Cucurbitacin I inhibits cell migration and invasion and enhances chemosensitivity in colon cancer

JUNMIN SONG[1], HONGXIANG LIU[1], ZHEN LI[1], CHAO YANG[1] and CHAOJIE WANG[2]

[1]Department of General Surgery, First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052; [2]Department of Oncology, Henan Provincial People's Hospital, Zhengzhou 450003, P.R. China

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Abstract. Colorectal cancers are the third most common types of cancers worldwide. Surgical resection is unable to eliminate tumors completely due to metastasis. A demand for new chemotherapeutic tools exists. In the present study, we examined the chemopreventive potential of cucurbitacin I, a natural component extracted from plants of the *Cucurbitaceae* family, in the colon cancer cell line COLO205. We hypothesized that cucurbitacin I would prevent colon cancer cell migration and invasion, and sensitize colon cancer cells to chemotherapy. Our data demonstrated that exposure of the COLO205 cells to cucurbitacin I significantly decreased cell viability. Furthermore our data demonstrated for the first time that in the COLO205 cells, cucurbitacin I could suppress the cell migration and invasion, and harbor chemosensitization activity against colon cancer. The anticancer activity of cucurbitacin I was accomplished by downregulating p-STAT3 and MMP-9 expression. Collectively, our results suggest that cucurbitacin I may be a potent adjuvant chemotherapeutic agent for colon cancer with anti-migration, anti-invasion and chemosensitizing activities.

Introduction

Colorectal cancers are the third most common types of cancers worldwide (1-3). Surgical resection is unable to eliminate tumors completely since metastasis occurs at the time of diagnosis in approximately three-fifths of the patients. The 5-year survival rate is also very low due to metastasis (4-6). At present, there is no effective adjuvant chemotherapy, and rationally designed new adjuvant therapeutic tools need to be developed to manage the metastatic process in colorectal cancer patients. In the past several decades, a large number of substances derived from plants has been studied in antitumor research fields and many have proven to exhibit chemopreventive properties (7-10), which could be used as adjuvant chemotherapy.

Cucurbitacin I, also known as elatericin B, is a member of a family of natural occurring compounds with potent antitumor activity in many human cancers, including glioblastoma, adenocarcinoma of the lung and breast cancer cells (11-13). The cucurbitacin family comprises a group of triterpenoid compounds originally isolated from the plants of the *Cucurbitaceae* family. Recently, these anticancer compounds have been observed in many plant families, including *Cruciferae*, *Cucurbitaceae* and *Scrophulariaceae*, and have been used as traditional or folk medicines for centuries in China, India, Brazil and Peru (13,14). Previous studies have demonstrated that cucurbitacin I inhibits the JAK/STAT3 pathway in a number of cancer cell lines and *in vivo* tumor models (11,15). Upon inhibition of STAT3-dependent gene transcription, cucurbitacin I elicits antiproliferative effects in glioma, lung and breast cancer cells with activated STAT3 (13,16).

Limited research has been carried out concerning the effect of cucurbitacin I on colon cancer. A recently published study demonstrated that cucurbitacin I induced cell death and G2/M phase cell cycle arrest in SW480 cells (17). Our results in the present study confirmed this effect of cucurbitacin I on colon cancer cells. However, how cucurbitacin I influences colon cancer cell migration and invasion is still elusive. In the present study, we investigated the effect of cucurbitacin I on colon cancer cell migration and invasion, and whether cucurbitacin I enhances the chemosensitivity of the colon cancer cells. Furthermore, we also investigated the molecular mechanisms of cucurbitacin I function.

Materials and methods

Materials and cell culture. Cucurbitacin I was purchased from Calbiochem (Jersey City, NJ, USA) and dissolved in dimethyl sulfoxide (DMSO) at the concentration of 10 mM. The antibodies used in the study included: anti-phospho-STAT3-Tyr705 (Cell Signaling Technology, Danvers, MA, USA), anti-MMP-9 (Abcam, Cambridge, MA, USA) and anti-actin (Santa Cruz Biotechnology, Dallas, TX, USA). Secondary antibodies were purchased from Jackson ImmunoResearch (Baltimore, MD, USA).

Correspondence to: Dr Junmin Song, Department of General Sugery, First Affiliated Hospital of Zhengzhou University, 1 East Jianshe Road, Zhengzhou 450052, P.R. China
E-mail: songjunmin99@163.com

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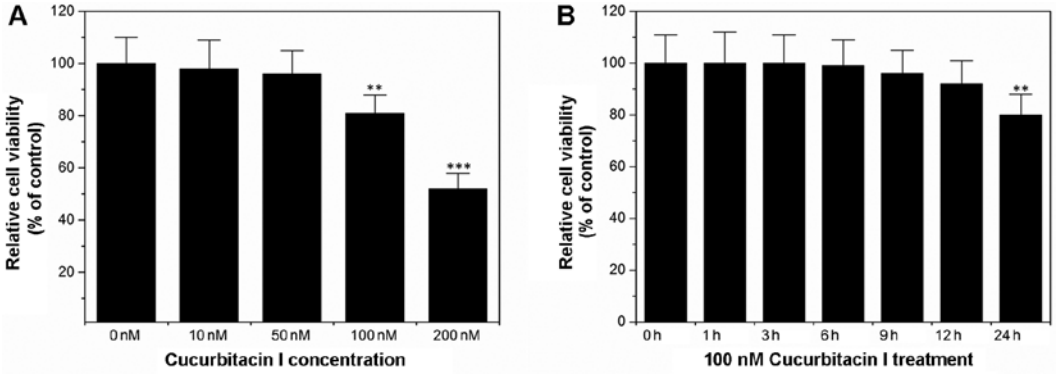

Figure 1. Cucurbitacin I induces colon cancer cell death. The COLO205 cells were treated with different doses of cucurbitacin I for (A) 24 h or with (B) 100 nM cucurbitacin I for different times. The cell viability was determined by the CCK-8 Kit. The cell viability was presented as a percentage of the control. **P<0.05 and ***P<0.01.

The COLO205 colon cancer cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium. All media were supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Madison, WI, USA), and 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA).

Cell viability assays. Cell viability was measured by the CCK-8 Kit (Dojindo, Kumamoto, Japan). Briefly, cells were plated at the density of $1x10^4$ cells/well in a 94-well plate. The following day, the cells were incubated with either DMSO or increasing concentrations of cucurbitacin I for 24 h. The 1/10 volume of CCK-8 solution of cultured medium was added to the cells. Then, the cells were further incubated at 37˚C for 2 h. The optical density (OD) at 450 nm was measured by using a VICTOR™ X plate reader (PerkinElmer, Waltham, MA, USA). The percentage of cell viability was calculated as $OD_{drug}/OD_{control}$ x 100%.

Cell migration and invasion assays. The *in vitro* migration assays were performed as previously described with some modifications (18,19) using Transwells (8-μm pore size; BD Corporation, Franklin Lakes, NJ, USA). The COLO205 cells were added to the upper inserts of the chamber (200 μl serum-free medium containing $2x10^4$ cells), and 600 μl medium with 1% FBS was added to the lower well. After 6 h of incubation, the cells were removed from the upper surface of the filter with a cotton swab and the cells that migrated through the inserts were fixed with methanol and stained with crystal violet. The migrated cells were counted under a microscope (TS100; Nikon, Tokyo, Japan) and the migration ability of the control group was set as 100%. The migration ability of the treated group was calculated as the migrated cell number of the drug-treated group/the migrated cell number of the control group x 100%. Each experiment was performed in triplicate and this experiment was repeated three times.

Cell invasion assays were performed as the migration assays except that the Transwells used in the invasion assays were Matrigel-coated, while in the migration assays the Transwells remained uncoated.

Western blotting. The cell lysates were prepared as previously described (20). After DMSO or cucurbitacin I treatment for 24 h, the cells were washed with ice-cold phosphate-buffered saline (PBS) three times and lysed in RIPA lysis buffer supplemented with a proteinase inhibitor (both from Beyotime, Nanjing, China) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Then, the cell lysates were harvested. Fifty grams of whole cell lysates was electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electro-transferred to poly-vinylidene fluoride (PVDF) membranes and probed with an appropriate primary antibody. Then the blots were next probed with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and were visualized by an enhanced chemiluminescence assay kit (ECL kit; Applygen Technology, Beijing, China). Membranes were also probed with an anti-actin antibody to monitor the sampling difference.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation (SD) and were analyzed by the Student's t-test. A difference was considered to be statistically significant at P<0.05.

Results

Cucurbitacin I suppresses colon cancer cell proliferation, migration and invasion in vitro. As shown in Fig. 1A, cucurbitacin I inhibited colon cancer cell COLO205 proliferation in a dose-dependent manner. These results confirmed a previous report by other researchers (17). However, to date it is not clear how cucurbitacin I affects colon cancer cell migration and invasion. In order to answer this question, Transwell assays were performed. Since Transwell assays take ~6 h, we firstly determined the cell viability after treatment with 100 nM cucurbitacin I for 6 h. As indicated in Fig. 1B, treatment with 100 nM cucurbitacin I for 6 h had no statistically significant influence on the cell viability as compared with the control. Thus, the parameters of 100 nM cucurbitacin I and the 6 h treatment were chosen for the Transwell assays. As illustrated in Fig. 2, 100 nM cucurbitacin I treatment for 6 h reduced COLO205 colon cancer cell migration to ~50% as compared with the control (P<0.001). Cell invasion assays showed that 100 nM cucurbitacin I also inhibited COLO205 colon cancer cell invasion *in vitro* (Fig. 3).

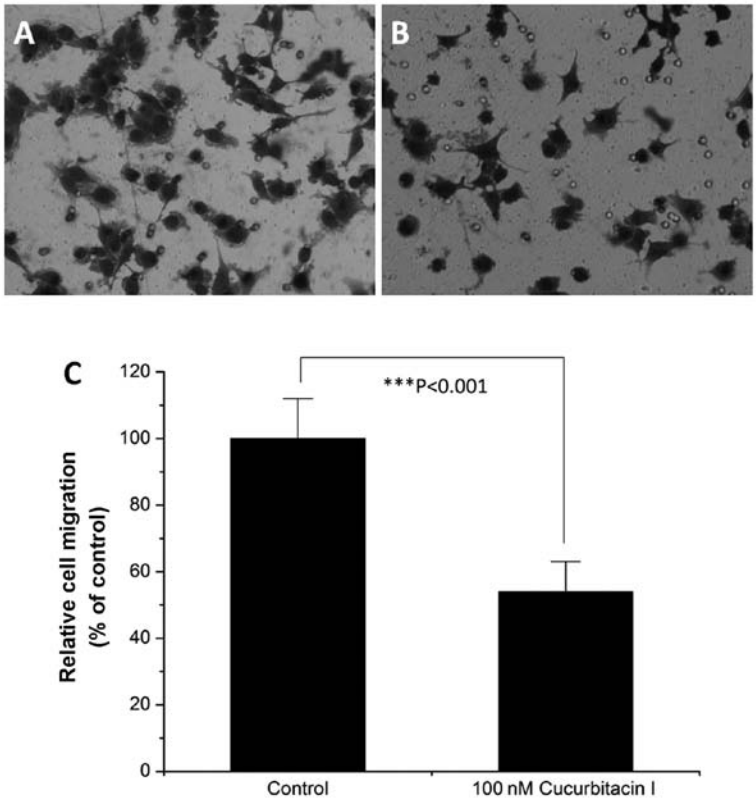

Figure 2. Cucurbitacin I inhibits colon cancer cell migration. (A) Control; (B) 100 nM cucurbitacin I treatment. The COLO205 cells were seeded onto the upper Transwell inserts of the chamber without Matrigel at the same cell density of $2x10^4$ cells. Six hours later, the cells that invaded through the inserts were stained and counted under a light microscope. (C) Finally, data were calculated and cell migration was presented as a percentage of the control. Similar results were obtained from three independent experiments.

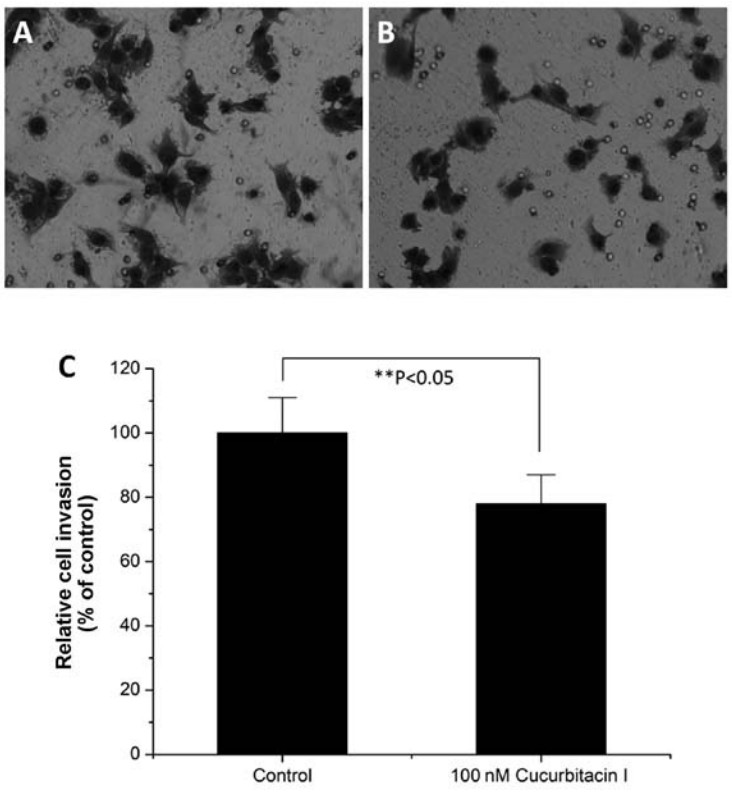

Figure 3. Cucurbitacin I suppresses colon cancer cell invasion. (A) Control; (B) 100 nM cucurbitacin I treatment. The COLO205 cells were seeded onto the upper Transwell inserts of the Matrigel chamber at the same cell density of $2x10^4$ cells. Six hours later, the cells that invaded through the Matrigel-coated inserts were stained and counted under a light microscope. (C) Finally, data were calculated and cell invasion was presented as a percentage of the control. Similar results were obtained from three independent experiments.

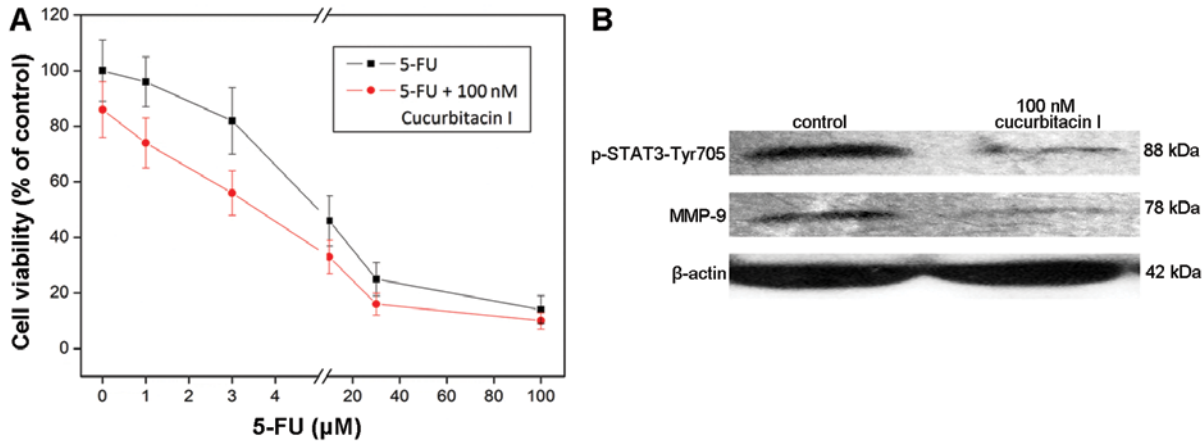

Figure 4. (A) Cucurbitacin I sensitizes colon cancer cell line COLO205 to 5-FU treatment. COLO205 colon cancer cells were seeded onto a 96-well plate at the same density ($1x10^4$ cells) and then treated with 5-FU alone or the combination of 5-FU and 100 nM cucurbitacin. Twenty-four hours later, the cell viability was determined using the CCK-8 Kit. The cell viability was presented as a percentage of the control. This experiment was repeated three times with similar results. (B) Cucurbitacin I inhibited the expression of activated STAT3 in COLO205 cells. COLO205 cells were treated with 100 nM cucurbitacin I for 24 h and then harvested for western blot analysis. The expression levels of phospho-STAT3-Tyr705 were compared between the control and cucurbitacin I-treated COLO205 cells. Actin is shown as a loading control. Similar results were obtained in three independent experiments.

Cucurbitacin I sensitizes colon cancer cell to chemotherapeutic agents. As confirmed above by our results and a number of previous reports (11,13,17,21,22), cucurbitacin I inhibits tumor cell proliferation, migration and invasion, which makes cucurbitacin I a promising antitumor target (23). However, to date it is not known whether cucurbitacin I has a sensitizing effect on colon cancer cells to chemotherapy. In the present study, we combined the chemotherapy with the cucurbitacin I treatment. As shown in Fig. 4A, the cell death was further enhanced by the combination treatment of 5-fluorouracil (5-FU) and 100 nM cucurbitacin I, which proved that cucurbitacin I sensitized the colon cancer cell line COLO205 to 5-FU treatment.

Cucurbitacin I suppresses STAT3 activation and decreases MMP-9 expression. Previous reports have shown that cucurbitacin I suppresses STAT3 activation in other cancer cell lines, such as breast cancer (24) and leukemia cells (25). In the present study, we determined the effect of cucurbitacin I on STAT3 in colon cancer cells. As indicated in Fig. 4B, 100 nM cucurbitacin decreased the protein level of phospho-STAT3, which proved the inhibitory effect of cucurbitacin I on STAT3 activation.

MMP-9 is an important enzyme for tumor cell invasion (26). As cucurbitacin inhibited colon cancer cell invasion, we determined whether cucurbitacin I treatment could change the MMP-9 expression level. Western blotting results showed that the MMP-9 expression level was decreased by 100 nM cucurbitacin treatment (Fig. 4B).

Discussion

At present, natural chemical compounds are important targets in anticancer research due to the drug resistance and toxic side-effects of current chemotherapy. Herbal medicine has attracted increased attention of medical scientists (27). Cucurbitacin I is a natural component extracted from plants of the *Cucurbitaceae* family, and exerts anticancer activities in glioblastoma, adenocarcinoma of the lung and breast cancer cells (11-13). The molecular mechanisms of cucurbitacin I function involve the inhibition of STAT3 activation (13,24), and interference with actin dynamics (28,29). A recently published study (17) demonstrated that in SW480 cells cucurbitacin I decreased the cell viability and cell proliferation by cleavage of caspase-3, -7, -8 and -9 and polyADP ribose polymerase and induced G2/M cell cycle arrest by downregulation of the cell cycle proteins including cyclin B1 and A, CDK1 and CDC25C. In the present study, we confirmed the inhibitory effect of cucurbitacin I on colon cancer cell growth as previously reported (17). Our results further showed that cucurbitacin I inhibited colon cancer cell migration and invasion *in vitro*, and sensitized colon cancer cells to 5-FU treatment.

Transcription factor STAT3 has been implicated in the promotion of growth and progression of many human cancers including gastric cancers (30-37). STAT3 is both a cytoplasmic signaling molecule and a nuclear transcription factor, which belongs to the seven-member Stat gene family of transcription factors (38). STAT3 becomes active by phosphorylation of a specific tyrosine residue in the carboxy-terminal domain by a tyrosine kinase (pTyr705). After phosphorylation, STAT3 homodimerizes and translocates to the nucleus where it binds to specific STAT3 response elements of target gene promoters to regulate transcription (39). Transcription factor STAT3 is constitutively active in many human cancers (30,40). In the present study, we firstly observed that in colon cancer cells cucurbitacin I suppressed phosphorylation of STAT3 (pTyr705). As STAT3 activation was involved in tumor metastasis (31), inhibition of cucurbitacin I on STAT3 activation could explain its inhibitory effect on colon cancer cell migration and invasion. Previous findings showed that, in medulloblastoma-derived cancer stem cells, cucurbitacin I enhanced chemoradiosensitivity by inhibiting STAT3 phosphorylation (40). In our results we observed that cucurbitacin I sensitized colon cancer cells to chemotherapy, which may

be promoted by inhibiting STAT3 activation. Lastly, we also observed that cucurbitacin I decreased the MMP-9 expression which is an important enzyme for cell invasion (26).

In conclusion, the present study showed that cucurbitacin I exhibited inhibitory effects on colon cancer cell proliferation, migration and invasion, which may be accomplished by down-regulating phosphorylation of STAT3 and MMP-9 expression. Our results also indicated that cucurbitacin I could sensitize colon cancer cells to chemotherapy.

Acknowledgements

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