and Cdc42 stimulated by PDGF in LoVo cells.

inhibited by treatment with 1 μM or higher concentration of CTXG, and the inhibitory effect is in a dose-dependent manner.

3.6. CTXG inhibits AP-1 targeting genes in SW620 cells

AP-1 has been demonstrated to play a key role in CTXG contribution, here we also explored whether CTXG affected the AP-1 downstream functional proteins involved in invasion and metastasis, including MMP-2, MMP-9 and CXCR4. Results revealed that 1 μM CTXG significantly inhibited MMP-2 and MMP-9 expression which is regulated by AP-1, but exhibited little effect on CXCR4 which is not regulated by AP-1 (Fig. 7C).

4. Discussion

Malignant tumor invasion and metastasis, which require the degradation of extracellular matrix, are associated with increased

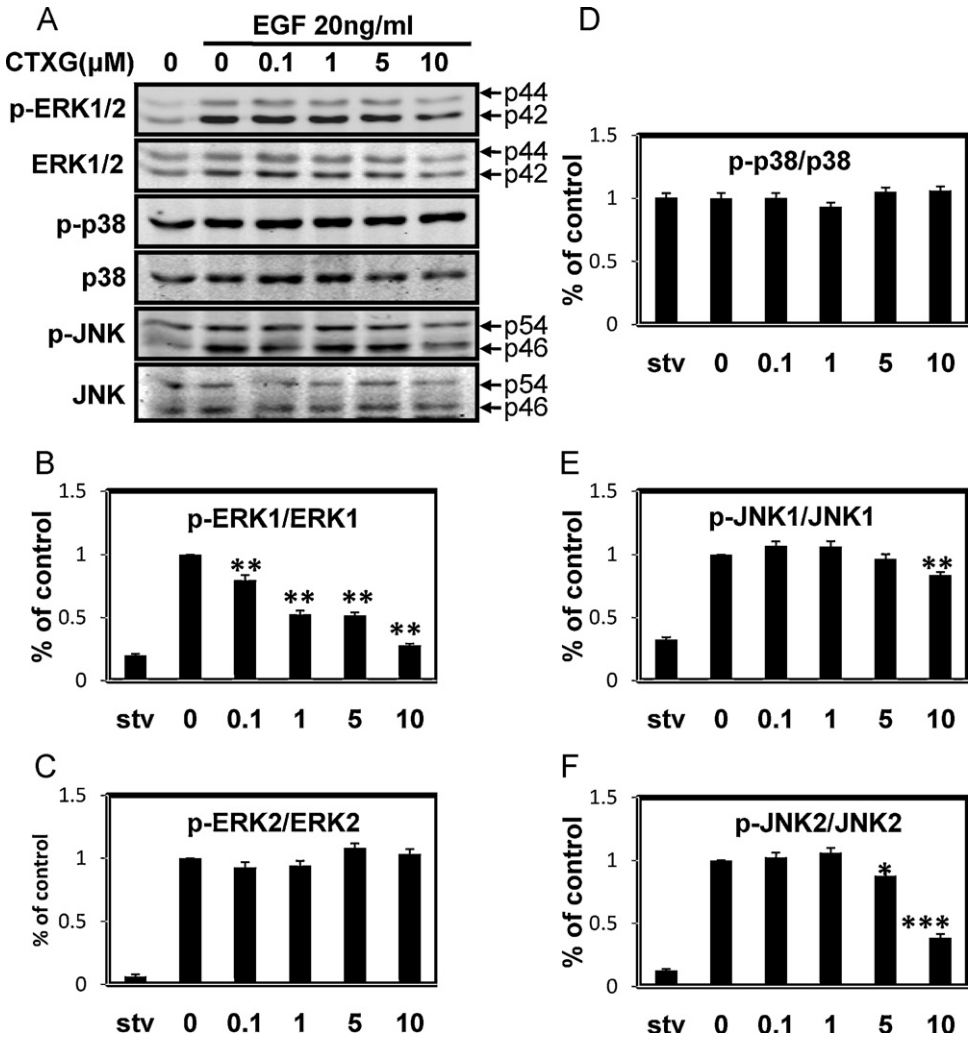

Fig. 6. Effect of CTXG on MAP kinases. (A) Effect of CTXG on the activity of ERK1/2, JNK and p38 MAPK. (B) Densitometric analysis of bands of phosphory-Erk1. (C) Densitometric analysis of bands of phosphory-Erk2. (D) Densitometric analysis of bands of phosphory-p38 MAPK. (E) Densitometric analysis of bands of phosphory-JNK1. (F) Densitometric analysis of bands of phosphory-JNK2. *Columns*, mean from three different experiments with 3 duplicates; *bars*, SE (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus PDGF alone).

expression of MMPs [27]. Recently, the anti-invasive effects of phytochemical polyphenols has drawn a lot of attention [28]. Xanthones, which consist of a large group of polyphenol, have been found to influence some steps in cancer progression beyond their traditional use [29,30]. CTXG is a natural bioactive xanthone extracted from *C. tricuspidata* [3]. Our previous study has demonstrated that CTXG exerted anti-proliferative effect on colorectal carcinoma cell HCT-116, hepatocellular carcinoma cell SMMC-7721 and SGC-7901, and also gastric carcinoma cell BGC-823 [3]. However, little information is known about its function in cancer cell invasion. In this study, we report the biological functions of CTXG as an inhibitor of invasion for the first time. Our results indicate that CTXG inhibits proliferation, migration and invasion in human colorectal carcinoma SW620 cells in a dose-dependent manner. Results from gelatin zymogram revealed that CTXG reduced the activation of MMP-2 secreted by SW620 cells. In addition, GST-PBD/RBD pull down assay indicated that CTXG significantly suppressed the activity of Rac1 and Cdc42 but had subtle effect on RhoA. AP-1, an important transcriptional factor involved in the regulation of various steps of tumor cell metastasis [31], was also inhibited by CTXG on the transcriptional activity. The expression of AP-1 targeted genes also decreased along with the suppression on AP-1 activation. Therefore, these results suggest that anti-cancer activity of CTXG may be associated with AP-1 signaling pathway.

Cell proliferation is known to be very important in the process of tumor metastasis [27]. To assess the anti-metastasis activity of CTXG on SW620, we measured its effects on cell proliferation. As expected, CTXG effectively induced cell growth inhibition of cultured colorectal carcinoma cell SW620 at concentrations above 1 μM (Fig. 1). In addition, cell viability assay revealed that CTXG exerted low cytoxicity at tested concentrations. These results suggest the anti-metastatic effect of CTXG may be attributed to its anti-proliferative activity to some extent. Cell migration and invasion of cancer cells are key steps in tumor metastasis [27,30]. The effects of CTXG on cell migration and invasion were assessed by scratch migration assay and Matrigel Boyden chamber invasion assay, respectively. In our *in vitro* assays, CTXG significantly inhibited both EGF-induced cell migration and invasion at over 0.1 μM ($P < 0.001$), with a half-maximal inhibition at a concentration between 0.1 and 1 μM (Fig. 2). Compared with the concentration achieving half-maximal inhibition in cell proliferation, CTXG is more effective in cell migration and invasion, indicating that the anti-migration and anti-invasion function could be more important to its anti-metastatic activity.

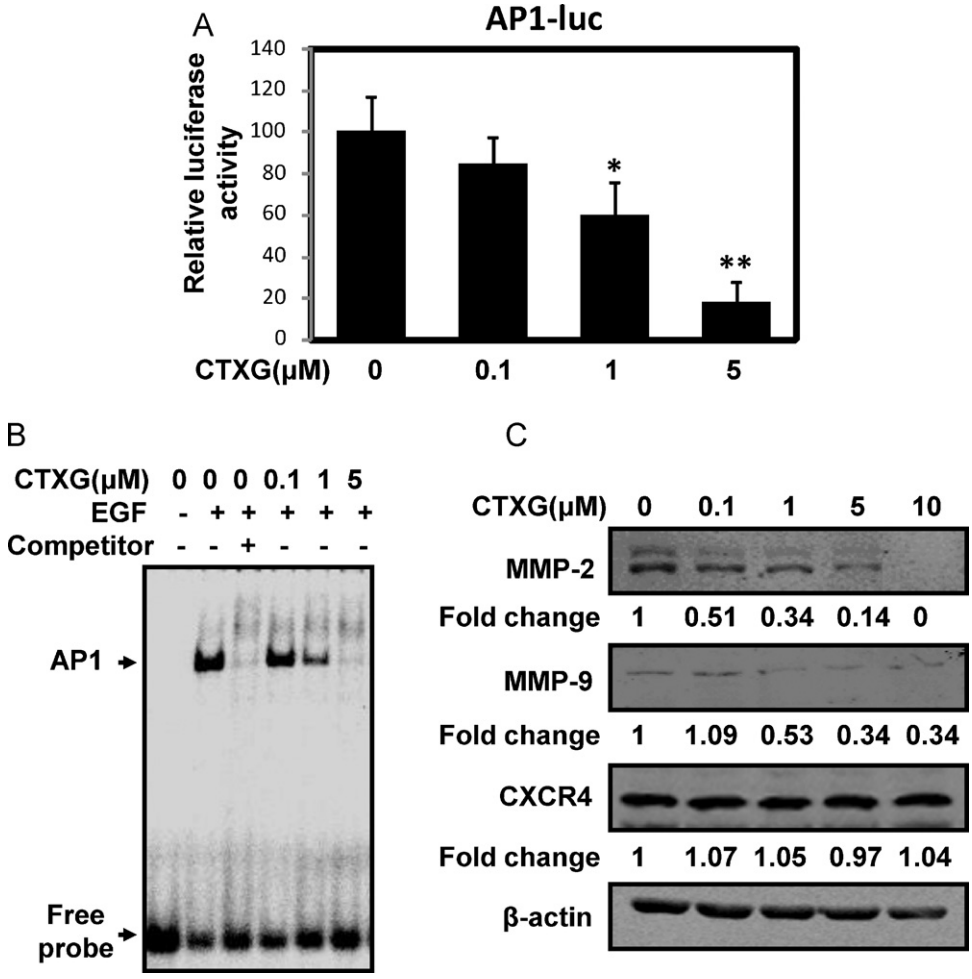

Fig. 7. CTXG inhibits AP-1 transcriptional and DNA-binding activity. (A) pAP1-luciferase vector transfected 293 T cells were treated with different concentrations of CTXG for 24 h. The relative transcriptional activity of AP-1 was measured by luciferase assay as described in Section 2. *Columns*, mean of AP-1 luciferase activities calculated from three independent experiments; *bars*, SE (*$P < 0.05$; **$P < 0.01$ versus control). (B) SW620 cells were treated with various concentrations of CTXG for 24 h, and then nuclear extracts were prepared and analyzed for AP-1 DNA binding activities using ^{32}P labeled AP-1 specific oligonucleotide. (C) CTXG inhibits the expression of MMP-2 and MMP-9 but not CXCR4.

MMPs, a family of zinc-dependent endopeptidases, have been reported to be playing a crucial role in ECM degradation, which is known to associate with tissue repair, cancer cell invasion, metastasis and angiogenesis [32,33]. Among several MMPs, MMP-2 has been demonstrated to be a critical factor in tumor invasion [26]. MMP-2 is secreted by tumor cells as a pro-enzyme (pro-MMP-2) and activated in the extracellular milieu to execute their proteolytic activity, then accordingly enables cells to invade into the target organ and develop tumor metastasis [34–36]. Previous study showed that MMP-2 deficient mice could not proceed to tumor metastasis [34]. Besides, inhibiting MMP-2 expression or interfering with MMP-2 functions by depressing its activity can reverse cell pro-metastasis motility and invasion. Therefore, MMP-2 could potentially be an important target in cancer therapy. However, many MMP inhibitors have been investigated in early clinical studies with disappointing results due to their low efficacy and various side effects [37]. Thus, searching for novel drugs targeting MMP proteins with high efficacy and specificity became critical to conquer the clinical difficulties. MMP-2 activity is known to be regulated on at least three levels: transcription, proteolytic activation of the zymogen form and inhibition of the active enzyme [38]. In this study, we found CTXG could suppress both the activation and expression of MMP-2 at 0.1–5 μM, indicating MMP-2 is a rational molecular target of CTXG for modulating metastasis in SW620 cells. We detected MMP-2 level with two different methods, gelatin zymography (for activity) and western blot (for expression) of medium. Decreased level of MMP-2 was identified in both experiments (Fig. 3A). These results indicate that inhibitory activity of MMP-2 is both regulated in the activity and expression. In contrast, the activity and expression of MMP-9 in SW620 cell conditional medium were not detectable by either zymogram or western blot. However, western blot on cell lysate with anti-MMP-9 antibody revealed that the MMP-9 expression level was very low, which could explain why it is undetectable in cell conditional cultured medium. Despite the low level, the expression of MMP-9 was still inhibited by CTXG in a dose-dependent manner, suggesting MMP-9 is also a target of CTXG (Fig. 7C).

Rho small GTPases which constitute a subfamily of the Ras superfamily of monomeric GTP-binding proteins, act as molecular switches that trigger signaling cascades in response to upstream signals [39]. Besides, small GTPases have also been implicated to regulate gene transcription [40]. Since small GTPases are critical regulators of metastasis, drugs targeting small GTPases signaling are potential pharmacological and therapeutic agents [41]. RhoA, Rac1, and Cdc42 are the best studied and understood members of small GTPase family [39,42]. Rac1 was characterized as a signaling molecule that regulates cell shape and motility by promoting lamellipodia formation and membrane ruffling in the leading edge of cells [43]. It was also known to play a pivotal role in malignant

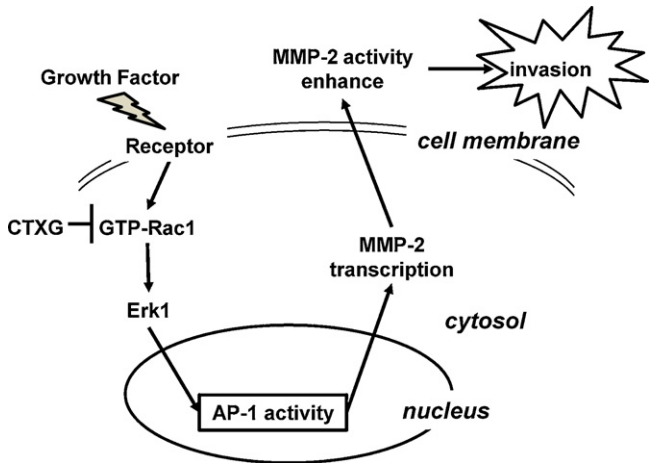

Fig. 8. Diagram of CTXG inhibiting tumor invasion through suppression of Rac1/AP-1 signaling.

tumor progression [44]. AP-1 is one of the downstream transcription factors regulated by Rac1. Abnormal activation of AP-1 has been implicated in malignant transformation, and tumor promotion [31,45]. Previous reports have demonstrated that some metastasis enhancing genes such as MMP-9 and MMP-2 have AP-1 transcription-binding motifs [31,45,46]. Therefore, signal pathways leading to the activation of AP-1 binding factors in tumor cells may contribute to MMPs transcription and metastasis enhancement. To investigate whether Rho small GTPases involved in the anti-metastasis action of CTXG, we carried out GST-PBD/RBD pull down assay to detect the activation of Rac1, Cdc42 and RhoA. GTP-Rac1 and GTP-Cdc42 were found to be down-regulated in cells treated with CTXG at 0.1 μM or higher concentrations (Figs. 4A–C and 5). In contrast, the effect of CTXG on RhoA was hardly detectable at tested concentrations (Fig. 4D and E), indicating that RhoA may not be pivotal to CTXG anti-invasive function. Thus, CTXG may inhibit colorectal cancer invasion mainly by Rac1 and Cdc42 signaling (Figs. 4 and 5). Besides, the inhibitory effect of CTXG on phosphory-Erk1 indicates that Erk1 may also contribute to CTXG function (Fig. 6). The inhibition of CTXG on phosphorylation of JNK1 and JNK2 at high-dose indicates that JNKs may be involved but not key factors in CTXG functioning. Since AP-1 may be one of the prime components of the intracellular signaling pathways responsible for the up-regulation of MMP-2 and MMP-9, we carried out luciferase reporter assay and EMSA to assess the effect of CTXG on AP-1 transcriptional activity. Results show that AP-1 transcriptional activity (Fig. 7A) and DNA binding activity (Fig. 7B) were inhibited markedly by CTXG in a dose-dependent manner, suggesting CTXG could exert its anti-metastasis activity by regulating gene expression through suppressing the activity of AP-1. Additionally, we performed western blot assay on cell lysate to detect the expression level of AP-1 target genes including MMP-2, MMP-9 and non-AP-1 target gene CXCR4. Consistent with the result of gelatin zymography assay, the expression of MMP-2 in SW620 cells was significantly reduced by treatment with various concentrations of CTXG. Besides, the expression of MMP-9 also decreased markedly in CTXG treated cells, while the expression of CXCR4, which is not regulated by AP-1, had little change (Fig. 7C) as expected.

Taken together, we demonstrated that CTXG inhibits colorectal carcinoma cell SW620 proliferation, migration and invasion by targeting MMP-2 through regulating the activation of Rac1/Cdc42 and downstream transcription factor AP-1, thus leading to suppression of metastasis (Fig. 8). The therapeutic potential of CTXG for controlling tumor metastasis was implied by our study for the first time.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgments

This work is supported by the Research Platform of Cell Signaling Networks (06DZ22923), Shanghai Scientific Development Foundation (074319104) from the Science and Technology Commission of Shanghai Municipality, and the Key National Scientific and Technological Program "Major New Drug Development" of the Ministry of Science and Technology of the PR China (No. 2009ZX09103-437).

References

[1] Hou A, Fukai T, Shimazaki M, Sakagami H, Sun H, Nomura T. Benzophenones and xanthones with isoprenoid groups from Cudrania cochinchinensis. J Nat Prod 2001;64:65–70.
[2] Fukai T, Yonekawa M, Hou AJ, Nomura T, Sun HD, Uno J. Antifungal agents from the roots of Cudrania cochinchinensis against Candida, Cryptococcus, and Aspergillus species. J Nat Prod 2003;66:1118–20.
[3] Zou YS, Hou AJ, Zhu GF, Chen YF, Sun HD, Zhao QS. Cytotoxic isoprenylated xanthones from Cudrania tricuspidata. Bioorg Med Chem 2004;12:1947–53.
[4] Tian YH, Kim HC, Cui JM, Kim YC. Hepatoprotective constituents of Cudrania tricuspidata. Arch Pharm Res 2005;28:44–8.
[5] Kim TJ, Han HJ, Hong SS, Hwang JH, Hwang BY, Yoo HS, et al. Cudratricusxanthone A isolated from the root bark of Cudrania tricuspidata inhibits the proliferation of vascular smooth muscle cells through the suppression of PDGF-receptor beta tyrosine kinase. Biol Pharm Bull 2007;30:805–9.
[6] Jeong GS, An RB, Pae HO, Chung HT, Yoon KH, Kang DG, et al. Cudratricusxanthone A protects mouse hippocampal cells against glutamate-induced neurotoxicity via the induction of heme oxygenase-1. Planta Med 2008;74:1368–73.
[7] Jeong GS, Lee DS, Kim YC. Cudratricusxanthone A from Cudrania tricuspidata suppresses pro-inflammatory mediators through expression of anti-inflammatory heme oxygenase-1 in RAW264.7 macrophages. Int Immunopharmacol 2009;9:241–6.
[8] Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics, 2001. CA Cancer J Clin 2001;51:15–36.
[9] Hawk ET, Limburg PJ, Viner JL. Epidemiology and prevention of colorectal cancer. Surg Clin North Am 2002;82:905–41.
[10] McCracken M, Olsen M, Chen Jr MS, Jemal A, Thun M, Cokkinides V, et al. Cancer incidence, mortality, and associated risk factors among Asian Americans of Chinese, Filipino, Vietnamese, Korean, and Japanese ethnicities. CA Cancer J Clin 2007;57:190–205.
[11] Yamamoto M, Kikuchi H, Ohta M, Kawabata T, Hiramatsu Y, Kondo K, et al. TSU68 prevents liver metastasis of colon cancer xenografts by modulating the premetastatic niche. Cancer Res 2008;68:9754–62.
[12] Hayashi K, Zhao M, Yamauchi K, Yamamoto N, Tsuchiya H, Tomita K, et al. Cancer metastasis directly eradicated by targeted therapy with a modified Salmonella typhimurium. J Cell Biochem 2009;106:992–8.
[13] Royston D, Jackson DG. Mechanisms of lymphatic metastasis in human colorectal adenocarcinoma. J Pathol 2009;217:608–19.
[14] Wen J, Matsumoto K, Taniura N, Tomioka D, Nakamura T. Inhibition of colon cancer growth and metastasis by NK4 gene repetitive delivery in mice. Biochem Biophys Res Commun 2007;358:117–23.
[15] Soreide K, Janssen EA, Korner H, Baak JP. Trypsin in colorectal cancer: molecular biological mechanisms of proliferation, invasion, and metastasis. J Pathol 2006;209:147–56.
[16] Yujiri T, Nawata R, Takahashi T, Sato Y, Tanizawa Y, Kitamura T, et al. MEK kinase 1 interacts with focal adhesion kinase and regulates insulin receptor substrate-1 expression. J Biol Chem 2003;278:3846–51.
[17] Black PC, Agarwal PK, Dinney CP. Targeted therapies in bladder cancer—an update. Urol Oncol 2007;25:433–8.
[18] Binker MG, Binker-Cosen AA, Richards D, Oliver B, Cosen-Binker LI. EGF promotes invasion by PANC-1 cells through Rac1/ROS-dependent secretion and activation of MMP-2. Biochem Biophys Res Commun 2009;379:445–50.
[19] Yi T, Yi Z, Cho SG, Luo J, Pandey MK, Aggarwal BB, et al. Gambogic acid inhibits angiogenesis and prostate tumor growth by suppressing vascular endothelial growth factor receptor 2 signaling. Cancer Res 2008;68:1843–50.
[20] Wang L, Kuang L, Pan X, Liu J, Wang Q, Du B, et al. Isoalvaxanthone inhibits colon cancer cell proliferation, migration and invasion through inactivating Rac1 and AP-1. Int J Cancer 2010;127:1220–9.
[21] Johansson S, Smedsrod B. Identification of a plasma gelatinase in preparations of fibronectin. J Biol Chem 1986;261:4363–6.

[22] Shan D, Chen L, Njardarson JT, Gaul C, Ma X, Danishefsky SJ, et al. Synthetic analogues of migrastatin that inhibit mammary tumor metastasis in mice. Proc Natl Acad Sci USA 2005;102:3772–6.
[23] Mitchell DC, Abdelrahim M, Weng J, Stafford LJ, Safe S, Bar-Eli M, et al. Regulation of KiSS-1 metastasis suppressor gene expression in breast cancer cells by direct interaction of transcription factors activator protein-2alpha and specificity protein-1. J Biol Chem 2006;281:51–8.
[24] Frische R, Klopffer W, Rippen G, Gunther KO. The Environmental Model Segment Approach for estimating potential environmental concentrations. I. The model. Ecotoxicol Environ Saf 1984;8:352–62.
[25] Jung SH, Woo MS, Kim SY, Kim WK, Hyun JW, Kim EJ, et al. Ginseng saponin metabolite suppresses phorbol ester-induced matrix metalloproteinase-9 expression through inhibition of activator protein-1 and mitogen-activated protein kinase signaling pathways in human astroglioma cells. Int J Cancer 2006;118:490–7.
[26] Dashevsky O, Varon D, Brill A. Platelet-derived microparticles promote invasiveness of prostate cancer cells via upregulation of MMP-2 production. Int J Cancer 2009;124:1773–7.
[27] Sheng S, Qiao M, Pardee AB. Metastasis and AKT activation. J Cell Physiol 2009;218:451–4.
[28] Duthie SJ. Berry phytochemicals, genomic stability and cancer: evidence for chemoprotection at several stages in the carcinogenic process. Mol Nutr Food Res 2007;51:665–74.
[29] Pandey MK, Sung B, Ahn KS, Kunnumakkara AB, Chaturvedi MM, Aggarwal BB. Gambogic acid, a novel ligand for transferrin receptor, potentiates TNF-induced apoptosis through modulation of the nuclear factor-kappaB signaling pathway. Blood 2007;110:3517–25.
[30] Hung SH, Shen KH, Wu CH, Liu CL, Shih YW. Alpha-mangostin suppresses PC-3 human prostate carcinoma cell metastasis by inhibiting matrix metalloproteinase-2/9 and urokinase-plasminogen expression through the JNK signaling pathway. J Agric Food Chem 2009;57:1291–8.
[31] Sliva D. Signaling pathways responsible for cancer cell invasion as targets for cancer therapy. Curr Cancer Drug Targets 2004;4:327–36.
[32] Bogenrieder T, Herlyn M. Axis of evil: molecular mechanisms of cancer metastasis. Oncogene 2003;22:6524–36.
[33] Vihinen P, Kahari VM. Matrix metalloproteinases in cancer: prognostic markers and therapeutic targets. Int J Cancer 2002;99:157–66.
[34] Itoh Y, Nagase H. Matrix metalloproteinases in cancer. Essays Biochem 2002;38:21–36.
[35] Kitamura T, Taketo MM. Keeping out the bad guys: gateway to cellular target therapy. Cancer Res 2007;67:10099–102.
[36] Bjorklund M, Koivunen E. Gelatinase-mediated migration and invasion of cancer cells. Biochim Biophys Acta 2005;1755:37–69.
[37] Dorman G, Cseh S, Hajdu I, Barna L, Konya D, Kupai K, et al. Matrix metalloproteinase inhibitors: a critical appraisal of design principles and proposed therapeutic utility. Drugs 2010;70:949–64.
[38] Polette M, Nawrocki-Raby B, Gilles C, Clavel C, Birembaut P. Tumour invasion and matrix metalloproteinases. Crit Rev Oncol Hematol 2004;49:179–86.
[39] Barber MA, Welch HC. PI3K and RAC signalling in leukocyte and cancer cell migration. Bull Cancer 2006;93:E44–52.
[40] Brown MA, Jones KA, Nicolai H, Bonjardim M, Black D, McFarlane R, et al. Physical mapping, cloning, and identification of genes within a 500-kb region containing BRCA1. Proc Natl Acad Sci USA 1995;92:4362–6.
[41] Fritz G, Kaina B. Rho GTPases: promising cellular targets for novel anticancer drugs. Curr Cancer Drug Targets 2006;6:1–14.
[42] Wennerberg K, Der CJ. Rho-family GTPases: it's not only Rac and Rho (and I like it). J Cell Sci 2004;117:1301–12.
[43] Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 1992;70:401–10.
[44] Kimmelman AC, Hezel AF, Aguirre AJ, Zheng H, Paik JH, Ying H, et al. Genomic alterations link Rho family of GTPases to the highly invasive phenotype of pancreas cancer. Proc Natl Acad Sci USA 2008;105:19372–7.
[45] de la Lastra CA, Villegas I. Resveratrol as an anti-inflammatory and anti-aging agent: mechanisms and clinical implications. Mol Nutr Food Res 2005;49:405–30.
[46] Mook OR, Frederiks WM, Van Noorden CJ. The role of gelatinases in colorectal cancer progression and metastasis. Biochim Biophys Acta 2004;1705:69–89.