Chrysanthemum indicum ethanolic extract inhibits invasion of hepatocellular carcinoma via regulation of MMP/TIMP balance as therapeutic target

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Abstract. Hepatocellular carcinoma (HCC) is an aggressive cancer with a dismal outcome largely due to metastasis and postsurgical recurrence. Thus, the inhibition of invasion and metastasis is of great importance in its therapies. Medicinal plants or ethnopharmacology used in folklore medicine continue to be an important source of discovery and development of novel or potential therapeutic agents for treatment of cancer. *Chrysanthemum indicum*, one of the medicinal plants or ethnopharmacology, is being used for treatment of many diseases including cancer. However, this plant molecular mechanisms underlining the anti-metastatic effects have not been well documented. In this study, *Chrysanthemum indicum* ethanolic extract (CIE) significantly suppressed proliferation and invasion of MHCC97H cells, one of the HCC cell lines with high metastatic potential, in a dose-dependent manner. CIE markedly decreased MMP-2 and MMP-9 expression, increased simultaneously TIMP-1, and TIMP-2 expression further restoring their balance in the cancer cells. The present study indicates that CIE reduced MHCC97H cell metastatic capability, in part at least, through decrease of the MMP expression, simultaneous increase of the TIMP expression, further restoring their balance as therapeutic target in HCC. It is suggested that *Chrysanthemum indicum* is a potential novel therapeutic medicinal plant for treatment of HCC or cancer invasion and metastasis.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in China, where it ranks as the second leading cause of cancer mortality (1) and approximately accounts for half in the world (2,3). Features of HCC are an aggressive cancer with a dismal outcome largely due to metastasis and postsurgical recurrence (4). Thus, the inhibition of invasion and metastasis is of great importance in the HCC therapies.

Tumor invasion and metastasis, which, respectively, refer to the spread and growth of cancer cells from a primary neoplasm to distant sites, represent a multistep process that depends on the activities of many factors associated with the proteolytic degradation of extracellular matrix (ECM) components (5). Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, play a crucial role in ECM degradation associated with cancer cell invasion, metastasis and angiogenesis (5,6). Among them, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are significantly up-regulated in malignant tumors and cause invasion and metastatic spread of cancer cells by degrading type IV collagen, a major component of the basement membrane. Activities of MMPs are controlled by their endogenous inhibitors, which are known as tissue inhibitor of metalloproteinases (TIMPs) such as TIMP-1 and TIMP-2 in cancer cells (7). It has been suggested that the balance of MMPs and TIMPs was broken. Therefore, direct inhibition of MMPs but increase of TIMPs in cancer could emerge as a particularly attractive target for therapeutic intervention in tumor invasion and metastasis.

Less effective management that decreases MMP expression, but increases simultaneously TIMP expression, and further reverses the imbalance between them in HCC invasion is currently available. Medicinal plants or ethnopharmacology used in folklore medicine continue to be an important source of discovery and development of novel or potential therapeutic agents in cancer proliferation and invasion (8-10), thereby indicating that medicinal plants or ethnopharmacology could exhibit anti-metastatic properties against MMP/TIMP imbalance of cancer. Particularly, in

East Asia such as China, Korea, and Japan, abundant experience and substantial clinical data on the management of HCC invasion and metastasis have been documented with traditional Chinese medicine (TCM), especially medicinal plants, which have been developed over a period of thousands of years (11-13).

The Compositae plant, flowers of *Chrysanthemum indicum* (Chrysanthemi Indici Flos), one of the TCM and medicinal plants, is widely used in the treatment of hypertension, colitis, pneumonia and carbuncle in China. Mounting evidence indicates that *Chrysanthemum indicum* possesses anti-inflammatory, antioxidant, immunomodulation and anti-microbial activities (14,15). Accumulating evidence shows that *Chrysanthemum indicum* is capable of inhibiting proliferation of human PC3, HL 60 and HeLa cancer cells in a dose- and time-dependent manner (16). Furthermore, our previous study found that *Chrysanthemum indicum* extract (CIE) exerted significant apoptotic effect through the mitochondrial-dependent capase-3 pathway and arrested the cell cycle by regulation of cell cycle related proteins including P21 and CDK4 on MHCC97H cells without impairment of normal cells (17). Intriguingly, in clinic, Xiang *et al* (18) found that patients receiving *Chrysanthemum indicum*, as one of main components, combined with other TCM, a 5-year overall survival rate of 70% and a complete response rate of 60%, and combination with chemotherapeutic agents, a 5-year overall survival rate of 77% and a complete remission rate of 80%, were achieved, respectively, in post-operative patients with metastatic breast cancer without adverse effects. Bi *et al* (19) demonstrated that with *Chrysanthemum indicum* combined with TCM, a response rate of 67% were obtained in management of esophageal carcinoma patients with advanced stage, and without myelosuppression and toxicities of liver and kidneys. These studies suggest that *Chrysanthemum indicum* is one of novel anti-metastatic drugs with low toxicity and high efficacy. However, studies of anti-metastatic properties of this herb hardly exist.

In the present study, we investigated the anti-metastatic properties of CIE using the MHCC97H cell line, a typical human HCC cells with high metastatic potential (20,21) that highly expressed MMPs (21,22), and commonly used in the study of antitumor invasion. Furthermore, we determined whether CIE inhibits the migration of MHCC97H cells through a Matrigel-coated membrane, possible modulating the effects of CIE toward the expression of MMPs and TIMPs.

Materials and methods

Cell culture and reagents. Human MHCC97H, a HCC cell line with high metastatic potential, was obtained from Liver Cancer Institute of Fudan University (Shanghai, China). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazo-lium bromide (MTT) was purchased from (Sigma Chemical Co., St. Louis, MO). DMEM, fetal bovine serum (FBS) and Trypsin was obtained from (Gibco BRL, Grand Island, NY, USA). Anti-MMP-2 and MMP-9, and anti-ß-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-TIMP-1 and TIMP-2 were purchased from Biosynthesis Biotechnology Company (Beijing, China). MHCC97H cells were cultured in DMEM supplemented with 10% FBS, growth in a 37˚C humidified incubator containing 5% CO_2 and 95% subcultured with 0.25% Trypsin-0.02% EDTA.

Preparation of Chrysanthemum indicum extract. The high quality flowers of *Chrysanthemum indicum* were dried under sunlight for 2 days, and processed for powder. As described in detail previously (17), briefly, the powdered herbs were extracted twice with 95% ethanol (with 2 h reflux), and the extract was then concentrated under reduced pressure. The concentrated extract was filtered, lyophilized, and stored at -20˚C. The yield of dried extract from starting crude materials was 13.3%. The lyophilized powder was dissolved in phosphate buffered solution (PBS) and then filtered through a 0.2-μm syringe filter.

Cell inhibition assay. MHCC97H cells were seeded into 96-well plates at a density of $5x10^4$/well. For the cell inhibition assay, cells were incubated with different concentrations of CIE 200, 400, 800, 1200, 1600 μg/ml or vehicle alone for 24 h, following by a MTT assay as described (23). After 24 h MTT solution (5 mg/ml) was then added to each well, and the plates were further incubated at 37˚C for 4 h in a humidified incubator with 5% CO_2. At the end of incubation period, the medium was discarded and 200 μl DMSO/well was added to a 96-well plate to solubilize formazan crystals. The optical density was determined at 570 nm with a microplate autoreader (Biotech Instruments, VT, USA).

Invasion assays. Briefly, as described previously (24), matrigel-coated filter inserts (8 μm pore size) that fit into 24-well invasion chambers were obtained from Becton-Dickinson (NJ, USA). MHCC97H cells were resuspended in conditioned medium ($5x10^4$ cells/200 μl), and then added to the upper compartment of the invasion chamber in the presence of various concentration of CIE (400, 800, 1200 μg/ml) or vehicle. Conditioned medium (500 μl) was added to the lower compartment and was incubated at 37˚C for 24 h in 5% CO_2. After incubation, the cells on the upper side of the filter were removed using cotton swabs and were fixed, mounted and stained according to the manufacturer's instructions. Cells that invaded through the matrigel and were located on the underside of the filter were counted. Three to five invasion chambers were used per condition. The values obtained were calculated by averaging the total number of cells from three filters.

Immunofluorescent staining of MHCC97H cells. To detect the effect of CIE on MMP-2, MMP-9, TIMP-1 and TIMP-2 expression in MHCC97H cells, the cells were pretreated with CIE (800 μg/ml) for 24 h. After the treatment, the cells were fixed with 4% formaldehyde-PBS for 15 min. The cell membranes were fenestrated with 0.3% Triton-100-PBS, and nonspecific binding sites were blocked with 10% goat serum. The cells were incubated with rabbit anti-human MMP-2 and MMP-9 (1:400) or anti-human TIMP-1 and TIMP-2 antibody (1:200) at 4˚C overnight and then incubated with the appropriate fluorescence-labeled secondary antibody conjugated to fluorescein isothiocyanate (FITC) at room

temperature for 1 h. The immunolabeled cells were observed under fluorescence confocal microscopy (Leica TCS SP2-AOB).

Isolation of RNA and semiquantitative RT-PCR. When $1x10^6$ MHCC97H were incubated with various concentrations of CIE (400, 800, 1200 μg/ml) or vehicle for 24 h, total cellular RNA was isolated from cells using the TRIzol kit (Takara, Biomedicals), as reported previously (25). Total RNA (2 μl) was reverse transcribed to 10 μl cDNA with 1 mM oligo (dT), 0.5 mM dNTP, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1 unit RNase inhibitor. The PCR reaction proceeded in 'Ready To Go' PCR Beads (Takara, Bio-medicals). The PCR cycles were 94˚C for 5 min, 58˚C for 2 min, and 72˚C for 3 min. The PCR products (179-bp ß-actin fragment; 498-bp MMP-2 fragment; 204-bp MMP-9 fragment; 276-bp TIMP-1 fragment; 430-bp TIMP-2 fragment) were eletrophoresed on 1.5% agarose gel after 30 cycles and visualized by ethidium bromide staining. Amplification of ß-actin served as a control for sample loading and integrity. The sense and anti-sense primer sequences were ß-actin: ATCGTGCGTGACTTAAGGAG and GGAAGGAAGGCT GGAAGAG; MMP-9: GACGCCGCTCACTTACTC and GGAACCACGACGCCCTTGC; MMP-2: CAAGTGGTCC TGTAATATGG and GTCATCATCGTAGTTGGCTGTGG; TIMP-1: GTTGTGCGTGGCGATAG and TGTGGGACC TGTGGAGTA; TIMP-2: CACCCACAGACGGCCTTCTG CAAT and AGTGTAGGTCTTGGTGAAGCC.

Western blotting. MHCC97H cells ($1x10^6$/well) were treated with CIE (400, 800, and 1200 μg/ml) or vehicle for an appropriate time. For MMP-2, MMP-9, TIMP-1 and TIMP-2 Western blotting, as described previously (25), cells were lysed in a sample buffer followed by denaturation. Protein concentration was measured using Bradford assay and equal amounts of protein (50 μg) were subjected to SDS-PAGE on 12% gel. The proteins were then electrophoretically transferred to nitrocellulose membranes. Membranes were first blocked with 5% nonfat dry milk/PBS + 0.1% Tween-20 (W/V) and immunolabelled using primary antibodies. Goat anti-rabbit HRP conjugated antibodies (Cell Signaling Technology) were used as secondary antibodies and detected with enhanced chemiluminescence (ECL) (Amersham, USA). Equal loading of each lane was evaluated by immunoblotting the same membranes with ß-actin antibodies after detachment of previous primary antibodies. Photographs were taken and optical densities of the bands were scanned and quantified with the Gel Doc 2000 (Bio-Rad).

Gelatin zymography analysis on the enzyme activities of MMP-2 and MMP-9. Gelatin zymography was performed as described previously (26). Briefly, MHCC97H cancer cells treated with CIE (400, 800, and 1200 μg/ml) or without were incubated in serum-free medium for 24 h. Then the samples of conditioned media were collected and diluted in a sample buffer containing and loaded without boiling separating gel containing 0.1% (W/V) gelatin. After electrophoresis with a constant voltage of 100 V, the gels were soaked in 0.25% Triton X-100 (2 times for 30 min) at room temperature and rinsed in distilled water. The gel slab was cut into slices

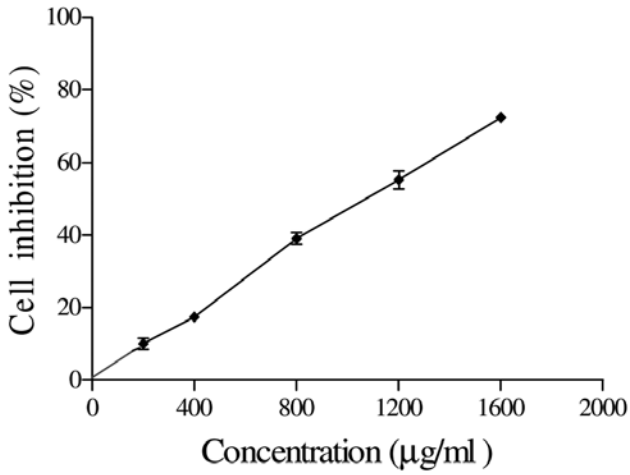

Figure 1. Anti-proliferative effect of *Chrysanthemum indicum* ethanolic extract (CIE) on MHCC97H cells at $5x10^4$ cells/well which were cultured with CIE (200, 400, 800, 1200, 1600 μg/ml) in DMEM-10% fetal bovine serum for 24 h. Cells proliferation were assayed by MTT. Values represent means ± SEM from three independent experiments.

corresponding to the lanes and incubated at 37˚C for 24 h in an incubation buffer. The gel was then stained for 30 min in 0.1% (W/V) Coomassie blue R-250 in 30% methanol and 10% acetic acid, and destained in the same solution without the Coomassie blue dye. Proteolysis was detected as a white zone in a dark field, and the intensity of the bands obtained from the zymogram studies was estimated with a Scion Image instrument (Scion Corp., MD, USA).

Reverse zymography analysis on the enzyme activities of TIMP-1 and TIMP-2. Reverse zymography was used to detect TIMP-1 and TIMP-2 activity as described previously (27). Briefly, after MHCC97H cancer cells treated with CIE (400, 800, and 1200 μg/ml) or without were incubated in serum-free medium for 24 h, equal protein concentrations of conditional culture medium were then subjected to SDS-10% polyacrylamide gel electrophoresis on gels containing 1 mg/ml of gelatin. After electrophoresis was completed, gels were washed in 2.5% Triton X-100 and incubated for 1 h at 37˚C with conditioned media from phorbol 12-myristate 13-acetate (PMA)-activated MHCC97H cells, containing a mixture of activated MMPs. Then gels were incubated with 50 mM Tris-HCl, 50 mM NaCl, 10 mM $CaCl_2$, and 0.05% Brij for 18 h at 37˚C, stained with Coomassie blue, and destained in acetic acid/methanol. Under these conditions, TIMPs inhibit gelatin digestion by activating MMP sand produce dark blue bands against a bright background. For quantification, densitometric scanning was performed by using a Bio-Rad GS 690 Image Analysis software system.

Statistical analysis. All measurements were performed in triplicate and repeated 3 times. Results are expressed as means ± SEM. Data were analyzed using ANOVA and Dunnett's multiple comparison tests. $P<0.05$ was considered significant.

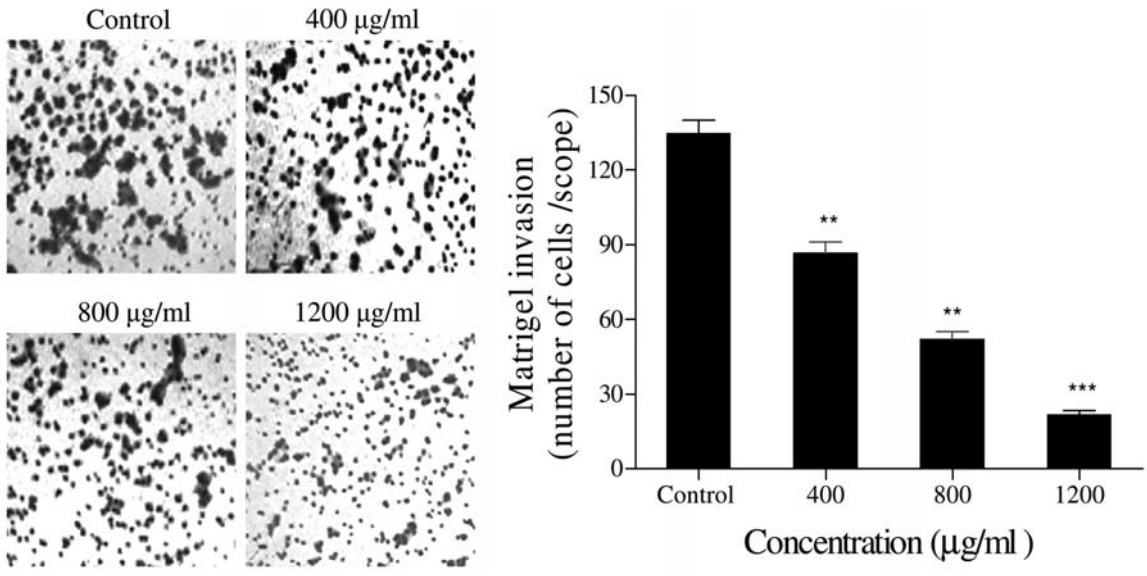

Figure 2. Effect of CIE on MHCC97H cells invasion at $5x10^4$ cells/200 μl cultured with or without CIE (400, 800, and 1200 μg/ml) in DMEM-10% fetal bovine serum for 24 h. Cell invasion was determined by transwell assay. Values represent means ± SEM from three independent experiments (**P<0.01, ***P<0.001 vs. control group).

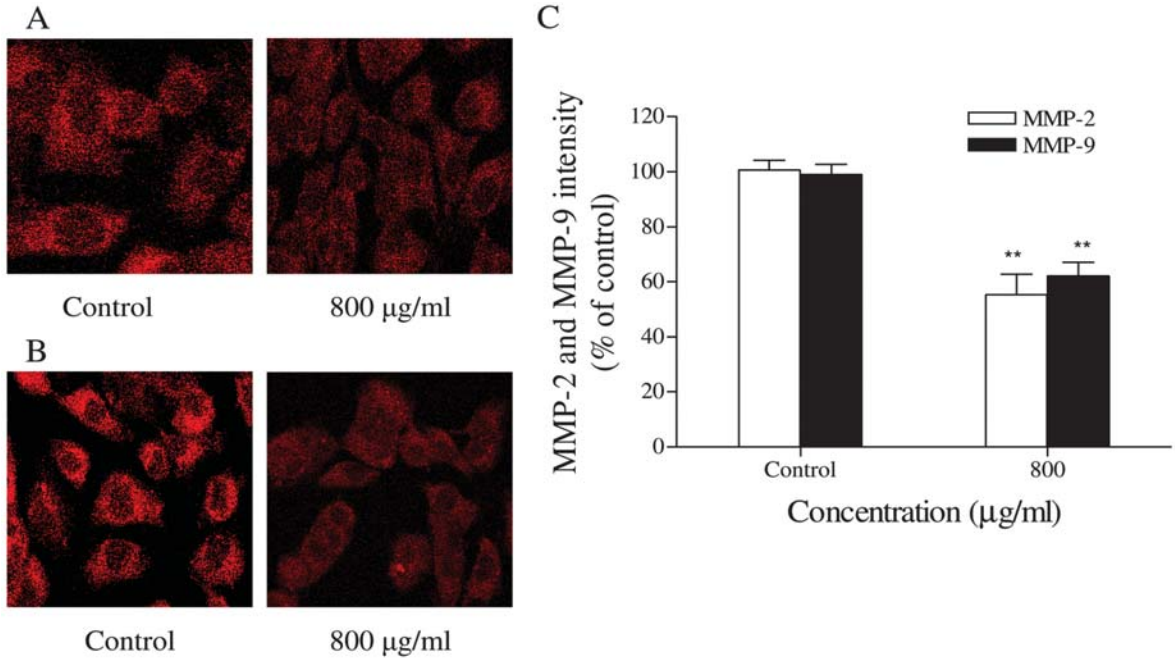

Figure 3. Immunofluorescence analysis of MMP-2 and MMP-9 expression in CIE treated MHCC97H cells or without. MHCC97H cells were pretreated for 24 h with concentrations of 800 μg/ml CIE and then expression of MMP-2 (A) and MMP-9 (B) was identified with immunocytofluorescence and observed by confocal microscope. Fluorescence intensity of MMP-2 and MMP-9 was also detected (C). Values represent means ± SEM from three independent experiments (**P<0.01 vs. control group).

Results

CIE inhibited proliferation in HCC cells. In this study, the effect of CIE on cell proliferation toward MHCC97H cells was measured by MTT assay. The effects of 200-1600 μg/ml CIE on cell growth after 24 h are shown in Fig. 1. After 24 h of incubation, CIE significantly suppressed MHCC97H cell growth in a concentration-dependent manner with cell numbers markedly reduced compared to control. CIE at various concentrations significantly increased inhibition of cell proliferation compared with control, by 12.8, 19.2, 41, 59, and 74.3%, respectively.

CIE attenuated HCC cell invasion. The potential effect of CIE on cell migration was tested by counting MHCC97H cells that migrated through the Matrgel coated-membrane. As shown in Fig. 2, cell migrations were 86.7±7.5, 52±5.6, 21.7±3.1 in 400, 800, 1200 μg/ml dose of CIE, respectively,

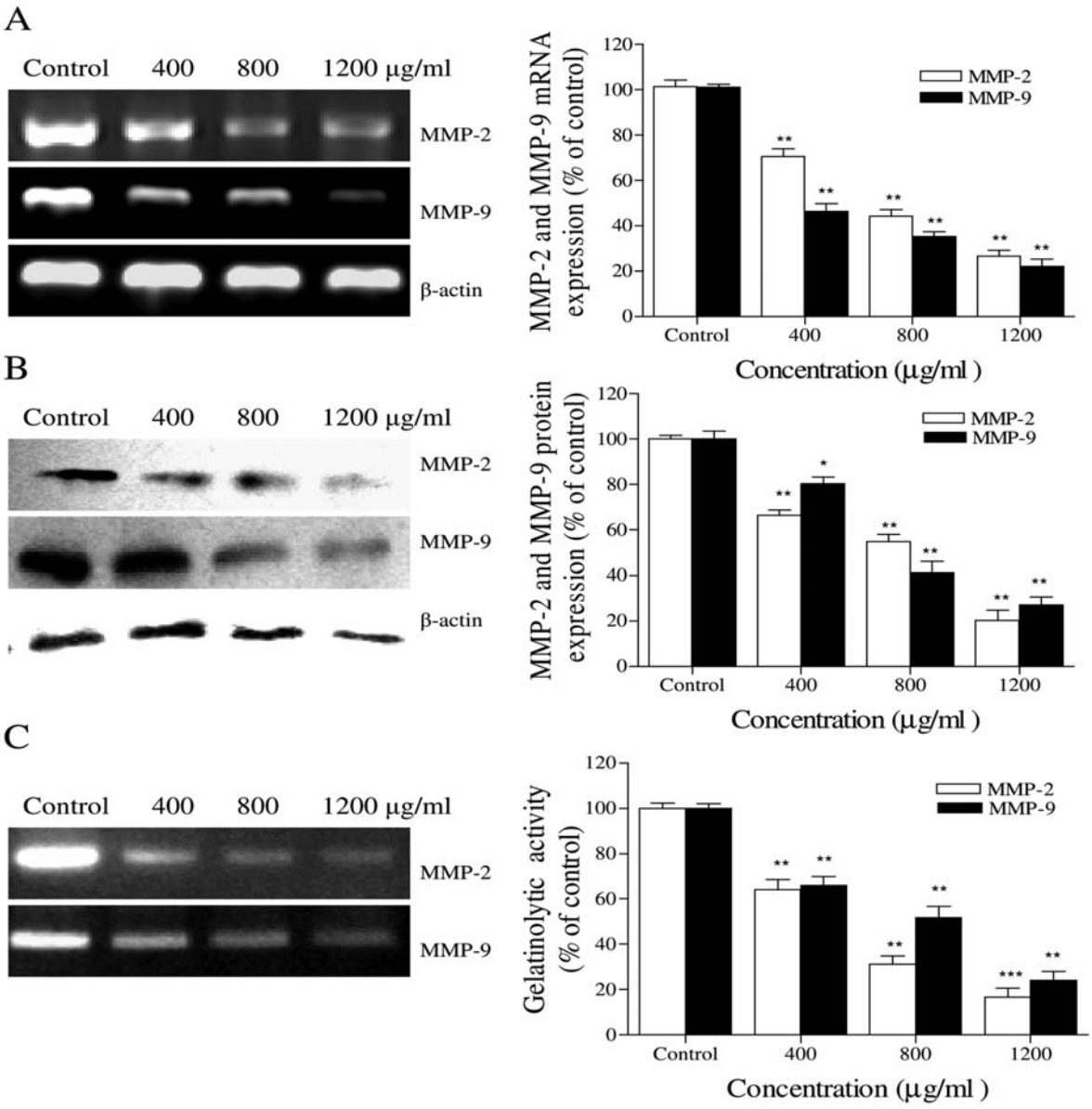

Figure 4. Effect of CIE on MMP-2 and MMP-9 mRNA, protein and enzyme activity in MHCC97H cells. MHCC97H cells ($1x10^6$ cells/well) were incubated for 24 h with CIE (400, 800, and 1200 μg/ml) or without. The mRNA (A), protein (B) and enzyme activity (C) expression of MMP-2 and MMP-9 were determined by RT-PCR, Western blot and gelatin zymography analysis. Values represent means ± SEM from three independent experiments (*P<0.05, **P<0.01, ***P<0.001 vs. control group).

whereas control group was 134.7±9.3. A significant decrease in the number of cancer cells migrating through the filters was observed after treatment with CIE (400, 800 and 1200 μg/ml) for 24 h (P<0.01). Furthermore, lesser cell counts were found along with the higher dosage of herbs added when MHCC97H cells were incubated with an increased dose of CIE.

CIE reduces MMP-2 and MMP-9 expression in MHCC97H cells with luciferase assay. On the basis of the above-mentioned cell invasion results, we tested the effect of CIE on the expression of MMP-2 and MMP-9 to investigate their possible action mechanisms of antimetastasis. The cells were pretreated with concentrations of CIE (800 μg/ml) for 24 h, and then MMP-2 and MMP-9 expression was identified with immunocytofluorescence and observed by confocal microscope. Compared with control group (100%), MMP-2 and MMP-9 expression was suppressed by 38% and 55%, respectively, by treatment with CIE at 800 μg/ml (Fig. 3, P<0.01).

CIE decreased MMP-2 and MMP-9 mRNA, protein and enzyme activity expression. Prompted by the above-mentioned results with the immunofluorescent method, we further analyzed the effect of CIE on MMP-2 and MMP-9 mRNA, protein and enzyme activity expression in MHCC97H cells. When MHCC97H cells were incubated with CIE (400, 800 and 1200 μg/ml) or without for 24 h, the amount of MMP-2 and MMP-9 mRNA, protein and enzyme activities was measured by RT-PCR, Western blotting and gelatin zymography, respectively. These results showed that CIE markedly suppressed MMP-2 and MMP-9 mRNA, protein

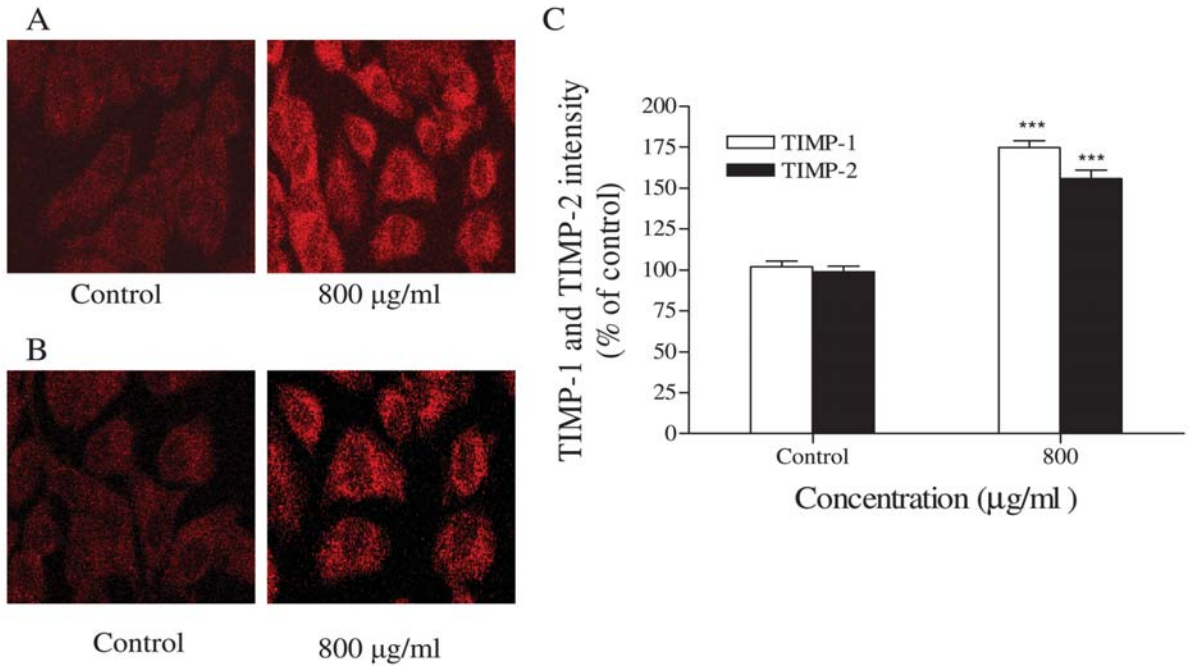

Figure 5. Immunofluorescence analysis of TIMP-1 and TIMP-2 expression in CIE treated MHCC97H cells or without. MHCC97H cells were pretreated for 24 h with concentrations of 800 μg/ml CIE and then expression of TIMP-1 (A) and TIMP-2 (B) were identified with immunocytofluorescence and observed by confocal microscope. Fluorescence intensity of TIMP-1 and TIMP-2 was also detected (C). Values represent means ± SEM from three independent experiments (***P<0.001 vs. control group).

and enzyme activity in a dose-dependent manner (Fig. 4, P<0.05). CIE at 1200 μg/ml inhibited MMP-2 and MMP-9 mRNA expression by 31.5% and 26%, reducing their protein expression by 27.6% and 33.8%, and attenuated their activity by 16.8% and 24.1%, respectively.

CIE promotes TIMP-1 and TIMP-2 expression in MHCC97H cells with luciferase assay. As mentioned above, CIE is able to decrease the MMP-2 and MMP-9 production. To confirm whether CIE inhibits MMPs production via increasing TIMP expression, expression of TIMP-2 and TIMP-9 in MHCC97H cells were also identified with immunofluorescent staining and observed by confocal microscope. Representative graphs from each group are shown in Fig. 5. Compared with the control group (100%), stronger TIMP-1 (175%) and TIMP-2 (156%) staining in the cytoplasm were seen in treatment with CIE concentrations of 800 μg/ml for 24 h (P<0.001).

CIE increases TIMP-1 and TIMP-2 mRNA, protein and enzyme activity expression. TIMPs are preferably bound to the active center and inhibit MMP protease activity, thereby suppressing tumor invasion and metastasis. The balance between both molecules finally determines the net proteolytic activity. To examine whether CIE had decreased MMPs and increased simultaneously the expression and activity of their natural inhibitors or not, we tested both TIMP-1 and TIMP-2 mRNA, protein and enzyme activities. As shown in Fig. 6A and B, there was a significant increase in TIMP-1 and TIMP-2 mRNA, protein expression in MHCC97H cells treated with 400, 800 and 1200 μg/ml CIE in comparison with the control group (P<0.05). In addition, to test whether CIE also affects TIMP-1 and TIMP-2 enzyme activity, we performed reverse zymography analysis. The amount of enzyme activity for TIMP-1 and TIMP-2 were dramatically enhanced with CIE in a concentration-dependent manner (Fig. 6C, P<0.05). Specifically, CIE at 1200 μg/ml increased TIMP-1 and TIMP-2 mRNA expression by 203% and 367%, enhanced their protein expression by 219% and 244%, and improved their activity by 98% and 99%, respectively.

Effect of CIE on restoring the MMP-TIMP expression imbalances in MHCC97H cells. Several investigations suggest that the imbalance of MMPs/TIMPs could contribute to cancer invasion and metastasis and the poor prognosis. To verify whether CIE could act on MMPs/TIMPs of cancer cells, we tested the expression ratio of MMPs:TIMPs in MHCC97H cells treated with CIE (0, 400, 800 and 1200 μg/ml). As shown in Fig. 7, the mRNA, protein, and enzyme activity levels of MMP-2 and MMP-9 significantly decreased in 400-1200 μg/ml group when compared with the untreated group. However, the mRNA, protein, and enzyme activity levels of TIMP-1 and TIMP-2 were also increased simultaneously under 400-1200 μg/ml conditions. Therefore, in contrast to control group, the ratio of MMP:TIMP, as shown representatively in MMP-2/TIMP-2 and MMP-9/TIMP-1, reduced followed by the increased concentration of CIE (P<0.05).

Discussion

Emerging studies report that CIE can inhibit proliferation of cancer cells, induce cell apoptosis including in HCC cells (16,17) and have shown anti-metastatic properties in clinic without side effects (18,19). CIE with low toxicity and high efficacy, one of the most active fields in cancer research, has been identified by our study (17) and clinical testing (18,19).

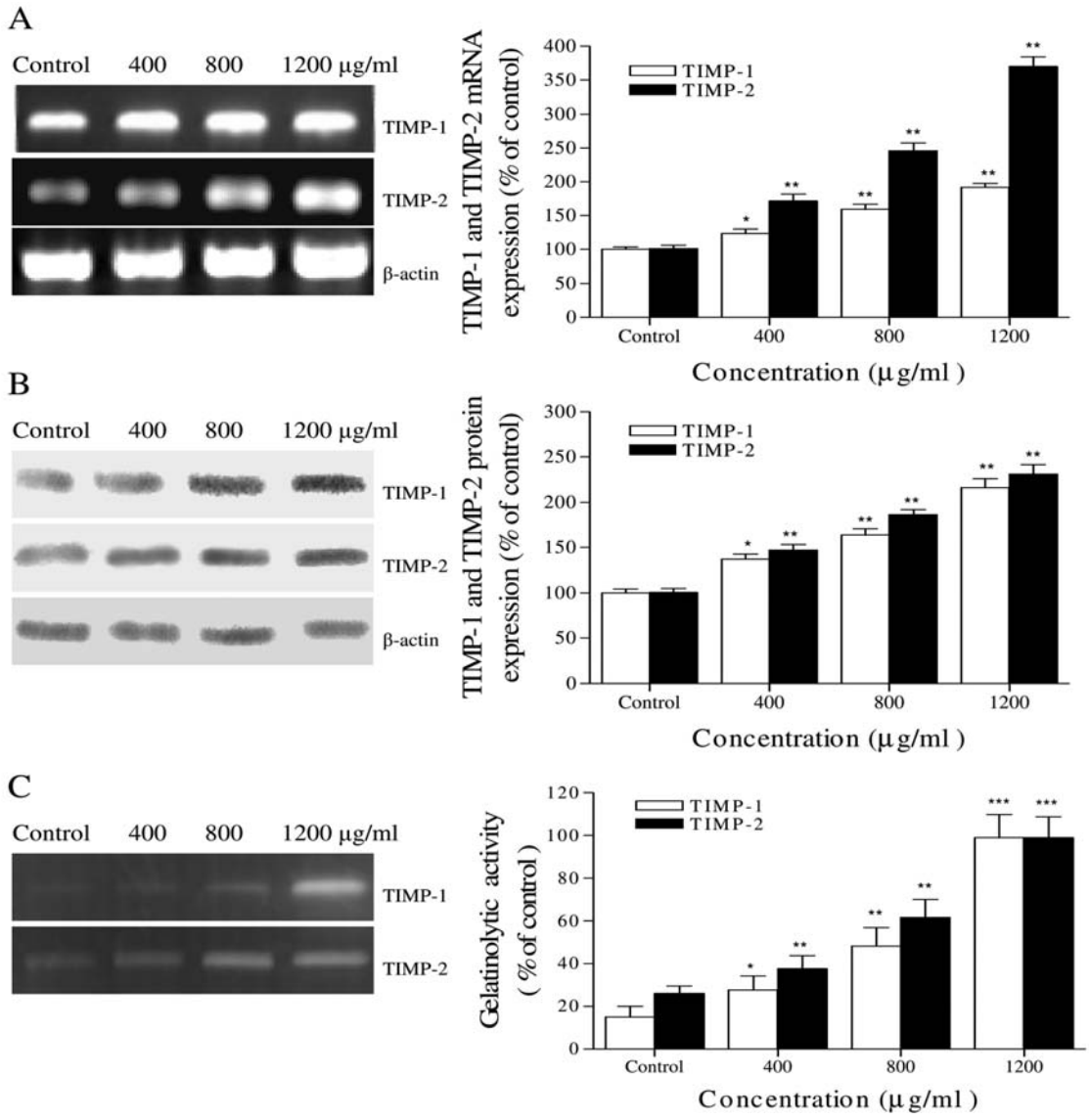

Figure 6. Effect of CIE on TIMP-1 and TIMP-2 mRNA, protein and enzyme activity expression in MHCC97H cells. MHCC97H cells ($1x10^6$ cells/well) were incubated for 24 h with CIE (400, 800, and 1200 μg/ml) or without. The mRNA (A), protein (B) and enzyme activity (C) expression of TIMP-1 and TIMP-2 were determined by RT-PCR, Western blot and reverse gelatin zymography analysis. Values represent means ± SEM from three independent experiments (*P<0.05, **P<0.01, ***P<0.001 vs. control group).

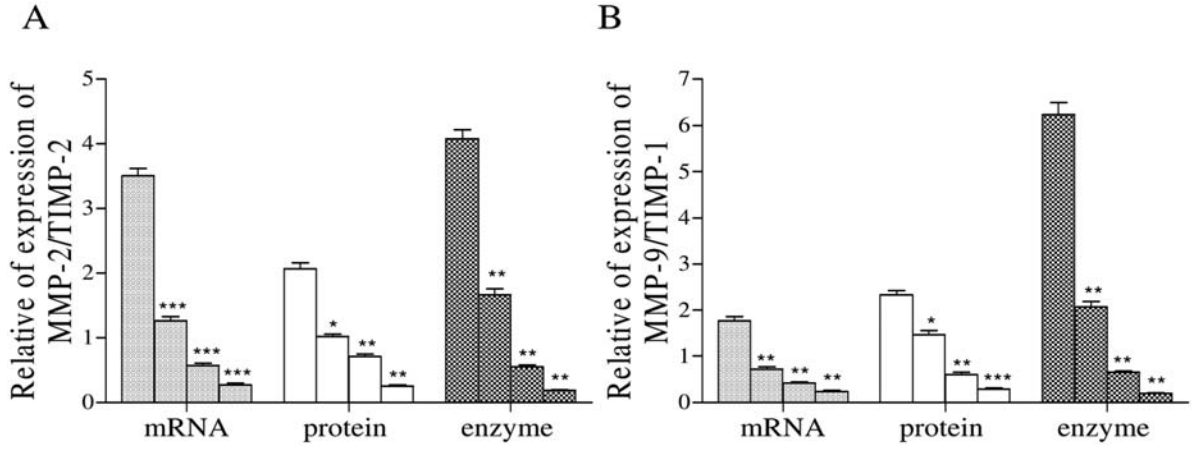

Figure 7. Effect of CIE on restoring the disoriented MMP and TIMP expression balances in MHCC97H cells. After MHCC97H cells were incubated for 24 h with CIE (400, 800, and 1200 μg/ml) or without, the relative expressions of MMPs and TIMPs were detected. Then the ratio values of MMPs/TIMPs in mRNA, protein expression and enzyme activity were calculated. Values represent means ± SEM from three independent experiments (*P<0.05, **P<0.01, ***P<0.001 vs. control group).

However, its molecular mechanisms underlining the anti-metastatic effects have not been well documented. In our experiments, as shown in Fig. 1, CIE has a significant inhibition of viability effect on MHCC97H cells. Further investigations verified that CIE significantly decreased the migration of MHCC97H cells through a Matrigel-coated membrane (Fig. 2), indicating that CIE had a potent anti-metastatic effect. Although the detailed mechanisms of metastasis has not been completed elucidated, the involvement of MMPs and TIMPs have been implicated.

Proteolysis of ECM components has been widely documented to be a critical step in the tumor metastasis cascade. Although the breakdown of ECM is achieved by several MMPs, notably the gelatinases or type IV collagenase MMP-2 and MMP-9 appear more important to ECM. Additionally, accumulating studies have demonstrated that activity of type IV collagenase (MMP-2 and MMP-9) is the highest in HCC with metastasis, and elevated serum levels of type IV collagenase reflect and predict potential of invasion and metastasis in patient with HCC (28-30). Furthermore, overexpression of MMP-2 or MMP-9 exacerbates the ECM degrading of invasive HCC cancer, whereas their inhibition has been reported to attenuate the ECM degraded process (26,31,32), proposing that MMPs act as induced metastasis in cancer. In the present study, as shown in Figs. 3 and 4, CIE significantly reduced mRNA, and protein activity of MMP-2 and MMP-9 in MHCC97H cells, suggesting that the inhibited effect of CIE on HCC invasion is likely attributable to its anti-MMP properties. Moreover, previously, several natural plants including TCM, Chai-hu-jia-longgu-mu-li-tang (31), *Euonymus alatus* extract (24), and *Magnolia officinalis* extract (33), have exhibited a potent anti-invasive inhibition of the gelatinolytic activities such as MMP-9 and MMP-2 expression properties in cancer cells. Thus far, however, it is unknown whether herbs or medicinal plants are able to attenuate MMP expression, but promote simultaneously TIMP expression and further restore the MMP/TIMP balance.

MMP activity, however, is inhibited by TIMPs so that an extensive degradation is avoided. In contrast, serum and tissue levels including HCC tissue and nodules, and TIMPs were significantly higher in hepatocellular carcinoma patients without metastasis than in those with metastasis (32). Therefore, the role of TIMPs is intriguing, not only in view of its involvement in the MMP activation process, but also because of its inhibitory function of MMP proteolytic activity. Among the members of the TIMP family, particularly TIMP-1 and TIMP-2, can inhibit tumor growth, invasion and metastasis, and also suppress MMP expression (27,30,32). Increased MMP expression, but reduced TIMP expression, were also found in several HCC cell lines with high metastatic potential (21,22,34) including MHCC97H cells. Furthermore, increased TIMP expression reduced invasion and metastasis of cancer cells (5,27), indicating that TIMP exert anti-metastatic effects in cancer. In this study, in MHCC97H cells, the effect of CIE on TIMP-1 and TIMP-2 mRNA, protein and enzyme activity was determined at the same time by RT-PCR, Western blot and reverse zymography analysis, respectively. As illustrated in Figs. 5 and 6, CIE could enhance TIMP-1 and TIMP-2 expression in MHCC97H cells in a dose-dependent manner, indicating CIE treatment of HCC could act as a potential anti-metastatic effect involving TIMPs.

Under physiologic conditions, the regulation of MMPs and TIMPs that could be bound with the active site of MMPs in the ratio of 1:1, in normal cells and tissue, maintain their dynamic equilibrium (27). However, it has been proposed that for basement membrane and ECM degradation, and ultimately invasion and metastasis to occur, alterations exist in the relative levels of MMPs to their associated TIMPs leading to an imbalance causing increased type IV collagen degradation (30,32). The metalloproteinases that degrade type IV collagen, the principal component of the basement membrane, include MMP-2 and MMP-9 and their inhibitors TIMP-2 (for MMP-2) and TIMP-1 (for MMP-9) (30). Moreover, previous studies have displayed that the MMP: TIMP ratio in cancer cells was ~1 that corresponded to cancers of good prognosis (27,30). Based on the evidence above, it is proposed that restoring impairment of MMPs/TIMPs could exert anti-metastasis effect on cancer cells. Our experiments demonstrated that CIE inhibits cancer cell invasion by suppressing MMP-2 and MMP-9 expression and by enhancing TIMP-1 and TIMP-2 expression, simultaneously. To combine the effects of CIE the decreased MMP expression and the increased TIMP expression, as shown in representatively for MMP-2/TIMP-2 and MMP-9/TIMP-1 (Fig. 7), our present results indicate that CIE could reverse the balance of the ratio between MMPs and TIMPs.

Collectively, at least in part, the results suggest that CIE contributes to a recuperation of the molar ratio imbalance between MMPs and TIMPs by decreasing expression of MMPs but augmenting expression of TIMPs. These phenomena indicate that CIE decreases invasion of hepatocellular carcinoma via regulation of MMP/ TIMP balance as therapeutic target.

In summary, the present study indicated that CIE inhibited the invasion and migration ability of MHCC97H cells, as assesses through a Matrigel-coated membrane invasion. These findings also showed that CIE could reduce cancer cell metastatic ability, in part at least, through decrease of the MMP expression, simultaneous increase of the TIMP expression, and further restoring their balance as a therapeutic target in HCC. *Chrysanthemum indicum* extract could be a novel therapeutic natural product for treatment of HCC or cancer invasion and metastasis without side effects or cytotoxicity.

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