Cucurbitacin I elicits anoikis sensitization, inhibits cellular invasion and in vivo tumor formation ability of nasopharyngeal carcinoma cells

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1Cancer Signaling Laboratory, 2Cancer Drug Testing Unit, Department of Clinical Oncology and 3Department of Surgery, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong Special Administrative Region, 4Department of Anatomy, University of Hong Kong, Hong Kong Special Administrative Region and 5State Key Laboratory of Oncology in Southeast Asia. Although NPC is rare in Western countries, NPC shares similarity with other head and neck cancers as being highly invasive and metastatic. The majority of NPC patients (60–70%) are presented with advanced disease (Stages III and IV) at a time of diagnosis. Currently, the standard concurrent chemoradiation therapy is highly effective against early stage NPC, however, this invasive therapy still fails ~30–40% of patients with disease recurrence or distant metastasis (1,2). Besides its strong geographic link to Southeast Asia, several epidemiological studies demonstrated that this invasive cancer of the nasopharynx is associated with several important risk factors, including Epstein-Barr virus infection (EBV is an oncogenic virus), family history, human leukocyte antigen class I genotype, male gender, age, consumption of preserved food, tobacco smoke, as well as exposure to environmental carcinogens (3). Among which, EBV infection is not only the strongest risk factor but also known to be etiologically contributing to NPC tumorigenesis (4). In fact, increased antibody titers against EBV capsid antigen and anti-EBV deoxyribonucleic acid antibody were found to predict NPC as their serum levels precede NPC development by several years (5). The common adult onset of NPC (peak occurrence at 45–54 years of age) (6) suggests cumulative genetic (possibly related to early and latent EBV infection) and environmental insults for disease disposition (6). In familial NPC, the first-degree relatives have significantly higher risk of developing NPC at earlier age (7), suggesting genetic factors and common exposure to environmental carcinogens. In view of its invasiveness and adulthood onset, as well as the identifiable high-risk groups, several cancer prevention efforts have been recently pursued with the aim of preventing this invasive cancer from occurring (8,9). Unlike other head and neck cancers, chemoprevention in NPC is still in its infancy, more studies are needed to figure out the best chemopreventive agent for NPC and to identify the best molecular target for prevention of NPC tumor formation.

Signal transducer and activator of transcription 3 (STAT3) is a pivotal oncogene known to regulate proliferation, apoptosis, metastasis, epithelial-mesenchymal transition and differentiation in cancer. Numerous studies demonstrated that STAT3 is a prominent target for anticancer therapy (10,11). Recent evidences arise indicating STAT3, in addition to be a target for anticancer therapy, may also be a crucial target for cancer prevention. Studies in skin cancer, breast cancer and lung cancer demonstrated an essential role of STAT3 in tumor formation, tumor initiation and promotion (12–18). In a chemical-induced skin tumorigenesis model, transgenic mice with epidermal-specific disruption of STAT3 showed a delay in tumor development and reduction of tumor formation (16). STAT3-deficient mice were completely resistant to skin tumor development when challenged with a tumor initiator (Dimethylbenz[a]anthracene) and tumor promoter (12-O-tetradecanoylphorbol 13 acetate) (13). On the contrary, transgenic mice with epidermal-specific expression of constitutive activated STAT3 (STAT3C) showed a dramatic shortened latency in tumor development and formation, which was accompanied by a more malignant tumor progression (15). Similarly in lung cancer, Li et al. (18) showed that expression of activated STAT3 mutant in alveolar epithelial cells directly caused spontaneous lung cancer in vivo. In breast cancer, inhibition of STAT3 expression (by STAT3 short hairpin RNA) significantly abrogated the ability of breast cancer cells to form breast tumors in the mammary fat pad of a syngeneic model (17). These findings agree with the early demonstration that sole activation of STAT3 (by expressing constitutive activating mutant of STAT3) was sufficient to induce transformation and tumor formation of fibroblasts in nude mice (19). Therefore, it is believed STAT3 may be a potential target for cancer prevention in human epithelial cancer (14). In NPC, STAT3 is activated or overexpressed in the majority of patients (>75% of cases) and its overexpression/activation is found to be clinically correlated with advanced disease (stages III and IV) (20,21). Our recent study demonstrated a direct contribution of STAT3

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**Abbreviations:** COX, Cyclooxygenase; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; EBV, Epstein-Barr virus; ECM, extracellular matrix; LMP1, latent membrane protein 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NPC, nasopharyngeal carcinoma; PARP, Poly (adenosine diphosphate-ribose) polymerase; STAT3, signal transducert and activator of transcription 3; TUNEL, terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling.

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activation to the intrinsic invasiveness of NPC cells (22). Although the role of STAT3 activation in NPC tumor formation/initiation is incompletely understood, its activation by EBV infection (23,24) or the EBV oncoprotein latent membrane protein 1 (LMP1) (24) seems to suggest its potential involvement in early tumorigenesis of NPC as EBV infection is believed to take part in NPC oncogenesis in early stages (25). In line with this are several studies hypothesizing the involvement of STAT3 in the induction of DNA lesion formation and possible maintenance of EBV latent infection in NPC, indicating STAT3 could be a potential target for cancer prevention in NPC (21,23).

Cucurbitacin I, also known as Elatercin B or JSI-124, belongs to a family of natural occurring compounds with potent anti-tumor activity in a number of human cancers, including glioblastoma, adenocarcinoma of the lung and breast cancer cells (26–28). The Cucurbitacin family comprises a group of triterpenoid compounds originally isolated as the ‘bitter-tasting’ components of the Cucurbitaceae plant family. Recently, this group of anticancer compounds has been found in many plant families, including Cruciferae, Cucurbitaceae and Scrophulariaceae, which have been used as traditional or folk medicines for centuries in China, India, Brazil and Peru (28,29). In addition to their prominent anticancer activities, Cucurbitacins are also known to have anti-inflammatory and anti-fungal activities (30–33). Cucurbitacin I (National Cancer Institute [NCI] Identifier: NSC 521777), isolated from Iberis amara seeds, was originally identified to be a potent selective inhibitor of the JAK/STAT3-signaling pathway by a large-scale screening study of the National Cancer Institute Structural Diversity Set (a library of 1992 compounds selected from the NCI drug depository) using a phosphotyrosine STAT3 high-throughput cytoblot assay (28). Cucurbitacin I (JSI-124) was then verified and demonstrated to have high specificity to JAK/STAT3 in vitro tumor models (26,34). Upon inhibition of STAT3-dependent gene transcription, this small molecule inhibitor elicits anti-proliferative effects in glioma, lung and breast cancer cells with activated STAT3 (28,35). Although sparse, there are some recent evidence of anti-invasion activity of Cucurbitacin I in keloid fibroblasts and a breast cancer model (36,37).

Whether this potent anticancer agent harbors additional anticancer activity against human cancers remains to be explored.

Given the importance of STAT3 in NPC invasion and possibly tumorigenesis, we set out to examine the chemopreventive potential of Cucurbitacin I in both in vitro and in vivo models of NPC. We hypothesized that this natural occurring JAK/STAT3 inhibitor, Cucurbitacin I, could prevent NPC invasion and tumor formation. Using two invasive NPC cell lines with elevated phospho-STAT3 expression, HK1-LMP1(B95.8) and CNE-2, we demonstrated for the first time anoikis-sensitization activity of Cucurbitacin I, in addition to its potent anti-invasion activity in NPC. We further demonstrated that brief exposure of NPC cells to this STAT3-inhibiting agent was sufficient to reduce significantly their in vitro clonogenicity, as well as in vivo tumorigenicity in nude mice. Further, pre-dosing of animals with Cucurbitacin I before tumor inoculation also suppressed growth of tumors in nude mice, demonstrating its chemopreventive activity in the in vivo models of NPC. Taken together, our results indicate that Cucurbitacin I may be an effective preventive agent for NPC with anti-invasion and anoikis-sensitization activities.

Materials and methods

Reagents

Cucurbitacin I (Cucumis sativus) was purchased from Calbiochem (Gibbstown, NJ). Antibodies used in the study include: anti-phospho-STAT3-Tyr705, anti-phospho-STAT3-Ser727, anti-STAT3, anti-cleaved Poly (adenosine diphosphate-ribose) polymerase (PARP) (Cell Signaling Technology, Danvers, MA), anti-Cyclin D1 (Thermo Scientific, Waltham, MA), anti-Mel-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-actin (Calbiochem). Secondary antibodies were purchased from Invitrogen (Carlsbad, CA).

Cell lines

The NPC cell lines, HK1 and CNE-2 were originally derived from NPC patients (38,39). Two HK1-LMP1 stable cell lines were established by retroviral transfection of the LMP1 genes (either the B95.8 or 2117 LMP1 variants) into the parental HK1, followed by puromycin selection. The prototype LMP1(B95.8) was cloned from the B95.8 cell line, whereas the 2117 LMP1 variant was isolated from an NPC xenograft (Xeno-2117). Both stable cell lines (HK1-LMP1(B95.8) and HK1-LMP1(2117)) were maintained in selection medium containing 100 μM puromycin. Morex, NJ. All NPC cultures were maintained at 37°C and 5% CO2 in cell culture incubator, in RPMI-1640 medium supplemented with 10% fetal bovine serum (Hyclone, Thermo Scientific, Waltham, MA), 100 U/ml penicillin and 100 μg/ml streptomycin and 1 mM sodium pyruvate (Invitrogen).

Western blotting for cell lysates

Cell lysates were prepared as described previously (40). For Cucurbitacin I(500nM) or dimethyl sulfoxide (DMSO) treatment, cell lysates were harvested at 48 h in the presence of serum. Fifty micrograms of total protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting.

Cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, cells were plated at a density of 1.3 × 104 cells per well in a 24-well plate. Next day, cells were then incubated with either DMSO or increasing concentrations of Cucurbitacin I (0, 25, 50, 100, 200, 500 and 100 nM) for 24 and 48 h. MTT solution (5 mg/ml; Invitrogen) was added onto the cells for the appearance of colored formazan product, which was then dissolved in DMSO, and subjected to colorimetric measurement at 570 nm with Victor plate reader (PerkinElmer, Waltham, MA). DMSO was used as blanking control. The percentage growth inhibition was calculated as (ODvehicle – ODdrug/ODvehicle) × 100%.

Matrigel invasion assay

NPC cell invasion was evaluated in vitro using Matrigel-coated modified Boyden inserts with a pore size of 8 μm (Becton Dickinson/Biocat, Bedford, MA). NPC cells were seeded onto the upper chamber at a density of 7 × 104 cells per chamber (for CNE-2) or 1 × 105 cells per chamber (for HK1-LMP1(B95.8)) and maintained in serum-free medium. The cell-containing chamber was immersed in a lower chamber containing complete medium, with DMSO (vehicle control) or Cucurbitacin I. Cells were incubated for 24 h at 37°C in a 5% CO2 incubator. Non-invaded cells retaining in the upper chamber were removed with a cotton swab. The invaded cells were stained with 1% Toluidine Blue O (Sigma, St Louis, MO) in 1% borax (USB, Cleveland, OH) and counted under the microscope (×200 magnification). Ten random fields were counted under a light microscope at ×200 magnification. Triplicate experiments were performed.

Assay for anoikis resistance

Anoikis is defined as detachment-induced apoptosis or cell death. Anchorage-dependent cells detaching from their surrounding extracellular matrix (ECM) will undergo anoikis. It is known that metastatic tumor cells may escape from anoikis (hence named anoikis resistance) and invade other organs. Anoikis resistance was assayed by the following: (i) Cell growth or survival in suspension culture (in detached status) in an ultra-low attachment cell culture plate covalently bounded with hydrogel that is hydrophilic and neutrally charged, which prevents cell attachment (Corning Life Sciences, Teterboro, NJ; Cat#3473). NPC cells were plated at a very low density (4 × 103 cells for HK1-LMP1(B95.8) and CNE-2; 1 × 104 cells for C666-1) in RPMI containing 10% fetal bovine serum, in the presence of DMSO or Cucurbitacin I. After 48 h, cells grew as suspension spheroids. Pictures were taken under light microscope at ×100 magnification. Anoikis was quantified by assessing the cell proliferation in suspension culture using a modified MTT assay for three dimensional culture. Briefly, 50 μl of MTT solution was added onto each well containing medium and incubated at 37°C for 4 h. The three dimensional aggregates were transferred to an eppendorf and pelleted down by centrifugation at 800g for 10 mins. All medium was removed and the cells/suspension spheroids were lysed overnight at 4°C with 400 μl of lysis buffer (99.4% DMSO, 0.6% acetic acid glacial, 10 g/l 100 ml sodium dodecyl sulfate). Colorimetric measurement was taken at 570 nm with Victor plate reader (Perkin-Elmer). Lysis buffer was used as blanking control. The percentage growth in suspension was calculated as ODdrug-treated/ODvehicle-treated × 100%; (ii) Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end label (TUNEL) assay for suspension culture: 2 days after plating (4 × 103 cells), cells in suspension spheroids were collected and spun onto Histobond slides by cytopinning at 400g for 5 mins. The slides were then air-dried at room temperature for 30 mins and stored at ~80°C until use. TUNEL staining was performed using In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer’s instruction. Briefly, cells
Cucurbitacin I suppresses NPC invasiveness, anoikis resistance and tumorigenicity

were fixed by ice-cold acetone:methanol (1:1) at −20°C for 10 mins, followed by permeabilization with 0.1% Triton X-100 and 0.1% sodium citrate on ice for 2 mins. Fluorescent TUNEL reaction was performed at 37°C for 1 h and the slides were mounted with 4′,6-diamidino-2-phenylindole (DAPI)-containing Fluoromount medium. TUNEL-positive cells and DAPI-labeled nuclei were imaged and recorded with fluorescein isothiocyanate and DAPI optics by epifluorescence microscopy at ×100 magnification. The number of TUNEL-positive cells and DAPI-positive nuclei were counted using the cell counting WCIF ImageJ bundles (version 1.37c, Wright Cell Image Facility, Toronto Western Research Institute, Toronto, Canada). Percent apoptotic cells were calculated as (TUNEL-positive/DAPI-positive cells) × 100%.

In vitro clonogenicity assay
HK1-LMP1(B95.8) and CNE-2 cells were briefly pretreated with DMSO or Cucurbitacin I (1 μM) for 2 h. The pretreated cells were thoroughly washed with serum-free medium for three times to remove all drugs in culture medium. HK1-LMP1(B95.8) (0.3 × 10^5) and CNE-2 (0.2 × 10^5) cells then were plated onto a 6-well tissue culture plate in complete medium and incubated at 37°C. Cells were allowed to grow from single cells to colonies in complete medium for 3–5 days. When large visible colonies were formed, cells were fixed and stained with 1% Toluidine Blue O (Sigma) in 1% borax (USB) and counted under the microscope (×50 magnification). Five random fields were counted under a light microscope at ×50 magnification. Triplicate experiments were performed.

In vivo studies
Six-week-old female athymic nude mice (nu/nu) were supplied by the Laboratory Animal Services Centre of the Chinese University of Hong Kong and were housed in individually ventilated cages under a 12 h light–dark cycle at a constant humidity and temperature. Food and water were provided ad libitum. All experiments were conducted under license from the Department of Health and according to approval given from the University Animal Experimentation Ethics Committee.

Pretreatment of NPC cells with Cucurbitacin I before tumor inoculation. NPC cells were briefly pretreated with DMSO or Cucurbitacin I (1 μM) for 2 h. The pretreated cells were thoroughly washed with HANKS buffer for three times to remove all drugs in culture medium, harvested and resuspended in HANKS for subcutaneous inoculation. For inoculation, 0.9 × 10^6 of viable HK1-LMP1(B95.8) or HK1-LMP1(C176) cells (assayed by trypan blue exclusion assay) were subcutaneously inoculated into the flanks of the nude mice using a Hamilton syringe. First appearance of tumor and tumor growth was carefully monitored every other day. A digital caliper was used for tumor measurement. Tumor volume was calculated as: volume = length × width^2/2.

Pre-dosing of animals with Cucurbitacin I before NPC tumor inoculation. Nude mice were given a total of three doses of Cucurbitacin I (1.3 mg/kg in DMSO) or DMSO control (once per day for 3 days, by intraperitoneal injection) before subcutaneous inoculation of tumor cells. Same as above, first appearance of tumor and tumor size was carefully monitored every day up to 7 days post-inoculation.

Statistical analysis
Statistical analyses were performed using PRISM 4 software (GraphPad, La Jolla, CA), the P-values were obtained by the Wilcoxon–Mann–Whitney test. Results were considered as statistically significant with a P < 0.05.

Results
Cucurbitacin I inhibited STAT3 activation and invasiveness of NPC cells
Given the importance of STAT3 in NPC invasion and possibly early tumorogenesis, we examined the chemopreventive potential of Cucurbitacin I in both in vitro and in vivo models of NPC. Cucurbitacin I is a potent inhibitor of JAK/STAT3, originally isolated from Iberis amara seeds. Using two invasive NPC cell lines with elevated phospho-STAT3 expression, HK1-LMP1(B95.8) and CNE-2, the effects of Cucurbitacin I on NPC cell invasion, proliferation, anoikis resistance and in vivo tumor-formation were investigated.

The EBV oncoprotein, LMP1, is known to induce STAT3 activation (24). Here, we found that this LMP1-induced STAT3 activation was correlated with increased cellular invasiveness in NPC cells when we compared the invasion activity of the parental HK1 versus HK1-LMP1(B95.8) and HK1-LMP1(B2117) stable cell lines (Figure 1A and B). Results from Matrigel invasion assay demonstrated that HK1-LMP1(B95.8) stable cell line was 94.56-fold more invasive than its parental counterpart, HK1 (Figure 1B). Similar results were observed with the HK1-LMP1(B2117) stable cell line. Treatment of HK1-LMP1(B95.8) cells with Cucurbitacin I (500 nM) completely abrogated STAT3 activation when compared with DMSO control (Figure 1C). Moreover, Cucurbitacin I inhibited invasion of HK1-LMP1(B95.8) cells through the Matrigel in a dose-dependent manner (Figure 1D). At 500 nM concentration, Cucurbitacin I reduced the invasiveness of HK1-LMP1(B95.8) cells by 62% (from 109.8 ± 3.721 invaded cells per field to 41.8 ± 3744 invaded cells per field, n = 10, P < 0.0001). Similar result was observed in another invasive NPC cell line with high level of STAT3 activation, namely CNE-2. CNE-2 cells expressed an elevated level of phospho-STAT3-Tyr705 when compared with a normal epithelial cell line, Het-1A (Figure 1E). Cucurbitacin I treatment also significantly reduced the cellular invasiveness of CNE-2 cells in a dose-dependent manner, with a 72.3% inhibition at 500 nM when compared with the DMSO control (from 97.9 ± 2.549 to 27.1 ± 1.76 invaded cells per field, n = 10, P < 0.0001) (Figure 1F). Our results demonstrated that Cucurbitacin I was able to inhibit cellular invasion of invasive NPC cells.

Cucurbitacin I-induced growth inhibition associated with PARP cleavage, Cyclin D1 and Mcl-1 downregulation
Cucurbitacin I has been found to have anti-proliferative effects in various non-EBV-associated human cancers, including glioblastoma, lung adenocarcinoma and breast cancer (26–28). Next, we examined if Cucurbitacin I would exert any effects on NPC cell growth, in addition to its anti-invasion effects. As shown in Figure 2A, Cucurbitacin I elicited potent dose-dependent anti-proliferative activity against these two invasive NPC cell lines, HK1-LMP1(B95.8) and CNE-2 (Figure 2A). A maximal growth inhibition of ∼72 and 65% was achieved at 48 h in HK1-LMP1(B95.8) and CNE-2 cells, respectively. Similar trend but lower maximal activity was observed at 72 h. This dose-dependent growth inhibition was associated with induction of PARP cleavage (a hallmark for apoptosis) in both cell lines at 24 and 48 h (Figure 2B). In addition to PARP cleavage, Cucurbitacin I-mediated growth inhibition of NPC cells was associated with down-regulation of cyclin D1 (a STAT3 target gene for proliferation), as well as Mcl-1 (an anti-apoptotic STAT3 target gene) in both cell lines (Figure 2B). Our results demonstrated good anti-proliferative activity of Cucurbitacin I in invasive NPC cells.

Anoikis resistance of NPC cells reversed by Cucurbitacin I
Anoikis is a normal cell death program initiated when cells are detached from their surrounding ECM. However, it is known that metastatic tumor cells can escape from anoikis (hence named anoikis resistance), survive in a detached status and invade other organs (41). NPC is highly invasive as lymph node invasion or metastasis occurs in as high as 60–70% NPC patients at the time of diagnosis (1). It is unclear if anoikis resistance plays a role in NPC metastasis. Both of these invasive NPC cell lines, HK1-LMP1(B95.8) and CNE-2, were found to have the ability to proliferate from very low density in suspension to large multicellular spheroids in detached state, which was coupled with low basal level of spontaneous apoptosis in such a state, suggesting these NPC cells are intrinsically resistant to anoikis (Figure 3A and B). Cucurbitacin I was found not only to significantly inhibit NPC cell growth in attached (Figure 2A) but also in detached state (Figure 3A and B). When compared with DMSO control, the number and size of large multicellular spheroids were reduced by Cucurbitacin I in a dose-dependent manner (Figure 3A). Using a MTT assay for three-dimensional culture, we demonstrated that the ability of HK1-LMP1(B95.8) and CNE-2 to proliferate in suspension was significantly reduced by 78.3 and 56.4% (at 500 nM dosage), respectively (Figure 3B). In order to examine if the reduced ability to proliferate in detached state was associated with apoptosis, the number of apoptotic cells in suspension culture was quantified by TUNEL assay. Cucurbitacin I, in addition to
inhibiting cell proliferation in detached state, it also induced significant apoptosis in such a status in both cell lines (Figure 3C). At 500 nM concentration, Cucurbitacin I induced apoptosis in ~45 and 34% of HK1-LMP1(B95.8) and CNE-2 cells, respectively. It is observed that HK1-LMP1(B95.8) was very sensitive to Cucurbitacin I as its inhibitory effect seemed to be saturated/maximal at 200 nM (as well as 500 nM). We further tested a lower dose of Cucurbitacin I (50 nM) and found that this much lower dose of Cucurbitacin I was able to impart a partial inhibition on the growth in suspension ability of HK1-LMP1(B95.8) without significantly inducing apoptosis in the

![Fig. 1](image-url)

Cucurbitacin I inhibited STAT3 activation and invasiveness of NPC cells. (A). Increased STAT3 activation (p-STAT3-Tyr705) in LMP1-expressing NPC cell lines. The expression levels of phospho-STAT3-Tyr705 were compared between the parental HK1 cells and the LMP1-expressing stable cell lines, HK1-LMP1(B95.8) and HK1-LMP1(2117). Actin was shown as loading control. Similar results were obtained in three independent experiments. (B) Increased levels of p-STAT3-Tyr705 expression correlated with increased invasiveness of NPC cells. HK1, HK1-LMP1(B95.8) and HK1-LMP1(2117) cells were seeded onto the upper inserts of the Matrigel chamber at the same density (7 × 10⁴ cells). Twenty-four hours later, cells invaded through the inserts were stained and counted under a light microscope. The average number of invaded cells per field was presented as mean ± SEM (n = 10 fields). Similar results were obtained in three independent experiments, The P value was shown, P < 0.0001. (C) Cucurbitacin I inhibited the expression of activated STAT3 in HK1-LMP1(B95.8) cells. Cucurbitacin I (500 nM) inhibited the expression of activated STAT3 (p-STAT3-Tyr705) in HK1-LMP1(B95.8) cells when compared with the vehicle control, DMSO upon 48 h of treatment. (D) Inhibition of STAT3 activation by Cucurbitacin I targeting significantly reduced the invasiveness of HK1-LMP1(B95.8) cells. HK1-LMP1(B95.8) cells were seeded onto the inserts of the Matrigel invasion chambers. The cell-containing chamber was then placed in the lower chamber containing complete medium (containing 10% fetal bovine serum) with DMSO or 200 nM or 500 nM of Cucurbitacin I. Twenty-four hours later, cells invading through the inserts were stained and counted. The average number of invaded cells per field was presented as mean ± SEM (n = 10 fields). Similar results were obtained in three independent experiments. The P value was shown, P < 0.0001. (E) Elevated expression of activated STAT3 (p-STAT3-Tyr705) in another invasive NPC cell line, CNE-2. The expression levels of phospho-STAT3-Tyr705 were compared between the normal epithelial cell line, Het-1A and CNE-2. Actin was shown as loading control. Similar results were obtained in three independent experiments. (F) Cucurbitacin I reduced the invasiveness of CNE-2 cells in a dose-dependent manner. Experiments were performed as in Figure 1D above. The average number of invaded cells per field was presented as mean ± SEM (n = 10 fields). Similar results were obtained in three independent experiments. The P value was shown, P < 0.0001.
TUNEL assay (Supplementary Figure 1 is available at Carcinogenesis Online). Our results strongly indicated that Cucurbitacin I can reverse the intrinsic anoikis resistance of NPC cells and induce anoikis sensitization (or detachment-induced apoptosis) in detached state. This was the first demonstration of the anoikis-sensitization activity of a specific JAK/STAT3 inhibitor, Cucurbitacin I, in human cancer.

Cucurbitacin I reduced in vitro clonogenicity and in vivo tumor formation ability of NPC cells

In a breast cancer model, STAT3 targeting by short hairpin RNA abrogated the ability of breast cancer cells to form tumor in mice (17), demonstrating its essential role in governing the in vivo tumorigenicity of breast cancer cells. Given the potential role of STAT3 in NPC tumorigenesis, we examined if inhibition of STAT3 activation by Cucurbitacin I would be sufficient to prevent NPC tumor formation/tumorigenicity, which has not been investigated before. First, using an in vitro clonogenic assay, we found that just brief exposure of NPC cells to Cucurbitacin I (1 μM) for 2 h was sufficient to significantly reduce both the number and size of tumor colonies of HK1-LMP1(B95.8) and CNE-2 cells (Figure 4A and B), suggesting that brief inhibition of STAT3 activation by Cucurbitacin I may inhibit NPC tumorigenicity. This was further confirmed in vivo, where brief exposure of HK1-LMP1(B95.8) and CNE-2 to Cucurbitacin I (1 μM for 2 h), but not DMSO, significantly attenuated the ability of these invasive NPC cells to form tumors in nude mice (Table I and Figure 5). We followed the appearance of NPC tumors in both the DMSO- and Cucurbitacin I-pretreated groups in the first week after inoculation. As shown in Table I, cumulative data demonstrated that Cucurbitacin I-pretreatment significantly reduced the in vivo tumor formation ability of both NPC cell lines, when compared with DMSO pretreatment. For both HK1-LMP1(B95.8) and CNE-2, the tumor incidence was 100% in the control groups and only ~44% in the Cucurbitacin I-pretreated groups. In addition to the reduction of tumor incidence, the average volume of palpable tumors for Cucurbitacin I-pretreatment group was also significantly smaller than the DMSO-pretreated group (5.47 ± 1.72 mm³ versus 43.99 ± 4.98 mm³ for HK1-LMP1(B95.8) and 3.81 ± 1.01 mm³ versus 70.75 ± 14.10 mm³ for CNE-2). In Figure 5, a longer time course study was conducted to follow the tumor growth rate of HK1-LMP1(B95.8) (Figure 5A) and CNE-2 (Figure 5B) upon Cucurbitacin I-pretreatment. For both cell lines, the suppression activity of Cucurbitacin I pretreatment sustained till 2 weeks, when the DMSO pretreatment tumors started to ulcerate and thus the experiments were terminated. In HK1-LMP1(B95.8), the average volume of Cucurbitacin I-pretreated tumors was 12.61 ± 10.05 mm³ versus 232.02 ± 26.96 mm³ for the DMSO control on day 13 (P < 0.0001) (Figure 5A). Similarly, the average volume of Cucurbitacin I-pretreated CNE-2 tumors was 2.26 ± 1.31 mm³ versus 415.22 ± 58.96 mm³ for the DMSO control on day 14 (P = 0.0002) (Figure 5B). This result suggested that STAT3 inhibition may inhibit the ability of NPC tumor seeding.

Pre-dosing of Cucurbitacin I in nude mice suppressed NPC tumor growth

Next, we examined if Cucurbitacin I pre-administration/pre-dosing into the nude mice prior to tumor inoculation will affect NPC tumor formation. As shown in Table II, three doses of Cucurbitacin I pre-administration/pre-dosing into the nude mice (1.3 mg/kg, once per day by intraperitoneal injection) prior to tumor inoculation was able to reduce the tumor incidence by ~20% in both HK1-LMP1(B95.8) and CNE-2 models when compared with the DMSO vehicle control. Moreover, the initial tumor size at first tumor appearance was significantly reduced by pre-dosing of animal with Cucurbitacin I (~52% and 38% in HK1-LMP1(B95.8) and CNE-2 models, respectively; Figure 5C). Furthermore, the suppressive effects of Cucurbitacin I pre-dosing were observed (with both tumor models) up to 7 days post-inoculation (Figure 5D and E). The results from this prevention model indicated that Cucurbitacin I harbors chemopreventive activity against NPC tumor formation.

Discussion

Although several important risk factors for the development of NPC have been identified, there is currently no concrete measure for NPC prevention, even in endemic regions. To prevent this highly invasive...
cancer from occurring, preventive approaches are highly desirable. In this study, we demonstrated for the first time that Cucurbitacin I, a natural product identified to be a selective inhibitor of JAK/STAT3-signaling pathway, significantly reduced the tumor-forming abilities/tumorigenicity of NPC cells in nude mice. In addition to its demonstrated anti-invasion activity in invasive NPC cell lines, our data provided the first evidence that Cucurbitacin I harbors anoikis-sensitization activity (i.e. sensitization to detachment-induced cell death) against human cancer. Taken together, our results suggested that Cucurbitacin I may be a potent chemopreventive agent for NPC with anti-invasion and anoikis-sensitization activities.

Cucurbitacin I, also known as JSI-124, was originally identified to have anti-STAT3 activity by large-scale screening of the NCI Diversity Set (a library of 1992 compounds selected from the NCI drug depository) using a phosphotyrosine STAT3 high-throughput cytoblot assay (28). Baskovich et al. further showed that this drug is highly selective for JAK/STAT3 pathway and showed no inhibitory effect on other major other mitogenic pathways including Akt, extracellular signal-regulated kinase and src pathways in both non-small cell lung cancer cells (A549) and breast cancer cells (MAD-MB-468). Agreeing with the previous findings in other cancer cell lines, we also found that Cucurbitacin I did not inhibit the level of p-src, p-Akt, p-extracellular signal-regulated kinase (p-p44/42 MAPK) even up to 1 μM concentration (Supplementary Figure 2 is available at Carcinogenesis Online). Furthermore, the anti-STAT3 specificity of this natural-occurring compound, Cucurbitacin I has then been demonstrated in several in vitro models as well (28). In several tumor models, it was shown that Cucurbitacin I only exhibited antitumor activity in in vivo xenograft models with constitutively activated STAT3, including breast cancer (MDA-MB-468), non-small cell lung cancer (A549), melanoma [including colon cancer, v-src-transformed NIH3T3, glioma (28,35)].

**Fig. 3.** Anoikis resistance of NPC cells was reversed by Cucurbitacin I. (A) Cucurbitacin I reduced the ability of HK1-LMP1(B95.8) and CNE-2 cells to proliferate and survive in suspension (or detached state) in ultra-low attachment cell culture plate (hydrophilic and neutral charged). Cells were seeded at a very low density of 4 x 10^3 in the presence of 10% fetal bovine serum for 2 days. Single cell suspension grew into large multicellular spheroids for both cell lines. Cucurbitacin I reduced the proliferation of two NPC cell lines in suspension as shown in the picture (A and B) MTT assay. (B) Cucurbitacin I reduced NPC cell proliferation in suspension as measured by MTT assay for three dimensional culture. The percentage growth in suspension was calculated as OD_{drug-treated} / OD_{vehicle-treated} x 100%. Similar results were obtained in three independent experiments. (C) Cucurbitacin I induced apoptotic cell death in suspension culture/spheroids of NPC cells. Cells/Spheroids in suspension were harvested for staining by TUNEL assay. The number of TUNEL-positive and DAPI-positive nuclei were counted and percent apoptotic cells were calculated as: (TUNEL-positive/DAPI-positive cells) x 100%. Similar results were obtained in three independent experiments. The reduction in cell proliferation in suspension was accompanied by induction of apoptosis in suspension indicated Cucurbitacin I was able to sensitize NPC cells for anoikis.
Due to the promising preclinical efficacy of this natural-occurring Cucurbitacin I against STAT3 (28,35), increasing number of efforts are sought out to see if it has other anti-tumor activity other than anti-STAT3 (28,35). Thus far, all current evidences indicated that the antitumor activity of Cucurbitacin I is related to JAK/STAT3 inhibition, except for the case of lymphoma, where Cucurbitacin I has been reported to downregulate both STAT3 and a fusion protein, nucleophosmin-anaplastic lymphoma kinase (the mechanism of nucleophosmin-anaplastic lymphoma kinase downregulation is unclear) (42). Nevertheless, Cucurbitacin I, thus far, has been shown to be a high-specific JAK/STAT3 inhibitor in various cancer models.

To date, there are only several attempts evaluating the efficacy of preventive agents for NPC, partly due to the lack of knowledge on potential molecular targets for NPC prevention. With the aim of targeting EBV, anti-viral agents, phosphonated nucleoside analogs were demonstrated to inhibit the growth of established NPC tumors in nude mice by inducing apoptosis (9,44,45). However, the toxicity profile of this class of agents, including nephrotoxicity and myelosuppression, may limit its clinical application at this stage (45). Cyclooxygenase (COX)-2 has also been explored for NPC prevention. Celecoxib, a COX-2 inhibitor can treat and prevent colorectal polyps in patients with familial adenomatous polyposis (46). Several studies were undertaken to examine the effect of celecoxib on preclinical and clinical models of NPC (8,47,48). Celecoxib has exhibited dose-dependent inhibition in the growth of NPC cells (8). Moreover, celecoxib was found to reduce angiogenesis and induce tumor transcriptional changes in NPC patients (48). Although the chemopreventive efficacy of celecoxib has not been evaluated in the prevention of NPC, it is reported severe cardiovascular toxicity of celecoxib (49) will impede the use of this agent for NPC prevention. Except for these studies, the exploration for chemopreventive agent for NPC is scarce. This current study demonstrated that in addition to its in vitro anti-proliferation and anti-invasion activity, Cucurbitacin I can also reduce the tumorigenicity of NPC cells in vivo, indicating this natural product with defined inhibitory activity against STAT3 activation, can be a potential chemopreventive agent for NPC.

STAT3 is a promising target for anticancer therapy. Recent studies suggest that targeting STAT3 pathway may have added benefits of cancer prevention as STAT3 is found to be critically involved in tumor formation, tumor initiation and promotion of several cancer models of skin, breast and lung (12–18). This natural occurring compound has been reported to have inhibit tumor cell proliferation or survival in various cancer systems, including lung, breast, colon, glioblastoma multiforme and melanoma (26,28,50,51). Although being infrequently looked at, the anti-invasion activity of Cucurbitacin I has been recently reported in a breast cancer cell line, MDA-MB-453-I4 (37). Other than these reported anticancer activities, it is unclear whether this natural occurring potent anticancer compound harbors additional anticancer activity or chemopreventive activity against human cancers. In this study, we found that Cucurbitacin I, can inhibit the invasiveness of NPC cell lines, in addition to its growth-inhibitory activity (Figure 1). Moreover, we showed that brief exposure to Cucurbitacin I can reduce the clonogenicity of NPC cells in vitro and significantly reduced their tumor-forming ability in nude mice when it is given prior to tumor inoculation (Figure 5C–E, Table II). More interestingly, we demonstrated that it can also sensitize NPC cells for anoikis. Anoikis is known as detachment-induced apoptosis/cell death. Normal cells (anchorage dependent) detaching from their surrounding ECM will be eliminated by anoikis. It is known that metastatic tumor cells may escape from anoikis (hence named anoikis resistance) and invade other organs. The anoikis-resistant property of cancer cells allows them to survive even after losing attachment to their surrounding ECM and thereby promotes metastases (41). Therefore, strategies inhibiting anoikis-resistance may reduce tumor metastasis. This was demonstrated by a recent study that when prostate tumor cells were pretreated with an anoikis sensitizer, anisomycin, the ability of prostate cancer cells to form metastases was greatly reduced (52). The molecular mechanism underlying anoikis resistance in cancer is not fully understood. The most well-documented...
Fig. 5. (A and B) Brief exposure to Cucurbitacin I reduced in vivo tumor formation ability of NPC cells. HK1-LMP1(B95.8) cells (Figure 5A) or CNE2 cells (Figure 5B) were briefly exposed to DMSO or Cucurbitacin I (1 \( \mu \)M) for 2 h in the presence of 10% fetal bovine serum. The pretreated cells were thoroughly washed with HANKS buffer for three times and then harvested, resuspended in HANKS for subcutaneous inoculation. For inoculation, \( 0.9 \times 10^6 \) of viable CNE-2 or \( 0.8 \times 10^6 \) of viable HK1-LMP1(B95.8) cells were subcutaneously inoculated into the flanks of the nude mice using a Hamilton syringe (\( n = 8 \) for each group). The first appearance of tumor and tumor growth was carefully monitored every other day. A digital caliper was used for tumor measurement. Tumor volume was calculated as follows: volume = length \( \times \) width\(^2\)/2. Photographs of the tumors were taken on day 13 for HK1-LMP1(B95.8) and day 14 for CNE-2. (C). Cumulative bar graph showing the initial tumor growth inhibition by pre-dosing of nude mice with three injections of Cucurbitacin I prior to tumor inoculation. HK1-LMP1(B95.8) tumors first appeared on day 3 (\( n = 21 \) for DMSO, \( n = 24 \) for Cucurbitacin I pre-dosed group), whereas CNE-2 tumors first appeared on day 4 (\( n = 18 \) for DMSO-pre-dosed group, \( n = 16 \) for Cucurbitacin I-pre-dosed group). (D and E) Pre-dosing of Cucurbitacin I in nude mice prior to tumor inoculation reduced the in vivo tumor formation ability of NPC cells. Nude mice were given a total of three doses of Cucurbitacin I (1.3 mg/kg in DMSO) or DMSO control, once per day for 3 days, by intraperitoneal injection prior to subcutaneous inoculation of tumor cells. For inoculation, \( 0.9 \times 10^6 \) of viable CNE-2 or
Cucurbitacin I suppresses NPC invasiveness, anoikis resistance and tumorigenicity

Table II. Pre-dosing of nude mice with Cucurbitacin I prior to tumor inoculation reduced the in vivo tumor formation ability of HK1-LMP1(B95.8) (A) and CNE-2 (B)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor incidence (%)</th>
<th>Average initial tumor volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) HK1-LMP1(B95.8)-initial tumor formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>20/21 (95.24)</td>
<td>14.38 ± 1.23</td>
</tr>
<tr>
<td>Cucurbitacin I</td>
<td>18/24 (75.0)</td>
<td>6.87 ± 0.76 (P &lt; 0.0001)</td>
</tr>
<tr>
<td>(B) CNE-2-initial tumor formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>18/18 (100)</td>
<td>42.49 ± 3.18</td>
</tr>
<tr>
<td>Cucurbitacin I</td>
<td>13/16 (81.25)</td>
<td>26.20 ± 2.871 (P = 0.0006)</td>
</tr>
</tbody>
</table>

Cumulative data showing the incidence of first tumor appearance and tumor size [day 3 for HK1-LMP1(B95.8), day 4 for CNE-2] in nude mice. Three doses of Cucurbitacin I (1.3 mg/kg in DMSO, once per day for 3 days by intraperitoneal injection) or DMSO was given prior to tumor inoculation. For CNE-2 and HK1-LMP1(B95.8), 0.9 x 10⁶ and 0.5 x 10⁶ viable cells were subcutaneously inoculated into the flanks of nude mice, respectively. Tumor appearance was monitored and tumor volume was measured every day with a digital caliper.

In this regard, Cucurbitacin I may offer a good choice for chemoprevention as it is found in many edible plants, including Cucurbitaeae and Scrophulariaceae, which have been used as traditional medicines for centuries (28,29).

In summary, our study demonstrated Cucurbitacin I, in addition to inhibiting NPC cell proliferation and invasion, can also inhibit NPC tumor formation in nude mice. Moreover, we reported for the first time, the anoikis-sensitization activity of Cucurbitacin I in human cancer. Taken together, our results indicate that Cucurbitacin I may be an effective preventive agent for NPC with anti-invasion activity. It is hoped that in the future, people in the high-risk group for developing NPC can be offered an affordable preventive agent to reduce the incidence of NPC, especially in the endemic regions.

Supplementary material
Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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References


