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Cucurbitacin E's Anti-Cancer Effects on HCT116 Human Colon Cancer Cells by Controlling Expression and Phosphorylation Levels of Caspase-9, eIF-2 α , and ATF-4

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Cucurbitacin E is a pivotal member of the cucurbitacin family and has been shown to have anti-cancer effects. However, until now, the anti-cancer effect and mode of action of cucurbitacin E in human colon cancer cells remain unclear. In this study, we investigated whether cucurbitacin E inhibits the growth of HCT116 human colorectal cancer cells. Treatment of cucurbitacin E at 1 μ M markedly reduced the survival of HCT116 cells. Moreover, treatment of cucurbitacin E at 1 μ M caused nuclear DNA fragmentation in HCT116 cells, pointing out its apoptosis-inducing effect. Treatment of cucurbitacin E at 1 μ M also led to the activation of caspase-9 and poly(ADP-ribose) polymerase (PARP) cleavage without affecting expression of death receptor (DR)-4/5 in HCT116 cells. Furthermore, while treatment of cucurbitacin E at 1 μ M had no effect on expression of Mcl-1, it largely increased expression and phosphorylation of eukaryotic translation initiation factor-2 α (eIF-2 α) and activating transcription factor-4 (ATF-4) in HCT116 cells. Treatment of cucurbitacin E at 1 μ M further up-regulated phosphorylation of extracellular signal-regulated kinase-1/2 (ERK-1/2), but not c-Jun N-terminal kinase1/2 (JNK-1/2), in HCT116 cells. However, treatment with PD98059, an inhibitor of ERK-1/2, that strongly blocked activation of ERK-1/2 had no effect on reduction of survival of HCT116 cells treated with cucurbitacin E at 1 μ M. Taken together, these findings demonstrate that cucurbitacin E at 1 μ M has strong anti-survival and pro-apoptotic effects on HCT116 cells, which are mediated through control of the expression and phosphorylation levels of caspase-9, PARP, eIF-2 α , and ATF-4.

Keywords: Apoptosis, ATF-4, Caspase-9, Cucurbitacin E, eIF-2 α , HCT116

Introduction

Colon cancer is one of the most common epithelial cancers [1]. Epidemiologically, it is the third distributed cancer worldwide with high mortality rate in both genders [2]. Due to the high incidence rates of age-dependent colorectal cancer, several approaches have been determined to achieve positive anti-cancer effects, including surgery, chemotherapy, and radiotherapy. However, these therapies did not produce the desired treatment effect when applied alone [3]. Therefore, there is an urgent need to identify or develop more effective and non-toxic therapeutics for colon cancer.

Many therapeutic and chemopreventive drugs eliminate cancerous cells through induction of apoptosis, also named programmed cell death. It is documented that cancer cells undergoing apoptosis have many characteristics, including plasma membrane bleb, cell shrinkage, depolarization of mitochondria,

chromatin condensation, and DNA fragmentation [4,5]. There is a wealth of information that induction of cancer cell death and inhibition of cancer cell growth are largely influenced by activation of the caspase pathway, expression of death receptor (DR)-4/5, and endoplasmic reticulum (ER) stress [6-8]. Involvement of the family of mitogen-activated protein kinase (MAPK) in induction of apoptosis also has been reported [9-10].

Today, many researchers around the world are conducting a lot of research to identify natural substances that can inhibit cancer cell growth or induce cancer cell death with little or no side effects [11,12]. Cucurbitacin E is a member of the cucurbitacin family that is a group of tetracyclic triterpenoids extracted from cucurbitaceous plants [13]. Cucurbitacin E is found to have anti-cancerous, anti-viral, and anti-inflammatory effects [14-15]. Up to date, little is known about the relationship between cucurbitacin E and human colon cancer cells. In this study, we investigated the effect of cucurbitacin E on growth of HCT116 human colon cancer cells. Here we report, for the first time, that cucurbitacin E at 1 μ M has strong anti-survival and pro-apoptotic effects on HCT116 cells and the effects are mediated through regulation of the expression and phosphorylation levels of caspase-9, PARP, eIF-2 α , and ATF-4.

Materials and Methods

Materials

Cucurbitacin E was obtained from Selleckchem (Houston, TX). Primary antibody of procaspase-9 was bought from Enzo (Farmingdale, NY, USA). Anti-ATF-4 and Mcl-1 antibodies were purchased from Santa Cruz Biotechnology (Delaware, CA). Primary antibody for phospho (p)-eIF-2 α was bought from Abcam (Cambridge, MA, USA). Anti-PARP, Anti-p-ERK-1/2, anti-ERK-1/2, anti-p-JNK-1/2, and anti-JNK-1/2 antibodies were bought from Cell Signaling Technology (Danvers, MA, USA). Primary antibody of β -actin was purchased from Sigma (St. Louis, MO).

Cell culture

HCT116 human colon cancers (ATCC, Manassas, VA) were cultured in RPMI-1640 media supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Cell count assay and cell morphology analysis

HCT116 cells were seeded in a 24-well plate. After overnight incubation, cells were treated with vehicle control (DMSO; 0.1%) or cucurbitacin E at the indicated concentrations (0.01, 0.1, and 1 mM) for 24 h. The number of surviving cells was counted with the trypan blue exclusion method, which based on the principle that live cells have intact cell membranes and cannot be stained. Approximately 100 cells were counted in each evaluation. For cell morphology analysis, phase contrast images of the conditioned cells treated with cucurbitacin E were taken with a compound microscope (Nikon Eclipse TS200, Nikon Corp., Tokyo, Japan).

Measurement of DNA fragmentation

Measurement of DNA fragmentation was conducted, as mentioned in our previous study [16]. Briefly, HCT116 cells were seeded the day before treatment. Cells were treated with vehicle control (DMSO) or cucurbitacin E (0.01, 0.1, and 1 μ M) for 24 h. The conditioned cells were harvested, washed and lysed in a buffer [50 mM Tris (pH 8.0), 0.5% sarkosyl, 0.5 mg/mL proteinase K and 1 mM EDTA] at 55°C for 3 h, followed by the addition of RNase A (0.5 μ g/mL) and incubation at 55°C for 18 h. The lysate was centrifuged at 10,000 $\times$ g for 20 min. Genomic DNA was extracted with an equal volume of neutral phenol-chloroform-isoamyl alcohol mixture (25:24:1) and analyzed by electrophoresis on a 1.8% agarose gel. The DNA was visualized and photographed under UV illumination after staining with ethidium bromide (0.1 μ g/mL) by the Gel documentation system (Gel Doc-XR, Bio-rad, Hercules, CA, USA).

Preparation of whole cell lysates

HCT116 cells were seeded in 6-well plates the day before treatment. Cells were treated with cucurbitacin E (1 μ M) and/or other reagent (PD98059) or vehicle control (DMSO) for the designated times. At the designated time point, cells were washed twice with PBS and proteins were extracted using a modified RIPA buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, 1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, proteinase inhibitor cocktail (1X)]. The cell lysates were collected and centrifuged at 12,074 $\times$ g for 20 min at 4°C. The supernatant was saved, and its protein concentration was determined by bicinchoninic acid assay (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

Immunoblot analysis

Equal amounts of protein (50 µg) were separated via 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore) by electroplating. The membranes were washed with Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl, pH 7.5) supplemented with 0.05% (v/v) Tween-20 (TBS-T), followed by blocking with TBS-T containing 5% (w/v) non-fat dried milk. The membranes were probed overnight using antibodies against procaspase-9 (1:2,000), PARP (1:2,000), Mcl-1 (1:2,000), p-eIF-2α (1:2,000), T-eIF-2α (1:2,000), ATF-4 (1:2,000), p-ERK-1/2 (1:2,000), T-ERK-1/2 (1:2,000), p-JNK-1/2 (1:2,000), T-JNK-1/2 (1:2,000), or β-actin (1:10,000) at 4°C, followed by incubation with secondary antibodies conjugated to at room temperature for 2 h. The membranes were washed, and immune reactivities were detected by Super Signal™ West Pico PLUS ECL (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Equal protein loading was assessed via β-actin expression levels.

Reverse-transcription polymerase chain reaction (RT-PCR) analysis.

Total cellular RNA from conditioned HCT116 cells was isolated using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RT-PCR was performed as previously described (21). Briefly, equal amounts of total RNA (5 µg) were reverse-transcribed in a 40-µl reaction mixture containing 8 µl Molony Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) 5X buffer, 3 µl 10 mM dNTPs, 0.45 µl 40 U/µl RNase inhibitor, 0.3 µl 200 U/µl M-MLV RT (Promega Corporation) and 3.75 µl 20 µM oligo dT (Bioneer Corporation). Single-stranded cDNA was amplified by PCR using 4 µl 5X Green Go-Taq® Flexi reaction buffer, 0.4 µM 10 mM dNTPs, 0.1 µl 5 U/µl Taq polymerase, 1.2 µl 25 mM MgCl₂ (Promega Corporation), and 0.4 µl primer (20 pM/µl). The following primer pairs were used: DR-4 sense, 5'-CTGAGCAACGCAGACTCGCTGTCCAC-3' and antisense, 5'-AAGGACACGGCAGAGCCTGTGC-CAT-3'; DR-5 sense, 5'-AGCCGCTCATGAGGAAGTTGG-3' and antisense, 5'-GGCAAGTCTCTCTCCAGCGTCTC-3'; ATF-4 sense, 5'-TAGGGCCTCCTACCTTTGT-3' and anti-sense, 5'-GTGTCAACCGTGGTCAG-3'; Mcl-1 sense, 5'-ATCTCTCGGTACCTTCGGGAG-3' and anti-sense, 5'-ACCAGCTCCTACTCCAGCAAC-3' and actin sense, 5'-TCAAGATCATTGCTCCTCTG-3' and antisense, 5'-CTGCTTGCTGATCCACATCTG-3'. The PCR conditions were as follows: For DR-4 and DR-5: 35 cycles of denaturation at

95°C for 30 sec, annealing at 63°C for 30 sec and extension at 72°C for 30 sec; for ATF-4: 35 cycles of denaturation at 95°C for 45 sec, annealing at 54°C for 45 sec and extension at 72°C for 45 sec; for Mcl-1: 30 cycles of denaturation at 95°C for 45 sec, annealing at 56°C for 45 sec and extension at 72°C for 45 sec and for β-actin: 25 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec. β-actin was used as an internal control to evaluate the relative expressions of DR-4 and DR-5.

Statistical analysis

Cell count analysis was done in triplicates and repeated three times. Data were expressed as mean ± SE. The significance of difference was determined by One-Way ANOVA. All significance testing was based upon a *p* value of <0.05.

Results

Treatment with cucurbitacin E reduces survival of HCT116 human colon cancer cells in a concentration-dependent manner

Initially, we investigated the effect of cucurbitacin E (Fig. 1A) at different concentrations (0.01, 0.1, and 1 µM) for 24 h on growth (survival) of HCT116 cells by cell count analysis. As shown in Fig. 1B, treatment with cucurbitacin E reduced survival of HCT116 cells in a concentration-dependent manner. Apparently, the largest reduction of HCT116 cell survival was found in the 1 µM treatment of cucurbitacin E. Microscopic observation further revealed that treatment with cucurbitacin E dose-dependently interfered with growth of HCT-116 cells in which the largest reduction of HCT116 cell growth was also seen at the 1 µM of cucurbitacin E (Fig. 1C).

Treatment with cucurbitacin E at 1 µM induces nuclear DNA fragmentation, activation of caspase-9, and PARP cleavage in HCT116 human colon cancer cells

We next sought to explore the effect of cucurbitacin E at different concentrations (0.01, 0.1, and 1 µM) for 24 h on apoptosis in HCT116 cells by measuring nuclear DNA fragmentation, a hallmark of apoptosis. Notably, as shown in Fig. 2A, while treatment with cucurbitacin E at 0.01 or 0.1 µM for 24 h did not induce nuclear DNA fragmentation, that with cucurbitacin E at 1 µM for 24 h resulted in high accumulation of nuclear DNA fragmentation in HCT116 cells. Next, to understand molecular mechanisms underlying cucurbitacin E's apoptosis inducing effects herein, we promptly examined the effect of cucurbitacin E at different concentrations

Fig. 1. Effects of cucurbitacin E on survival and morphology of HCT116 human colon cancer cells. (A) The chemical structure of cucurbitacin E. (B) HCT116 cells were treated with cucurbitacin E or vehicle control (DMSO; 0.1%) at the designated concentrations for 24 h. The numbers of surviving HCT116 cells were measured by cell count assay. Cell count assay was performed in triplicate. Data are means $\pm$ SE of three independent experiments. * $p < 0.05$ compared to the value of cucurbitacin E free control at the designated time. (C) After the above-mentioned treatment conditions, images of the conditioned cells were obtained by phase contrast microscopy; the magnification rate, 200 $\times$. Each image is representative of three independent experiments.

(0.01, 0.1, and 1 μ M) for 24 h on expression levels of apoptosis-related factors, such as caspase-9, PARP, and DR-4/5, in HCT116 cells. As shown in Fig. 2B, data of Western blotting analysis demonstrated that treatment with cucurbitacin E led to a concentration-dependent activation of caspase-9, as assessed by its ability to decrease expression levels of pro-caspase-9, an indicator of caspase-9 activation, in HCT116 cells. Moreover, while treatment with cucurbitacin E at 0.01 or 0.1 μ M had no effect on expression levels of PARP, a downstream effector of caspase-9, that with cucurbitacin E at 1 μ M led to generation of partially cleaved PARP in HCT116 cells. By contrast, as shown in Fig. 2C, results of RT-PCR analysis displayed that treatment with cucurbitacin E at the doses tested had no effect on expression levels of DR-4/5 in HCT116 cells. Protein and mRNA expression levels of con-

trol actin remained unchanged under these experimental conditions. Because of the largest growth inhibitory and apoptosis inducing effects on HCT116, we chose the 1 μ M concentration of cucurbitacin E for further studies.

Treatment with cucurbitacin E at 1 μ M up-regulates expression and phosphorylation levels of eIF-2 α , and ATF-4 in HCT116 human colon cancer cells

We next investigated the treatment effect of cucurbitacin E at 1 μ M on expression and phosphorylation levels of other growth- and/or apoptosis-related proteins, including mitochondrial protein (Mcl-1) and ER stress/translation-related proteins (eIF-2 α , ATF-4), in HCT116 cells over time. Distinctly, as shown in Fig. 3A, treatment with cucurbitacin E at 1 μ M induced high phosphorylation levels of eIF-2 α , and

Fig. 2. Effects of cucurbitacin E on nuclear DNA fragmentation, expression levels of procaspase-9, PARP, and DR-4/5 in HCT116 human colon cancer cells. (A) HCT116 cells were treated with cucurbitacin E or vehicle control at the designated concentrations for 24 h. Extra-nuclear fragmented DNA was extracted and analyzed on a 1.8% agarose gel. (B) After above mentioned treatment conditions, whole cell lysates were prepared and analyzed by Western blotting using the respective antibody of procaspase-9, PARP, and Actin. (C) After the above-mentioned treatment conditions, total cellular RNA was prepared and analyzed by RT-PCR analysis using the respective primer of DR-4, DR-5, and Actin.

ATF-4 were observed in HCT116 cells treated with cucurbitacin E at 1 μM for the times tested. Apparently, a marked increase in phosphorylation levels of eIF-2 α and ATF-4 were observed in these cells even at 2 h treatment with cucurbitacin E at 1 μM . However, Mcl-1 protein expression levels were not changed in HCT116 cells treated with cucurbitacin E at 1 μM for the times tested. As shown in Fig. 3B, data of RT-PCR analysis further revealed that treatment with cucurbitacin E at 1 μM for 24 h did not change the mRNA expression levels of ATF-4 and Mcl-1 in HCT116 cells treated with cucurbitacin E at 1 μM for the times applied. Control actin mRNA expression remained constant under these experimental conditions.

Fig. 3. Effects of cucurbitacin E on expression and phosphorylation levels of eIF-2 α , ATF-4 and Mcl-1 in HCT116 human colon cancer cells. (A) HCT116 cells were treated with cucurbitacin E (1 μM) or vehicle control for the indicated times. At each time point, whole cell lysates were prepared and analyzed by Western blotting using the respective antibody of GRP78, eIF-2 α , ATF-4, and Mcl-1. (B) After the above-mentioned treatment conditions, total cellular RNA was analyzed by RT-PCR analysis using the respective primer of ATF-4, Mcl-1, and Actin.

Treatment with cucurbitacin E at 1 μM largely increased phosphorylation levels of ERK-1/2 in HCT116 human colon cancer cells, which appear to be unrelated with cucurbitacin E's anti-survival effect

We next investigated the effect of cucurbitacin E at 1 μM on expression and phosphorylation of levels of ERK-1/2 and JNK-1/2 in HCT116 cells over time. As shown in Fig. 4A, treatment with cucurbitacin E at 1 μM resulted in a time-dependent increase in phosphorylation levels of ERK-1/2 in HCT116 cells. However, there was no change in phosphorylation levels of JNK-1/2 in HCT116 cells treated with cucurbitacin E at 1 μM for the times tested. Total expression levels of ERK-1/2 and JNK-1/2 remained constant under these experimental conditions. Using PD98059, an inhibitor of ERK-1/2, we further determined the role of ERK-1/2 phosphorylation (activation) in cucurbitacin E-induced growth inhibition of HCT116 cells. Notably, as shown in Fig. 4B, there were substantial levels of phosphorylated ERK-1/2 in control HCT116 cells. As expected, single treatment of PD98059 at 50 μM strongly inhibited phosphorylation of ERK-1/2 in control or cucurbitacin E (1 μM)-treated HCT116 cells. However, as shown in Fig. 4C, results of cell count analysis demonstrated that treatment with PD98059 did not attenuate cucurbitacin E-induced reduction of HCT116 cell survival. Of note, single treatment of PD98059 significantly reduced survival of con-

Fig. 4. Effect of cucurbitacin E and/or PD98059 on expression and phosphorylation levels of ERK-1/2 and JNK-1/2 and survival of HCT116 human colon cancer cells. (A) HCT116 cells were treated with cucurbitacin E (1 μ M) or vehicle control for the indicated times. At each time point, whole cell lysates were prepared and analyzed by Western blotting using the respective antibody of ERK-1/2 and JNK-1/2. (B) HCT116 cells were treated with cucurbitacin E (1 μ M) or PD98059 (50 μ M), an inhibitor of ERK-1/2, for 24 h. Whole cell lysates were prepared and analyzed by Western blotting using the respective antibody of ERK-1/2. (C) HCT116 cells were treated with cucurbitacin E (1 μ M) or PD98059 (50 μ M) for 24 h. The numbers of surviving HCT116 cells were measured by cell count assay in triplicate. Data are means $\pm$ SE of three independent experiments. * p < 0.05 compared to the value of cucurbitacin E or PD98059 free control at the designated time.

trol HCT116 cells.

Discussion

Studies have previously shown that cucurbitacin E has anti-cancerous, anti-viral, and anti-inflammatory effects [13-15]. However, up to date, the anti-cancer effect and mechanism of action of cucurbitacin E in human colon cancer cells are not fully defined. In this study, we demonstrate that cucurbitacin E has strong and potent anti-survival and pro-apoptotic effects on HCT116 human colon cancer cells and the effects are associated with the ability of cucurbitacin E to control the expression and phosphorylation levels of caspase-9, PARP, eIF-2 α , and ATF-4.

It has been shown that cucurbitacin E has anti-cancer ef-

fects on multiple cancer cell types, such as T24 (bladder), LN-CaP (prostate), CaSki (cervix), and 95D (lung) cells [15,17-19]. Possible mechanisms underlying the anti-cancer effects of cucurbitacin E on these cancer cells include induction of apoptosis, cell cycle arrest, overexpression of p53 and p21, dephosphorylation of signal transducer and activator of transcription 3, down-regulation of cyclin-dependent kinase 1 and cyclin B, activation of caspases, up-regulation of DR-5, and oxidative stress. Through initial experiments, we have shown that treatment with cucurbitacin E at 1 μ M markedly reduces survival of HCT116 cells, pointing out its potent and strong anti-survival effect. Furthermore, in this study, we have demonstrated the ability of cucurbitacin E at 1 μ M to induce strong apoptosis of HCT116 cells, as judged by cellular increases in nuclear DNA fragmentation, activation of

caspase-9, and cleavage of PARP, hallmarks of cancer cell apoptosis. It is known that apoptosis induction is mainly mediated through the intrinsic (mitochondrial) and extrinsic (DR)-mediated pathways [20] in which either the mitochondria-mediated activation of caspase-9 or the DR-dependent activation of caspase-8 mediates events, respectively. Considering the present findings that treatment with cucurbitacin E at 1 μ M causes activation of caspase-9 but does not alter expression levels of DR-4/5 in HCT116 cells, cucurbitacin E-induced apoptosis in HCT116 cells herein is likely to be due to activation of the intrinsic (mitochondrial) pathway.

An interesting finding of the present study is cucurbitacin E regulation of ER stress in HCT116 cells. In cells, ER is the primary site for protein synthesis, protein folding, and trafficking. However, ER stress often occurs when cells have dysfunction of protein synthesis and/or excessive accumulation of misfolded and nonfunctional proteins in the ER. Excessive ER stress is closely linked to inhibition of cell growth and/or induction of cell apoptosis. Truly, it is demonstrated that induction of ER stress is common to many anti-cancer drugs and/or agents [21,22]. Increasing evidence illustrates that cells undergoing ER stress are characterized by increases in expression of molecular chaperones (e.g., GRP78) [23] and phosphorylation of eIF-2 α with global translation inhibition [24]. It also has been reported that ATF-4, a proapoptotic factor, mediates ER stress-induced cell death in tumor cells treated with the chemotherapeutic agents [25,26]. In the current study, we have shown that treatment with cucurbitacin E at 1 μ M increases not only expression levels of ATF-4 but also phosphorylation levels eIF-2 α in HCT116 cells, indicating cucurbitacin E-induced ER stress and translation inhibition in these cells. It thus seems that cucurbitacin E's anti-survival and pro-apoptotic effects on HCT116 cells are, in part, attributable to the ATF-4 and eIF-2 α -dependent induction of ER stress and translation interference.

There is a wealth of information that the family of MAPKs, composing of ERK-1/2, JNK-1/2, and p38 MAPK plays central roles in cell proliferation, differentiation, survival, and apoptosis [27,28]. Reportedly, while ERK-1/2 acts as a cell survival factor, JNK-1/2 and p38 MAPK mediate cell growth inhibition and/or cell death under cellular stressful conditions. In this study, we have demonstrated that cucurbitacin E treatment with 1 μ M strongly leads to strong increase in phosphorylation of ERK-1/2, but not JNK-1/2, in HCT116 cells, indicating the selectivity. However, data of pharmacological inhibition study herein have revealed that inhibition of ERK-1/2 activation by PD98059 does not attenuate the ability

of cucurbitacin E to reduce survival of HCT116 cells. These results suggest that cucurbitacin E's anti-survival and pro-apoptotic effects on HCT116 cells are independent of its ability to activate ERK-1/2 in these cells. Given that ERK-1/2 and PKB are known to counteract each other [29] and cucurbitacin E inhibits the proliferation of NCI-N87 human gastric cancer cells by suppressing PKB activation [30], it is speculative that cucurbitacin E may inhibit PKB, which results in activation of ERK-1/2 in HCT116 cells.

In summary, we demonstrate firstly that cucurbitacin E at 1 μ M has strong anti-survival and pro-apoptotic effects on HCT116 cells and the effects are mediated through activation of the caspase-9-mediated intrinsic pathway and induction of the eIF-2 α - and ATF-4-dependent ER stress and translation repression. Although there are still important issues that remain to be resolved, including cucurbitacin E's anti-colon tumor effects on animal models, our present findings suggest cucurbitacin E as a potential lead for the development of anti-colon cancer drug.

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Conflict of interest

All authors declare no conflicts-of-interest related to this article.

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