OXFORD

Carcinogenesis, 2017, Vol. 38, No. 2, 184–195

doi:10.1093/carcin/bgw124
Advance Access publication November 23, 2016
Original Manuscript

ORIGINAL MANUSCRIPT

Sestrin-3 modulation is essential for therapeutic efficacy of cucurbitacin B in lung cancer cells

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Abstract

Many purified compounds from dietary sources have been investigated for their anticancer activities. The main issue with most agents is their effectiveness at high doses which generally could not be delivered to humans through dietary consumption. Here, we observed that cucurbitacin B, a tetracyclic triterpenoid present in pumpkins, gourds and squashes, exhibits antiproliferative effects on human non-small cell lung cancer (NSCLC) cells at nanomolar concentrations. Treatment with cucurbitacin B (0.2–0.6 μM; 24 h) was found to result in decrease in the viability of EGFR-wild type (A549 and H1792) and EGFR-mutant lung cancer cells (H1650 and H1975) and reduction in cell-colonies but had only minimal effect on normal human bronchial epithelial cells. Treatment with cucurbitacin B also caused inhibition of PI3K/mTOR and signal transducer and activator of transcription (STAT)-3 signaling along with simultaneous activation of AMPKα levels in both EGFR-wild type and EGFR-mutant lung cancer cells. Cucurbitacin B caused specific increase in the protein and mRNA expression of sestrin-3 in EGFR-mutant lung cancer cells, but not in EGFR-wild type cells. Treatment with cucurbitacin B to sestrin-3 siRNA treated EGFR-mutant cells further amplified the decrease in cell-viability and caused more sustained G2-phase cell cycle arrest, suggesting that these effects are mediated partly through sestrin-3. We also found that sestrin-3 has a role in the induction of apoptosis by cucurbitacin B in both EGFR-wild type and EGFR-mutant lung cancer cells. These findings suggest novel mechanism by the modulation of sestrin-3 for the action of cucurbitacin B and suggest that it could be developed as an agent for therapy of NSCLC.

Introduction

Lung cancer is the primary cause of cancer death in both men and women in the USA and worldwide. The general prognosis is still very low despite of developments in the treatment due to improved surgical techniques, increased application of combined modality treatments and the use of new drugs. The epidermal growth factor receptor (EGFR) was the first member of cell surface receptors which was identified and cloned (1). It has been reported that EGFR controls cell proliferation, differentiation and apoptosis in normal cells. It also facilitates cell growth, differentiation and migration during histogenesis (2,3). The standard therapy for advanced non-small cell lung cancer (NSCLC) is based on the presence of EGFR mutations with a clinical response to the EGFR tyrosine kinase inhibitors (TKIs). The chemotherapeutic drugs gefitinib and erlotinib are given as first-line therapies for patients with advanced *EGFR* mutation-positive NSCLC. Testing for EGFR-mutations is now regularly done in clinical practice (4). However, despite the initial efficacy of the treatments, almost all patients acquire drug resistance and develop relapse after variable periods of time. Various mechanisms have been designated for the acquired resistance to EGFR-TKIs; however, the *EGFR* T790M mutation is the most common variation and is identified in about 50% of progressing tumors (4–6).

One limiting factor for the use of natural and dietary agents for cancer prevention and treatment is that they exert their effect at high concentrations which are not physiologically attainable

Received: June 6, 2016; **Revised:** October 26, 2016; **Accepted:** November 22, 2016

Abbreviations

4E-BP1	4E-binding protein 1
AMPK	AMP-activated protein kinase
ATCC	American Type Culture Collection
CT	Threshold cycle
EGFR	epidermal growth factor receptor
eIF	eukaryotic initiation factor
mTOR	Mammalian target of rapamycin
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide
NHBE	Normal Human Bronchial epithelial Cells
NSCLC	non-small cell lung cancer
STAT	signal transducer and activator of transcription
TKIs	tyrosine kinase inhibitors

(7). The cucurbitacins are highly diverse and oxygenated tetracyclic triterpenoids isolated from plants of Cucurbitaceae family which are well-known for the bitterness of edible products like pumpkins, gourds and squashes. Cucurbitacins are arbitrarily divided into twelve categories and structurally characterized by the tetracyclic cucurbitane nucleus skeleton: 19-(10→9β)-abeo-10α-lanost-5-ene (also known as 9β-methyl-19-nor lanosta-5-ene), with several oxygenation functionalities at different sites (Figure 1A) (8).

Cucurbitacin B is one of the most abundant and has been most widely used. It has been shown that cucurbitacin B had antiproliferative effects on several leukemia and lymphoma cell lines, and on primary mononuclear bone marrow cells derived from patients with acute myeloid leukemia or myelodysplastic syndrome (9). Treatment with cucurbitacin B has been shown to inhibit the growth of human hepatocellular carcinoma cells by suppression of signal transducer and activator of transcription (STAT)-3 phosphorylation (10). In pancreatic cancer cells, cucurbitacin B induced apoptosis by inhibition of the JAK/STAT pathway and enhanced the antiproliferative effect of the chemotherapeutic drug gemcitabine *in-vitro* (11) and *in-vivo* (12). Cucurbitacin B treatment also caused increased radiosensitization of human breast cancer cells, via G2/M phase cell-cycle arrest (13). Combination of cucurbitacin B with either docetaxel or gemcitabine synergistically inhibited the proliferation of human breast cancer cells and reduced tumor volume in immunodeficient mice (14). Recently, it has been reported that cucurbitacin B treatment inhibited orthotopic breast tumor growth through inhibition of HER2/integrin signaling (15).

Mammalian target of rapamycin (mTOR) belongs to the phosphatidylinositol 3-kinase (PI3K)-related family of kinases and is a downstream target of Akt. It has homology between the C-terminus domain of mTOR and the catalytic domain of PI3K and is significant for the oncogenic transformation induced by phosphatidylinositol-3-kinase (PI3K) and Akt (16,17). The mTOR cause phosphorylation of S6 kinase, which phosphorylates the ribosomal protein S6 and leads to initiation of protein translation. The eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) is also phosphorylated by mTOR and is disassociated from the eukaryotic translation initiation factor 4E (eIF-4E), leading to activation of protein translation (18,19). Several studies have reported the role of deregulated PI3K/Akt/mTOR signaling in lung cancer (20). The mTOR is phosphorylated in numerous lung cancer cell lines (21) and its activation has been reported to be more frequent in tumors with EGFR mutations or over-expression of PI3K/Akt (22). Therefore, inhibition of the PI3K/AKT/mTOR signaling represents a favorable approach for chemoprevention/chemotherapy of lung cancer. Sestrins belong to a family of stress-inducible proteins, comprising of sestrins-1, -2 and -3. It has been reported that sestrins inhibit mTOR complex 1 activity through the activation of AMPK (23). Sestrins regulate the mTORC1 axis; their accumulation stimulates AMPK and inhibits TORC1, possibly by interacting with AMPK or causing increase in the abundance of AMPK subunits (24,25). Though, the overexpression of sestrins can cause mTORC1 inhibition even in AMPK-deficient cells, mainly recognized by the ability of sestrins to bind the TORC1-regulating GAP activity toward Rags (GATOR)-2 protein complex, which was postulated to control trafficking of TORC1 to lysosomes. It has been suggested that the amino acid leucine specifically disrupts the association of Sestrin2 with GATOR2, therefore, clarifying how leucine and related amino acids stimulate TORC1 activity (25–27). The importance of sestrins as potential therapeutic targets for metabolic diseases such as diabetes has been discussed in a review by Dong (28). It has been reported that systemic deficiency of sestrin-2 cause glucose and insulin intolerance in high-fat-diet induced obese or genetic leptin-deficient ob/ob mouse models. This may be due to downregulation of AMPK and overactivation of mTORC1 (29). The hepatic sestrin-3 deficiency led to insulin resistance and glucose intolerance and hepatic sestrin-3 overexpression recovered insulin sensitivity and glucose homeostasis in liver-specific sestrin-3 knockout and transgenic mouse models fed either regular chow or high-fat diet (30).

Numerous studies have reported the anticancer activities of the dietary compounds, especially for lung cancer (7). Although, there are encouraging results from experimental studies, high doses and poor bioavailability limit their therapeutic usefulness. In this study, we report that cucurbitacin B at very low concentrations caused inhibition of PI3K/mTOR and STAT-3 signaling alongwith simultaneous activation of AMPKα levels in EGFR-wild type and mutant lung cancer cells. Mechanistically, cucurbitacin B modulated cell intrinsic inhibitor sestrin-3 in both EGFR-wild type and EGFR-mutant lung cancer cells.

Materials and methods

Chemicals, reagents and antibodies

PI3 Kinase p85, PI3 Kinase p110, p-Akt^{Ser473}, p-Akt^{Thr308}, p-mTOR^{Ser2448}, p-4EBP1^{Ser65}, p-eIF4E^{Ser209}, p-p70S6K, p-AMPKα^{Thr172}, p-PRAS40^{Thr246}, Rictor, Raptor, GβL, STAT-3 and p-STAT-3 antibodies were obtained from Cell Signaling Technology (Danvers, MA). Sestrin-3 was purchased from Abcam (Cambridge, MA). Anti-mouse and anti-rabbit secondary antibody horseradish peroxidase conjugates were obtained from Amersham Life Science Inc. (Arlington Height, IL). Cucurbitacin B was purchased from Sigma Chemical Co. (St. Louis, MO). The Annexin-V-FLUOS staining kit was purchased from Roche Diagnostics GmbH, Mannheim, Germany. BCA Protein assay kit was obtained from Pierce (Rockford, IL). Novex precast Tris-glycine gels were from Bio-Rad (Hercules, CA).

Cell culture and treatment

The cell-lines (A549, H1792, H1650 and H1975) were obtained form from American Type Culture Collection (ATCC; Manassas, VA). These cells were tested by ATCC for post-freeze viability, growth properties, morphology, mycoplasma contamination, species determination (cytochrome c oxidase I assay and short tandem repeat analysis), sterility test and human pathogenic virus testing. The cell-lines were straightaway resuscitated upon receiving and frozen in aliquots in liquid nitrogen and the cells were cultured within six months. Cells were also regularly tested for mycoplasma contamination by using MycoAlert Mycoplasma Detection Kit from Lonza (Basel, Switzerland). The A549 cells were cultured in F12K medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (P-S). H1792, H1975 and H1650 cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% P-S. Normal Human Bronchial epithelial Cells (NHBE) were obtained

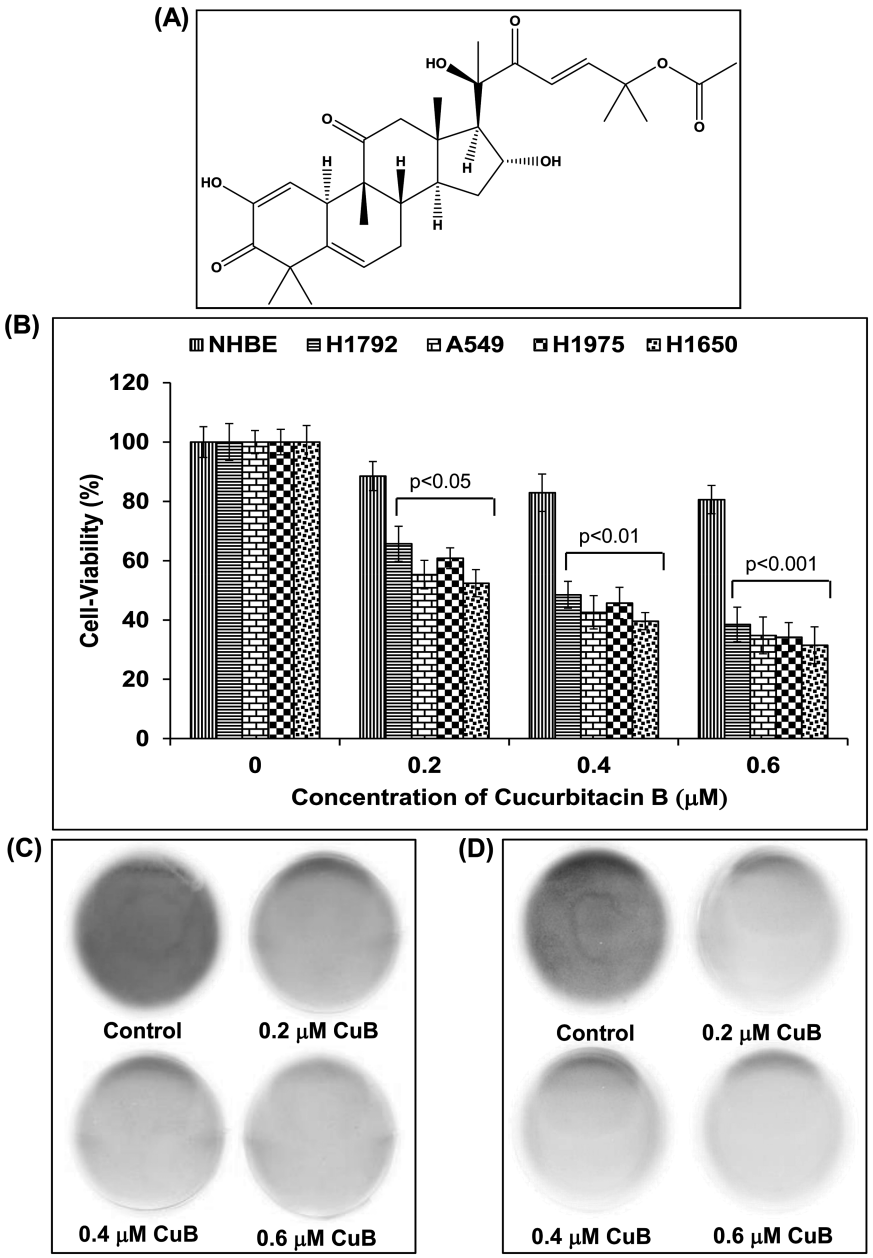

Figure 1. Structure of cucurbitacin B (CuB) and its effect on NSCLC cell-growth. (**A**) Structure of cucurbitacin B. (**B**) MTT Assay. As described in "Materials and methods", NHBE, A549, H1792, H1975 and H1650 cells were treated with cucurbitacin B (0.2–0.6 μM) for 24 h and the viability of cells was determined by the MTT assay. The data is expressed as the percentage of cell-viability and represent the mean ± SEM of three experiments in which each treatment was performed in multiple wells. (**C**) Effect of cucurbitacin B on colony formation in A549 cells. (**D**) Effect of cucurbitacin B on colony formation in H1650 cells. The cells were seeded in 6-well plates and treated with 0.2, 0.4 and 0.6 μM of cucurbitacin B as described in "Materials and Methods". At the end of the experiment, colonies were washed with 1X phosphate-buffered saline, stained with crystal violet and pictures were taken.

from Clonetics Airway Epithelial Cell Systems (Cambrex Bio Science, Walkersville, Inc) and cultured in Bronchial Epithelial Growth Media supplemented with growth factors (Cambrex Bio Science, Walkersville, Inc). The cells were maintained under standard cell culture conditions at 37°C and 5% CO_2 in a humid environment. Cucurbitacin B dissolved in dimethyl sulfoxide (final concentration 0.1% v/v) was used for the treatment of cells. The cells (60–70% confluent) were treated with cucurbitacin B (0.2–0.6 μM) for 24 h in complete growth medium.

Cell-viability

The effect of cucurbitacin B, on the viability of cells was determined by 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide assay. NHBE, A549, H1792, H1975 and H1650 cells were plated at 1 x 10^4 cells per well in 200 μl of complete culture medium containing cucurbitacin B (0.2–0.6 μM) in 96-well microtiter plates for 24 h. After incubation at 37°C in a humidified incubator, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT; 5 mg/ml in PBS) was added to each well and incubated for 2 h, after which the plate was centrifuged at 1800g for 5 min at 4°C. The supernatant was discarded and the pellet dissolved in 200 μl of DMSO and absorbance was recorded on a microplate reader at the wavelength of 540 nm. The effect of cucurbitacin B on growth inhibition was assessed as percent cell-viability where DMSO-treated cells were taken as 100% viable. DMSO at the concentrations used was without any effect on cell-viability.

Colony formation assay

NSCLC, A549 and H1650 cells were plated in 6-well plates at a very low density and treated with increasing concentrations of cucurbitacin B (0.2–0.6 μM) and maintained at 37°C in a humidified 10% CO_2 atmosphere.

Cells were allowed to grow for 14 days and fresh media containing cucurbitacin B was replaced every 3 days. At the conclusion of study, colonies were rinsed with 1X phosphate-buffered saline and stained with crystal violet.

Protein extraction and immunoblotting

Following the treatment of cells with cucurbitacin B (0.2–0.6 μM; 24 h), the media was aspirated, the cells were washed with cold PBS (pH 7.4), and ice-cold lysis buffer; 50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100 and 1 mM PMSF (pH 7.4) with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA) over ice for 30 min. The cells were scraped and the lysate was collected in a microfuge tube and passed through needle to break up the cell aggregates. The lysate was cleared by centrifugation at 14000g for 15 min at 4°C and the supernatant (whole cell lysate) was used or immediately stored at –80°C.

For immunoblotting, 30–50 μg protein was resolved over 8–12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (5% non-fat dry milk/1% Tween 20; in 20 mM TBS, pH 7.6) for 1 h at room temperature, incubated with appropriate monoclonal or polyclonal primary antibody in blocking buffer for one and half h to overnight at 4°C, followed by incubation with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate obtained from Amersham Life Science Inc. (Arlington Height, IL) and detected by chemiluminescence and autoradiography using Bio-Rad Gel-Doc (Bio-Rad Laboratories Inc., Hercules, CA).

Immunofluorescence analysis

Briefly, prior to staining, both cucurbitacin-B treated and untreated slides were fixed in ice-cold 4% paraformaldehyde (4% PFA) for 15 min at 4°C, followed by initial washing in PBS-Tween 20 (x% PBST). Post fixation both A549 and H1650 cells were washed 3 times with ice cold PBS. Further both types of slides were then blocked with normal serum block (10% normal goat serum) followed by incubation with different antibodies with appropriate dilutions ($pAKT^{ser473}$ 1:200; $pmTOR^{Ser2448}$ 1:250). After rinsing in PBST, slides were incubated with fluorescent-conjugated secondary antibody at 1:500 dilution in blocking buffer. Slides were then rinsed in PBST and were mounted with ProLong Gold Antifade reagent containing DAPI (Invitrogen, Carlsbad, CA) and left in dark overnight. Fluorescence imaging was performed using Nikon A1 confocal microscope (Nikon Instruments Inc., New York, USA). Images were acquired with ×20 objective at 1024 × 1024 resolutions. Image analysis was accomplished using the Nikon Elements software.

siRNA

ON-TARGET plus Human sestrin (SESN)-3 siRNA-SMARTpool (and a non-targeting control siRNA were purchased from Dharmacon (Lafayette, CO). For transfection, cells were incubated with sestrin-3-siRNA at a concentration of 100 nM. We used Lipofectamine RNAi max from Invitrogen (Carlsbad, CA) for H1650 cells and Transfection kit reagent from Lonza (Walkersville, MD) for A549 cells in antibiotic-free medium for 48 h.

Real-time qPCR analysis for mRNA expression

Briefly, RNA was extracted from the cells using RNeasy kit (Catalog No: 74134) from Qiagen (Valencia, CA), and reverse transcribed with iScript Reverse transcription supermix kit (Catalog No: 1708841) from Bio-Rad (Hercules, CA). cDNA (1–100 ng) was amplified in triplicate using human sestrin-3 primers (Catalog No: PPH15451A-200) from Qiagen (Valencia, CA). Threshold cycle (CT) values obtained from the instrument's software were used to calculate the fold change of the respective mRNAs. ΔCT was calculated by subtracting the CT value of the housekeeping gene from that of the mRNA of interest. ΔΔCT for each mRNA was then calculated by subtracting the CT value of the control from the experimental value. Fold change was calculated by the formula $2 - \Delta\Delta CT$.

Apoptosis assessment by Annexin-V-FLUOS staining

The Annexin-V-FLUOS staining kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to identify apoptotic cells within a cell population. The A549 and H1650 cells were incubated with SESN-3 siRNA for 72 h using the same methods as described above under siRNA heading and/or treated with cucurbitacin B (0.4–0.6 μM) for 24 h. The cells were washed, centrifuged and the cell-pellet was resuspended in Annexin-V-FLUOS labeling solution (prepared by prediluting Annexin-V-FLUOS labeling reagent in incubation buffer and adding Propidium iodide solution). The data was collected on a Becton-Dickinson FACSCalibur (San Jose, CA) and analyzed in FlowJo, Version 9.7 (FLowJo, LLC, Ashland, OR).

Cell-cycle analysis

The A549 and H1650 cells were incubated with sestrin-3 siRNA for 72 h using the same methods as described above under siRNA heading and/or treated with cucurbitacin B (0.4–0.6 μM) for 24 h. Cells were then trypsinized and fixed in 1% Paraformaldehyde. Cells were then washed thrice with PBS, centrifuged and the pellet was resuspended in chilled 70% ethanol. The Apo-Direct Kit (BD Pharmagen, CA) was used for labeling cells with FITC and PI. Flow cytometry was performed with a FACScan (Becton Dickinson, Germany) and the DNA histograms were further analyzed by using ModiFitLT software (Verily Software House, Topsham, ME, USA) for cell cycle analysis.

Statistical analysis

Results were analyzed using a two-tailed Student's *t*-test to assess statistical significance and p values <0.05 were considered significant.

Results

Inhibition of cell growth and colony formation by cucurbitacin B in EGFR-wild type and EGFR-mutant lung cancer cells

Our first experiment was to determine the effect of cucurbitacin B (CuB) on the growth of human EGFR-wild type (A549 and H1792) and mutant (H1975 and H1650) NSCLC cells. The dose-dependent effect of cucurbitacin B at concentrations of 0.2–0.6 μM on the growth of human NSCLC cells and NHBE cells was determined utilizing MTT assay. We found that treatment of both EGFR-wild type H1792 and A549 cells with cucurbitacin B (0.2–0.6 μM; 24 h) caused significant decrease in their viability by 34–61% and 45–65%, respectively (Figure 1B). There was also decrease in the viability of EGFR-mutant H1975 and H1650 cells by 39–66% and 49–69%, respectively, on treatment with cucurbitacin B. Importantly, there was insignificant effect on NHBE cells at the same doses of cucurbitacin B (Figure 1B). Based on these results, we selected EGFR-wild type A549 cells and EGFR-mutant H1650 cells for further experiments, since cucurbitacin B treatment caused maximum effect on cell-viability in these cells. We also investigated the effect of cucurbitacin B for decreasing the clonogenic potential of both EGFR-wild type A549 cells and EGFR-mutant H1650 cells which were treated with increasing concentrations of cucurbitacin B, every 3–4 days. Treatment with cucurbitacin B caused striking decrease in the number of colonies of both A549 cells (Figure 1C) and H1650 cells (Figure 1D) dose-dependently with almost complete inhibition at 0.6 μM after 14 days.

Inhibition of PI3K and phosphorylation of Akt protein expression by cucurbitacin B in EGFR-wild type and EGFR-mutant lung cancer cells

Several studies suggest that PI3K/ AKT signaling is frequently activated in NSCLC and is also involved in the promotion of cell survival, growth, proliferation and migration (31). Treatment with cucurbitacin B caused inhibition of the protein expression of both regulatory (p85) and catalytic (p110) subunits of PI3K in EGFR-wild type A549 cells (Figure 2A) and EGFR-mutant H1650 cells (Figure 2B), respectively. Phosphorylation of Akt is apparent in 50–70% of NSCLC, pointing to the fact that activation of the PI3K/Akt/signaling plays an important role in its development

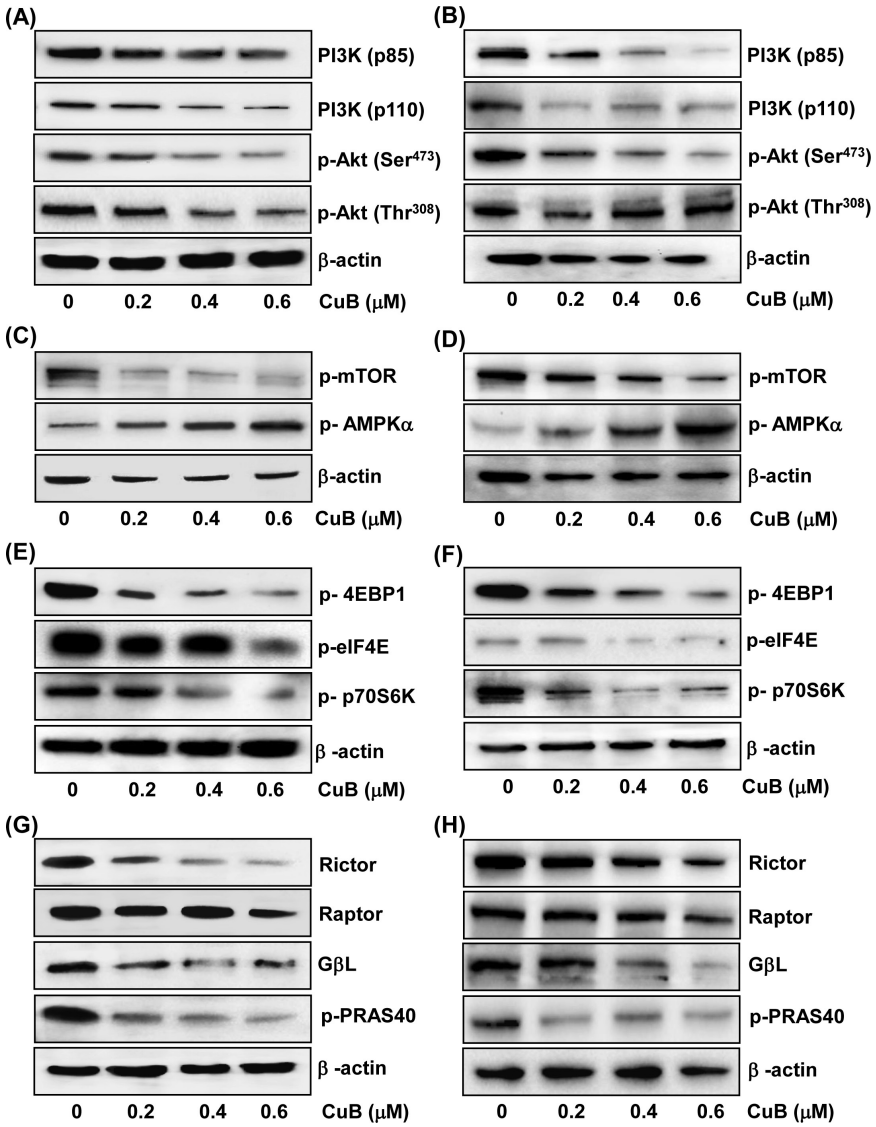

Figure 2. Effect of cucurbitacin B on the protein expression of PI3K, Akt, mTOR signaling and AMPKα in human lung cancer cells. (**A**) Effect of cucurbitacin B on the protein expression of PI3K and phosphorylation of Akt in A549 cells, and (**B**) H1650 cells. (**C**) Effect of cucurbitacin B on the phosphorylation of mTOR and AMPKα in A549 cells, and (**D**) H1650 cells. (**E**) Effect of cucurbitacin B on the phosphorylation of 4EBP1, eIF4E and p70S6K in A549 cells, and (**F**) H1650 cells. (**G**) Effect of cucurbitacin B on the protein expression of Rictor, Raptor, GβL and phosphorylation of PRAS40 in A549 cells, and (**H**) H1650 cells. As described in "Materials and Methods", the NSCLC cells were treated with cucurbitacin B (0.2–0.6 μM; 24 h) and then harvested. Total cell lysates were prepared and 40 μg protein was subjected to SDS-PAGE followed by immunoblot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for β-actin.

(32). There was also inhibition in the phosphorylation of Akt at both Ser^{473} and Thr^{308} on treatment with cucurbitacin B (0.2–0.6 μM) in A549 and H1650 lung cancer cells as shown in Figure 2A and B, respectively.

Inhibition of the phosphorylation of mTOR and activation of AMPKα by cucurbitacin B in EGFR-wild type and EGFR-mutant lung cancer cells

Activation of the PI3K pathway is involved in several types of cancers and is frequently connected with resistance to cancer therapies. The mTOR operates at a key link in the PI3K/Akt pathway and therefore, we evaluated the effect of the treatment with cucurbitacin B on the phosphorylation of mTOR. Treatment with cucurbitacin B (0.2–0.6 μM) caused dose-dependent inhibition in the phosphorylation of mTOR as shown in Figure 2C and D. The AMP-activated protein kinase (AMPK) is activated by cellular stress and is responsible for maintaining energy homeostasis (33). It is also an upstream regulator of mTOR signaling and acts as a tumor suppressor (34). We observed that there was a dose-dependent increase in the phosphorylation of AMPKα at 0.2–0.6 μM concentration of cucurbitacin B in A549 and H1650 NSCLC cells (Figure 2C and D). To further investigate cucurbitacin B acts on mTOR directly or via AMPK, we treated both EGFR-wild type A549 cells and EGFR-mutant H1650 human lung cancer cells with compound C, a well-known inhibitor of AMPK kinase activity. As shown in Supplementary Figure 1A and B, available at *Carcinogenesis* Online, treatment with compound C (20 μM) caused decrease in the phosphorylation of mTOR and protein expression of AMPKα. On addition of cucurbitacin B to compound C-treated cells, mTOR was further downregulated and there was increase in the expression of AMPKα. This demonstrates that cucurbitacin B acts on mTOR and not entirely via AMPK and other modes of actions are also involved.

Inhibition of the phosphorylation of mTOR target proteins by cucurbitacin B in EGFR-wild type and EGFR-mutant lung cancer cells

The mTOR functions at least as two distinct functional complexes mTORC1 and mTORC2. Activation of mTORC1 results in phosphorylation of its effectors, eukaryotic initiation factor (eIF), 4E-BP1 and P70S6K, whereas mTORC2 regulates phosphorylation of Akt (35). We examined the effect of cucurbitacin B (0.2–0.6 μM) on the phosphorylation of mTOR target proteins in EGFR-wild type A549 cells and EGFR-mutant H1650 human lung cancer cells. By immunoblot analysis, we found that treatment with cucurbitacin B consistently decreased the phosphorylation of 4E-BP1, eIF4E and p70S6K, best-known downstream effector molecules of mTORC1 in A549 (Figure 2E) and H1650 cells (Figure 2F).

Inhibition of the phosphorylation of mTOR-interacting proteins by cucurbitacin B in EGFR-wild type and EGFR-mutant lung cancer cells

The two mTOR complexes (mTORC1 and mTORC2) comprise of distinctive mTOR-interacting proteins that define their substrate specificity. The mTORC1 is composed of mTOR, G-protein β-subunit-like protein (GβL), raptor (regulatory associated protein of mTOR), PRAS40 (proline-rich Akt substrate 40 kDa) (36). The mTORC2 involves mTOR, GβL and rictor (rapamycin insensitive companion of mTOR) (37,38). Treatment of A549 and H1650 NSCLC cells with cucurbitacin B affected both mTOR complexes with inhibition of rictor, raptor, inhibition of the phosphorylation of PRAS40 and GβL as shown by immunoblot analysis in Figures 2G and H, respectively.

Inhibition of AKT and mTORC1 phosphorylation by cucurbitacin B in EGFR-wild type and EGFR-mutant lung cancer cells

Previous studies have suggested the role of PI3K/Akt and mTORC1 in both EGFR-wild type and EGFR-mutant lung cancer cells. In our immunofluorescence data, we clearly observed a higher proportion of phosphorylated Akt^{ser473} and $mTOR^{ser2448}$ (a readout for mTORC1 activity) in EGFR-mutant cells (Figure 3A, left and middle panels, respectively) when compared to wild type cells (Figure 3B, left and middle panels, respectively). However, we did not observe any nuclear localization of p-Akt^{ser473} in both EGFR-wild type and EGFR-mutant cells. Further, we observed nuclear localization of some p-$mTOR^{ser2448}$only in EGFR-mutant cells (Figure 3B, middle panel). This was in accordance with some previous studies that suggested nuclear localization of mTORC1 (39,40). This data correlates well with our western blot data where we have also observed increased induction on PI3K/Akt and mTORC1 in EGFR- mutant cells when compared with wild type cells. Treatment with cucurbitacin B dramatically reduces p-Akt^{ser473} and p-$mTOR^{ser2448}$ in both EGFR-wild type (Figure 3A, left and middle panels) and EGFR-mutant cells (Figure 3B, left and middle panels). We observed a common pattern of mTORC1 relocation, as most of residual mTORC1 seems to be localized at periphery of the cells after cucurbitacin B treatment in both EGFR-wild type and EGFR-mutant lung cancer cells. We also observed a clear reduction in cell size of both EGFR-wild type and EGFR-mutant cells after cucurbitacin B treatment. Since cell size is directly regulated by mTOR pathway it confirms the negative impact of cucurbitacin B on mTOR pathway. We also stained both EGFR-wild type and EGFR-mutant lung cancer cells with $pSTAT3^{Y705}$ as a positive control for cucurbitacin B (Figure 3A and B right panels, respectively).

Inhibition of the phosphorylation of STAT-3 by cucurbitacin B in EGFR-wild type and EGFR-mutant lung cancer cells

STAT-3 is a member of the STAT family of transcription factors which plays an important role in cancer-related inflammation (41,42) and is often deregulated in the development of lung cancer (43). STAT-3 has been reported to be activated in samples of lung adenocarcinoma patients and human lung cancer cell lines (44,45). It also plays an important role in promotion of NSCLC and in the course of acquired drug resistance (46,47). Treatment with cucurbitacin B (0.2–0.6 μM) dose-dependently decreased the phosphorylation of STAT-3 in both EGFR-wild type A549 cells (Figure 4A) and EGFR-mutant H1650 human lung cancer cells (Figure 4B).

Effect on sestrin-3 by cucurbitacin B in EGFR-wild type and EGFR-mutant lung cancer cells

Sestrins are highly conserved gene family found in all multicellular organisms of the animal kingdom. The invertebrate genome contains only a single sestrin (*Sesn*) gene whereas three sestrin genes are found in vertebrates (*Sesn1-3*) (48,49). It has been reported that sestrins can suppress mTOR complex 1 (mTORC1) activity via the activation of AMPK (23,50). FOXO1/3a-mediated transcriptional upregulation of sestrin3 has been shown to cause activation of the AMPK/TSC1/2 axis, leading to inhibition of mTORC1 activity (50).

There was no significant effect of cucurbitacin B on sestrins-1 and -2 in both EGFR-wild type and EGFR-mutant cells. However, we found that treatment with cucurbitacin B decreased the protein and mRNA expression of sestrin-3 in EGFR-wild type A549 cells (Figures 4C and E, respectively). However, treatment with cucurbitacin B caused specific increase in the protein and mRNA expression of sestrin-3 in EGFR-mutant lung cancer cells as shown in Figure 4D and F, and Supplementary Figure 2A and B, available at *Carcinogenesis* Online respectively, indicating that cucurbitacin B acts as activator of sestrin-3 in these cells. This is an important observation, as there is no published report of any known activator/inducer of sestrin-3 till date.

Effect on cell-viability of EGFR-wild type and EGFR-mutant lung cancer cells by knockdown of sestrin-3

To further investigate whether cucurbitacin B acts through sestrin-3, we knocked down sestrin-3 by siRNA in both EGFR-wild type A549 (Figure 4G) and EGFR-mutant H1650 (Figure 4H) lung cancer cells. There was no change in cell-viability on treatment with cucurbitacin B to sestrin-3 siRNA treated EGFR wild-type cells as compared with cells treated with cucurbitacin B alone, suggesting that effects in these cells are not mediated through sestrin-3 (Figure 4I). However, treatment with cucurbitacin B to sestrin-3 siRNA treated EGFR-mutant cells further amplified the decrease in cell-viability as compared with cells treated with cucurbitacin B alone, signifying that these effects are mediated partly through sestrin-3, in these cells (Figure 4J).

Effect of cucurbitacin B and knockdown of sestrin-3 on apoptosis of EGFR-wild type and EGFR-mutant lung cancer cells

To determine whether apoptosis is involved in the cucurbitacin B-induced cell-growth inhibition of EGFR-wild type A549 cells and EGFR-mutant H1650 cells, we first evaluated the effect of cucurbitacin B (0.4–0.6 μM) on the induction of apoptosis in these cells. As shown by flow cytometric evaluation, there was 10.8 and 18.8% late apoptosis on treatment of cells with 0.4 and 0.6 μM of cucurbitacin B, respectively, as compared to 1.51% in

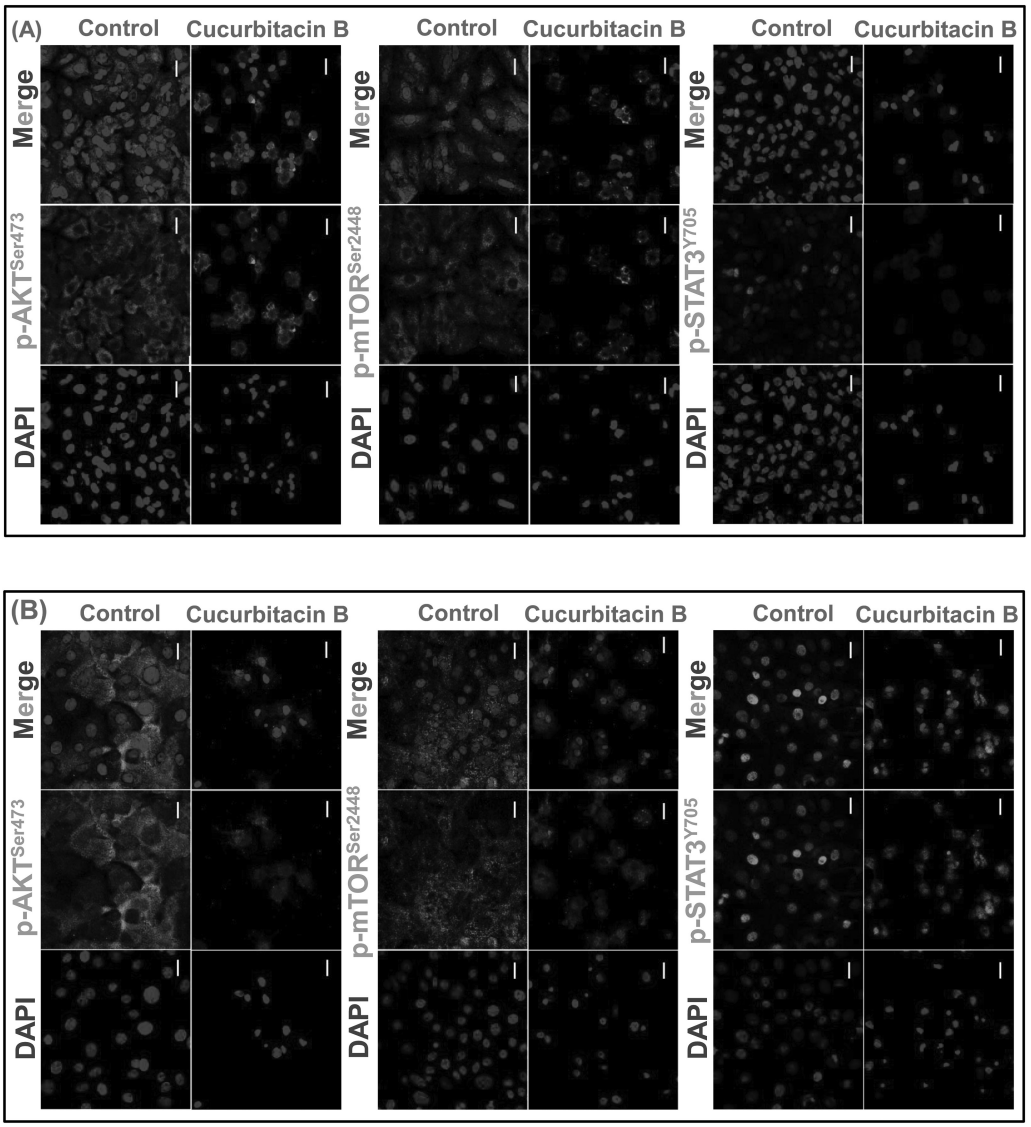

Figure 3. Effect of cucurbitacin B on Akt and mTORC1 phosphorylation in human lung cancer cells. (**A**) Representative immunofluorescent images of the effect of cucurbitacin B on Akt (left panel), mTORC1 (middle panel) and STAT-3 phosphorylation (right panel) in A549 cells, and (**B**) H1650 cells. The NSCLC cells were treated with vehicle control and cucurbitacin B (0.4 μM) for 24 h and stained for p-AKT^{ser473}, p-mTORC1 and pSTAT3^{Y705}. Bar = 50 mm.

control EGFR-wild type A549 cells (Figure 5A). Whereas, early apoptosis was 0.73% in control group, 4.05% in cells treated with 0.4 μM of cucurbitacin B and 8.59% in cells treated with 0.6 μM of cucurbitacin B, suggesting that cucurbitacin B induced dose-dependent late and early apoptosis in EGFR-wild-type cells (Figure 5A). Treatment of 0.4 and 0.6 μM of cucurbitacin B to sestrin-3 silenced EGFR-wild type cells, led to dramatic 62.3 and 66.5% late apoptosis, respectively, as compared to 19.8% in cells silenced with sestrin-3 only and without any cucurbitacin B treatment. Early apoptosis also increased from 5.14% in cells silenced with sestrin-3 only to 15.7% in sestrin-3 siRNA+0.4 μM and 25.3% in sestrin-3 siRNA+0.6 μM cucurbitacin B-treated cells (Figure 5A). This shows that although treatment with cucurbitacin B induced apoptosis in EGFR-wild type cells, silencing of sestrin-3 had dramatic effect on the induction of both late and early apoptosis, confirming that sestrin-3 has a role in these cells.

In contrast, there was no late apoptosis in EGFR-mutant H1650 cells (Figure 5B). In sharp contrast, when these cells were silenced with sestrin-3 and then treated with cucurbitacin B, there was 16.2% (0.4 μM CuB) and 18.5% (0.6 μM CuB) late apoptosis as compared with 7.1% in cells treated with sestrin-3 siRNA only (Figure 5B). The early apoptosis was modest 8.81 and 13.5% in cells treated with 0.4 and 0.6 μM of cucurbitacin B, respectively, as compared to 1.16% in control group (Figure 5B). There was more profound increase in early apoptosis in sestrin-3 silenced cells and treated with cucurbitacin B with 39.8% in cells treated with 0.4 μM CuB and 41.7% in cells treated with 0.6 μM CuB (Figure 5B). This further confirms that sestrin-3 has a prominent role in the induction of apoptosis in these cells, as silencing of sestrin-3 caused marked increase in both early and late apoptosis in these cells. The significant part of the observation is that treatment with cucurbitacin B decreased both protein and mRNA expression of sestrin-3 in EGFR-wild type cells and increased the expression in EGFR-mutant cells. In spite of this, sestrin-3 plays role in the induction of apoptosis in both types of lung cancer cells.

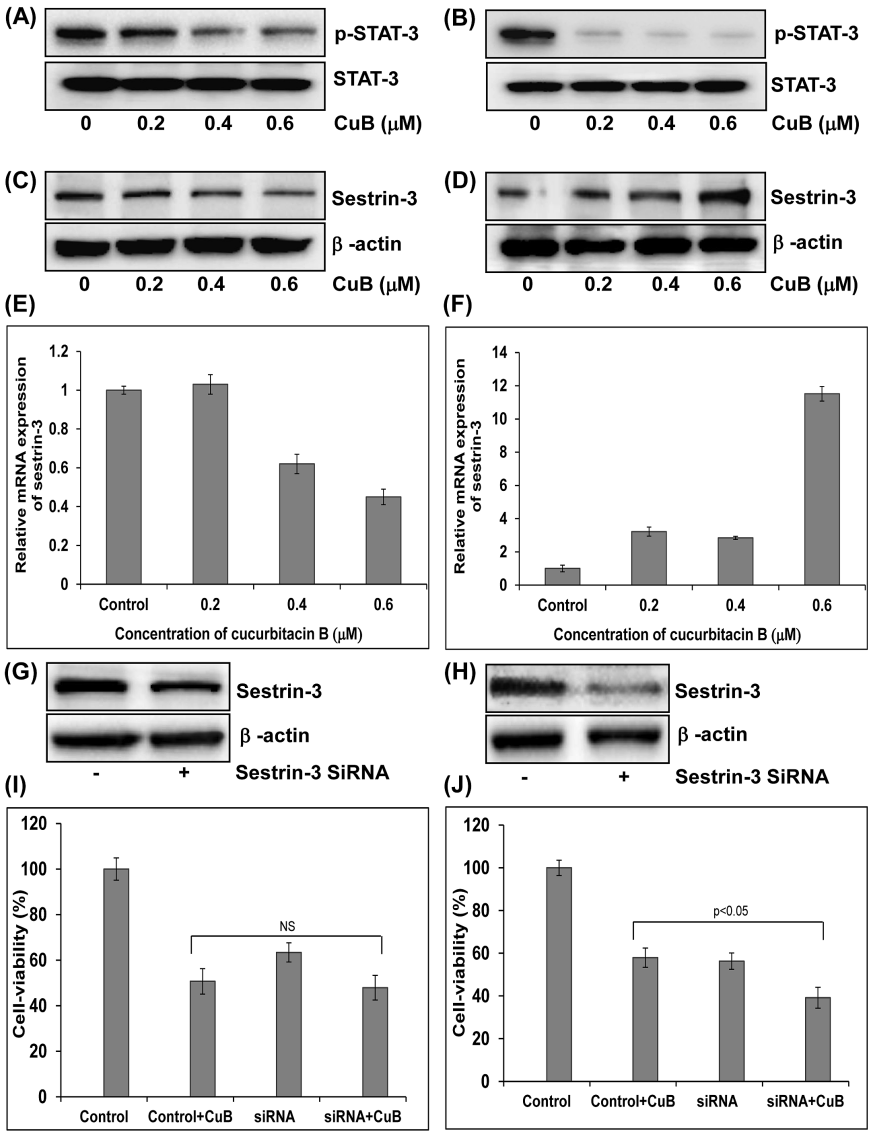

Figure 4. Effect of cucurbitacin B on the phosphorylation of STAT-3, activation and knockdown of Sestrin-3 in human lung cancer cells. (**A**) Effect of cucurbitacin B on the phosphorylation of STAT-3 in A549 cells. (**B**) Effect of cucurbitacin B on the phosphorylation of STAT-3 in H1650 cells. (**C**) Effect of cucurbitacin B on the activation of Sestrin-3 in A549 cells. (**D**) Effect of cucurbitacin B on the activation of Sestrin-3 in H1650 cells. As described in the in "Experimental Procedures", the NSCLC cells were treated with cucurbitacin B (0.2–0.6 μM; 24 h) and then harvested. Total cell lysates were prepared and 40 μg protein was subjected to SDS-PAGE followed by immunoblot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for β-actin. (**E**) Histogram showing the effect of cucurbitacin B in transcript levels of Sestrin-3 in A549 cells, and (**F**) H1650 cells. Expression of Sestrin-3 was analyzed by qRT-PCR. (**G**) Knockdown of Sestrin-3 in A549 cells, and (**H**) H1650 cells. Cells were incubated with Sestrin-3 siRNA and then harvested. Total cell lysates were prepared and 40 μg protein was subjected to SDS-PAGE followed by immunoblot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for β-actin. (**I**) Effect on cell-viability of A549 cells by knockdown of Sestrin-3. (**J**) Effect on cell-viability of H1650 cells by knockdown of sestrin-3. Cells were transfected with Sestrin-3 siRNA and/or treated with 0.4 μM of cucurbitacin B for 24 h and the viability of cells was determined by the MTT assay. The data is expressed as the percentage of cell-viability and represent the mean ± SEM of three experiments in which each treatment was performed in multiple wells.

Effect of cucurbitacin B and knockdown of sestrin-3 on cell-cycle in EGFR-wild type and EGFR-mutant lung cancer cells

To further explore if treatment with cucurbitacin B had any effect on the regulation of cell-cycle, we investigated its effect on distribution of cell cycle by flow cytometry after staining with propidium iodide. We observed that treatment of cells with either cucurbitacin B or knockdown of sestrin-3 had no effect on cell-cycle in EGFR-wild type lung cancer cells (data not shown). However, treatment with cucurbitacin B caused G2-phase cell cycle arrest in H1650 cells, which was not dose-dependent. However, in cells treated with sestrin-3 siRNA and cucurbitacin B, there was more sustained dose-dependent G2-phase cell cycle arrest (Figure 6A), suggesting that sestrin-3 might be involved partly, in these effects.

Discussion

One of the most limiting factors for the use of natural and dietary agents for cancer prevention and treatment in clinical trials is that they exert their effect at high concentrations which are generally not physiologically attainable. The main highlight of our study is that cucurbitacin B at very low concentrations (0.2–0.6 μM) displayed therapeutic effects in both EGFR-wild type and EGFR-mutant lung cancer cells through modulation of

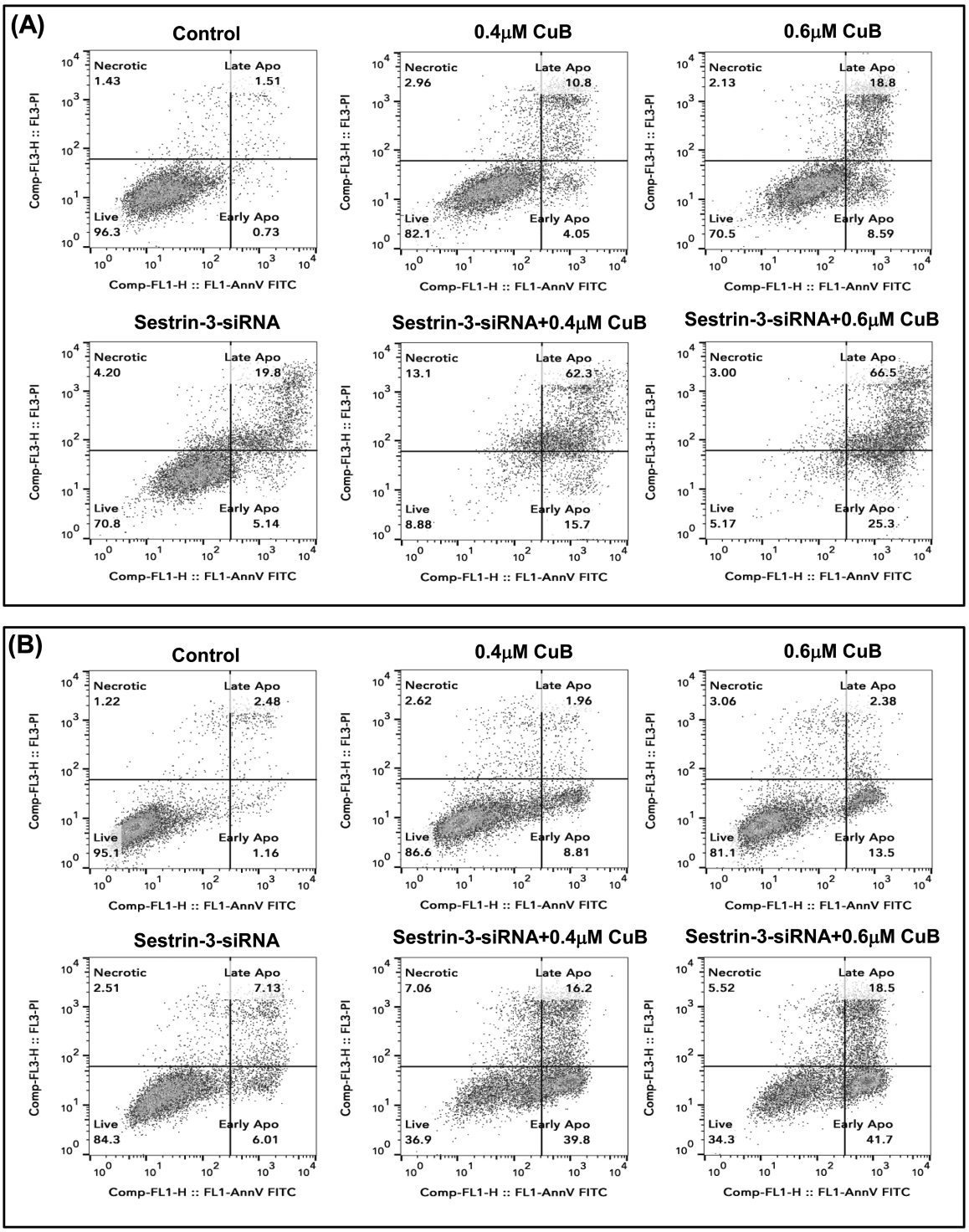

Figure 5. Effect of cucurbitacin B and knockdown of sestrin-3 on the induction of apoptosis in human lung cancer cells. (**A**) Effect of cucurbitacin B on apoptosis by annexin-V-FLUOS staining in A549 cells, and (**B**) H1650 cells. The cells were silenced with sestrin-3 siRNA and/or treated with cucurbitacin B (0.4–0.6 μM; 24 h) and resuspended in Annexin-V-FLUOS labeling solution. The data was collected on a Becton-Dickinson FACSCalibur (San Jose, CA) and analyzed in FlowJo, Version 9.7 (FLowJo, LLC, Ashland, OR).

sestrin-3. For NSCLC patients, the five-year survival rate is ~16%, even with advancement in diagnosis and treatment. The PI3K/mTOR pathway is involved in the regulation of cell proliferation, survival, differentiation, adhesion, motility and invasion (51). Deregulation of this pathway has been implicated in lung cancer with high grade tumors and advanced stages of the disease (52). Since the discovery of the first mTOR inhibitor rapamycin, the mTOR pathway has emerged as an attractive target for cancer therapy. Several inhibitors are being developed in recent years which target the molecular components of the PI3K/mTOR pathway (53–55). A probable mechanism of resistance to single agent PI3K/AKT/mTOR pathway inhibitors is the reactivation of upstream

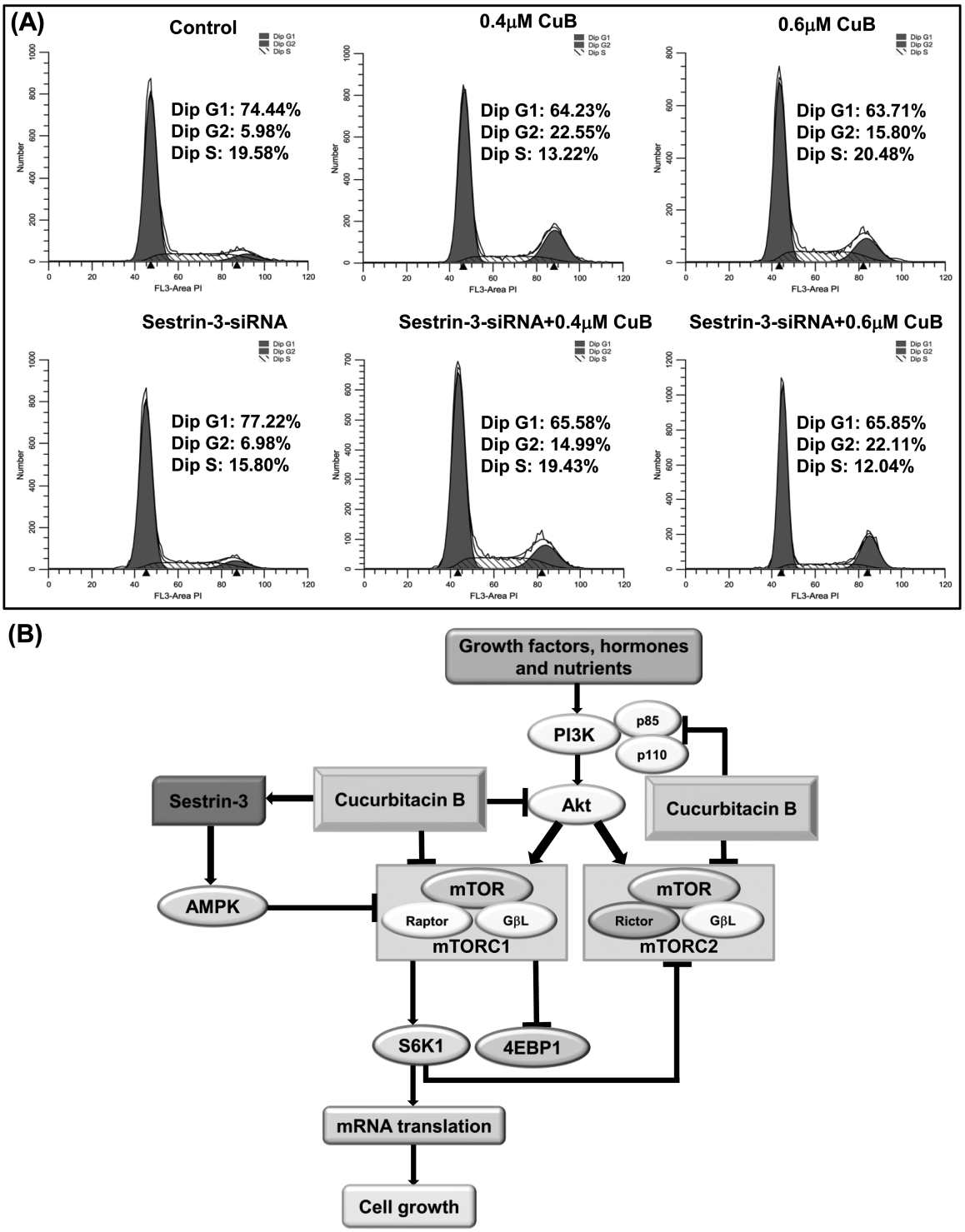

Figure 6. Effect of cucurbitacin B and knockdown of sestrin-3 on cell-cycle in human lung cancer cells. (**A**) Effect of cucurbitacin B on cell-cycle in H1650 cells. These cells were silenced with sestrin-3 siRNA and/or treated with cucurbitacin B (0.4–0.6 μM; 24 h), collected and stained with propidium iodide by using an Apo-Direct Kit obtained (BD Pharmagen, CA) as per manufacturer's protocol followed by flow cytometry. Following fluorescence activated cell sorter analysis, cellular DNA histograms were further analyzed by ModiFitLT V3.0. Further details are described in Materials and methods. (**B**) Figure depicting the effect of cucurbitacin B on prosurvival signaling pathways in human lung cancer cells.

signaling due to the release of negative feedback loops or the activation of signaling through alternative PI3K downstream targets. Therefore, it would be more beneficial to target different components of the PI3K/AKT/mTOR pathway. In the present study, we found that treatment of both EGFR-wild type and EGFR-mutant lung cancer cells with cucurbitacin B caused decrease in their viability along with minimal effect on NHBE cells. This specifies that cucurbitacin B was more specific towards cancerous cells. It was also shown that cucurbitacin B targeted PI3K/mTOR pathway by keeping the feedback loop in check (Figure 6B).

The PI3K signaling is often over-activated in several types of human cancers and plays an important part in the initiation and progression of NSCLC (56,57). It has been reported that activation of Akt occurs in 43–90% of NSCLC and ~50% in small cell lung cancer cases and the AKT1 mutations are present in 1% of all lung cancer cases (21). Akt facilitates most signals channeled through the PI3K pathway. We observed that treatment of both EGFR-wild type and EGFR-mutant lung cancer cells with cucurbitacin B caused inhibition of the expression of both regulatory and catalytic subunits of PI3K and inhibition of the phosphorylation of Akt at both Ser^{473} and Thr^{308}. Cucurbitacin B also caused inhibition of the phosphorylation of mTOR, suggesting that it targets PI3K/Akt/mTOR pathway. We also found a higher proportion of phosphorylated Akt and mTOR in EGFR-mutant cells as compared with EGFR-wild type cells. Treatment with cucurbitacin B caused decrease in the expression of p-Akt and p-mTOR in both EGFR-wild type and EGFR-mutant cells.

AMPK maintains cellular energy homeostasis and controls cell-proliferation, growth, and autophagy through the regulation of mTOR activity (58,59). The expression of high levels of p-AMPK was linked to improved survival in NSCLC patients with adenocarcinoma (60). Consistent with these studies, we found that in both EGFR-wild type and EGFR-mutant lung cancer cells cucurbitacin B caused increase in the expression of p-AMPKα and decrease in the expression of mTORC1 and mTORC2, two biochemically and functionally discrete signaling complexes in NSCLC cells. To ascertain whether these effects are facilitated in part through mTOR signaling, we have shown that there was further downregulation in the phosphorylation of mTOR and AMPKα proteins when cucurbitacin B was added to Compound C-treated cells, suggesting that these effects are in part, due to mTOR signaling. Cucurbitacin B treatment also led to decreased phosphorylation of the downstream targets of mTOR (4EBP1, eIF-4E and p70S6K), signifying that mTOR signaling was targeted by cucurbitacin B.

STAT-3 is implicated in survival, cell cycle progression and metastasis. Activation of STAT-3 in NSCLC has been associated with decreased survival and constitutively activated STAT-3 is increased in lung cancer cell-lines (45,61). We observed that treatment of both EGFR-wild type and EGFR-mutant cells with cucurbitacin B decreased the phosphorylation of STAT-3 dose-dependently.

Sestrins are highly conserved proteins across several species which are encoded by stress-sensitive genes. Sestrins also play an important role in the regulation of mTORC1 signaling, as their ectopic expression leads to inhibition of the phosphorylation of mTORC1 pathway effectors (24). We investigated the effect of cucurbitacin B on the activation of sestrin-3, as there is cross-talk between sestrin-3 and mTORC1/2 and we conducted studies concentrating on the link between sestrin-3 and lung cancer through the mTORC1/2 and AMPK axis. We found that treatment with cucurbitacin B caused specific increase in the protein and mRNA expression of sestrin-3 in EGFR-mutant lung cancer cells, but not in EGFR-wild type cells. Mechanistically, cucurbitacin B activated cell intrinsic inhibitor sestrin-3. These results were further confirmed by using sestrin-3 knockdown in cucurbitacin B-treated cells. Sestrin-3 knockdown significantly reduced the effect of cucurbitacin B on cell-viability in EGFR-mutant lung cancer cells.

The process of apoptosis protects the integrity of multicellular organisms and permits careful elimination of unwanted and damaged cells (62). We examined the effect of cucurbitacin B on the induction of apoptosis in EGFR-wild type and EGFR-mutant lung cancer cells by flow cytometry. We found that there was induction of early and late apoptosis on treatment of EGFR-wild type cells with cucurbitacin B. In sharp contrast, there was no late apoptosis and very modest early apoptosis in EGFR-mutant cells. We silenced sestrin-3 in both EGFR-wild type and EGFR-mutant cells and then treated the cells with cucurbitacin B, to determine if these differential effects of cucurbitacin B on apoptosis are due to sestrin-3. In both EGFR-wild type and EGFR-mutant cells, there was dramatic increase in the induction of early and late apoptosis, signifying the role of sestrin-3 in apoptosis in these cells. It is meaningful to note here that cucurbitacin B acts as an inhibitor of sestrin-3 in EGFR-wild type cells; whereas it is an inducer of sestrin-3 in EGFR-mutant cells, suggesting that sestrein-3 is acting through different mechanisms in EGFR-wild type and EGFR-mutant lung cancer cells. We also observed that treatment with either cucurbitacin B or knockdown of sestrin-3 had no effect on the distribution of cell-cycle in EGFR-wild type lung cancer cells. Though, in EGFR-mutant lung cancer cells, treatment with sestrin-3 siRNA and cucurbitacin B caused more sustained dose-dependent G2- phase cell cycle arrest, suggesting that sestrin-3 might have a role, in part, in these effects. These findings suggest novel mechanism for the action of cucurbitacin B by the modulation of sestrin-3 and suggest that it could be explored further as potential agent for therapy of NSCLC.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

Funding

American Cancer Society (RSG-15-013-01-CNE to N.K.); National Institutes of Health and National Cancer Institute (R01CA160867 and R01CA160867S1 to H.M.).
Conflict of Interest Statement: None declared.

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