Invest New Drugs
DOI 10.1007/s10637-015-0317-4

PRECLINICAL STUDIES

Cytotoxic effects of natural and semisynthetic cucurbitacins on lung cancer cell line A549

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Received: 9 November 2015 / Accepted: 17 December 2015

Summary Cucurbitacins and their derivatives are triterpenoids that are found in various plant families, and are known for their pharmacological and biological activities, including anti-cancer effects. Lung cancer represents a major public health problem, with non-small-cell lung cancer (NSCLC) being the most frequent and aggressive type of lung cancer. The objective of this work was to evaluate four cucurbitacins (CUCs) for their cytotoxic activity, effects on apoptosis induction, cell cycle progression, anti-migratory, and anti-invasive effects on the human NSCLC cell line (A549 cells). Our findings showed that these CUCs could suppress human NSCLC cell growth in vitro through their effects on the PI3Kinase and MAPK pathways, which lead to programmed cell death induction, as well as inhibition of cell migration and cell invasion. Additionally, these effects culminate in apoptosis induction and G2/M cell cycle arrest by modulating cyclin B1 expression, and in the mitigation of strategic steps of lung cancer metastasis, including migration and invasion of A549 cells. These results suggest that two natural (DDCB and CB) and two novel semisynthetic derivatives of cucurbitacin B (ACB and DBCB) could be considered as promising compounds with antitumor potential.

Keywords Cucurbitacins · Apoptosis · Invasion · Metastasis

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Introduction

Lung cancer remains the most common cancer worldwide, with 1.8 million cases diagnosed in 2012, representing 12.9 % of all new cancers. It was also the most common cause of cancer-related deaths, accounting for approximately 19.4 % of all cancer deaths in 2012 [1]. Despite recent advances in medicine, non-small cell lung cancer (NSCLC) continues to be a major health issue, and the search for new safe and effective chemotherapeutic agents has received increasing attention [2].

Approximately 80 % of all pulmonary malignancies are non-small-cell lung cancers. Of these patients, more than 65 % present locally advanced and/or metastatic disease [3]. The basic stages of invasion and metastasis involve the detachment of tumor cells from the extracellular matrix (ECM), invasion of surrounding tissues and basal lamina, intravasation into the blood stream, survival and transport through the blood stream, migration, arrest, and extravasation at a distal site, and the formation of a metastatic lesion. These steps require fundamental mechanisms such as angiogenesis, degradation of matrix barriers, disruption of cell-cell and cell-matrix adhesion, and stimulation of cell motility [4].

The increasing knowledge of cell signaling has led to strategies to target the signaling pathways, which are altered in tumor cells. The mutation or misregulation of multiple key signaling transduction pathways, defects in cell cycle progression, and resistance to apoptosis have frequently been attributed to the uncontrolled proliferation in lung tumor cells,

Published online: 16 January 2016

reinforcing the need to characterize new compounds that can successfully cause the tumor cells to differentiate in a way that induces programmed cell death and inhibits metastasis [5, 6].

Compounds derived from nature have been important sources of new drugs, and also serve as templates for synthetic or semisynthetic modifications. Numerous successful anticancer drugs currently in use are derived from nature, and some of their analogues are under clinical trials [7].

Cucurbitacins, which are widely distributed in plants, belong to a class of triterpenoids that are characterized by a tetracyclic cucurbitane skeleton, with a variety of oxygenation functionalities at different positions [8]. Several plants used in traditional medicine to treat a variety of tumors are rich in cucurbitacins, a fact that has led to several studies on their potential use as anti-cancer agents, as shown in recently published reviews [9, 10]. We recently described new cytotoxic cucurbitacins isolated from *Wilbrandia ebracteata* Cogn. [11], and elucidated the apoptotic mechanism in NSCLC cells for the most active compound [12]. We also described new semisynthetic derivatives of cucurbitacin B [13], for which we investigated the structure-activity relationship (SAR/QSAR) in the cytotoxic activity in a human lung adenocarcinoma cell line (A549) [14]. Subsequently, we elucidated the in vitro mechanism of cell death induced by a new semisynthetic derivative, named DACE, as well as its in vivo effect in a transgenic mouse lung cancer model expressing a mutated and constitutively active c-RAF kinase (c-RAF-1-BxB) [15]. The synergistic effect of DACE and chemotherapy drugs on the human lung adenocarcinoma cell line A549 was also demonstrated recently by our research group [16]. In the present work, we evaluated four cucurbitacins: two natural molecules, the cucurbitacin B and the 22-deoxy-22-hydroxy-25-deacetoxy-cucurbitacin B (named here as CB and DDCB, respectively), and two novel semisynthetic derivatives, the 2, 16-diacetyl-cucurbitacin B and the 2-deoxy-2-bromo-cucurbitacin B (named here as ACB and DBCB, respectively) (Fig. 1) for their cytotoxic activity, effects on apoptosis induction, cell cycle progression, and anti-migratory and anti-invasive effects on non-small cell lung cancer cells (NSCLC, A549 cells).

Material and methods

Compounds

The novel compound 22-deoxy-22-hydroxy-25-deacetoxy-cucurbitacin B (DDCB) and the well-known cucurbitacin B (CB) were isolated from *Wilbrandia ebracteata* Cogn., as previously described by our research group [11]. The new semisynthetic derivatives of cucurbitacin B, the 2,16-diacetyl-cucurbitacin B (ACB) and the 2-deoxy-2-bromo-cucurbitacin B (DBCB) were both obtained as also described by our group in a previous study [13] (Fig. 1). The anticancer drug paclitaxel, purchased from Sigma-Aldrich, St. Louis, MO, USA, was used as positive control.

Cell line

Human NSCLC cells (A549 cells, ATCC: CCL185) were cultured in Minimal Essential Medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS), and maintained at 37 °C in 5 % CO_2.

Cell viability assay

The effects of the treatment with CUCs on the proliferation of A549 cells were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, MO, USA) assay, based on the ability of live cells to cleave the tetrazolium ring to a molecule that absorbs at 540 nm [17]. Briefly, cells were seeded in 96-well culture plates (1×10^4 cells/well) and, after 24 h, were treated with different concentrations of the CUCs; 48 h later the medium was replaced by 50 μL of MTT reagent (1 mg/mL) and the cells were incubated for further 4 h. The viable cell number was correlated with the production of formazan, which was dissolved by dimethyl sulfoxide (DMSO), and optical densities (O.D.) were measured in a Spectra Max M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 540 nm. Afterwards, the inhibitory concentration (IC_{50}) of each compound was calculated as the concentration that inhibited cell proliferation by 50 %, when compared to untreated controls. The DMSO concentration showed no interference with cell growth (data not shown). Paclitaxel (at 0–10 μM) was used as positive control.

Cell cycle analysis by flow cytometry

To determine cell cycle progression, A549 cells (5×10^5) were treated with the CUCs for 24 h, and their effects on the cell cycle were evaluated by propidium iodide staining (PI-Sigma-Aldrich), as previously described [18]. Flow cytometry analyses were performed on a FACS Canto II cytometer (Becton Dickinson, BD, USA), and the events were acquired for each group. The percentages of cells in each phase of the cell cycle were determined using the free software Flowing 2.5 (University of Turku, Finland). At least three independent experiments were performed for each test condition.

Apoptosis analysis by flow cytometry

Apoptotic populations of control (DMSO)-or 12 h CUCs-treated cells were quantified using a dual-staining Annexin V-FITC/PI apoptosis detection kit (Sigma-Aldrich), according to the manufacturer's instructions. Briefly, adherent A549 cells (5×10^5/ six-well) were treated with the CUCs for 12 h.

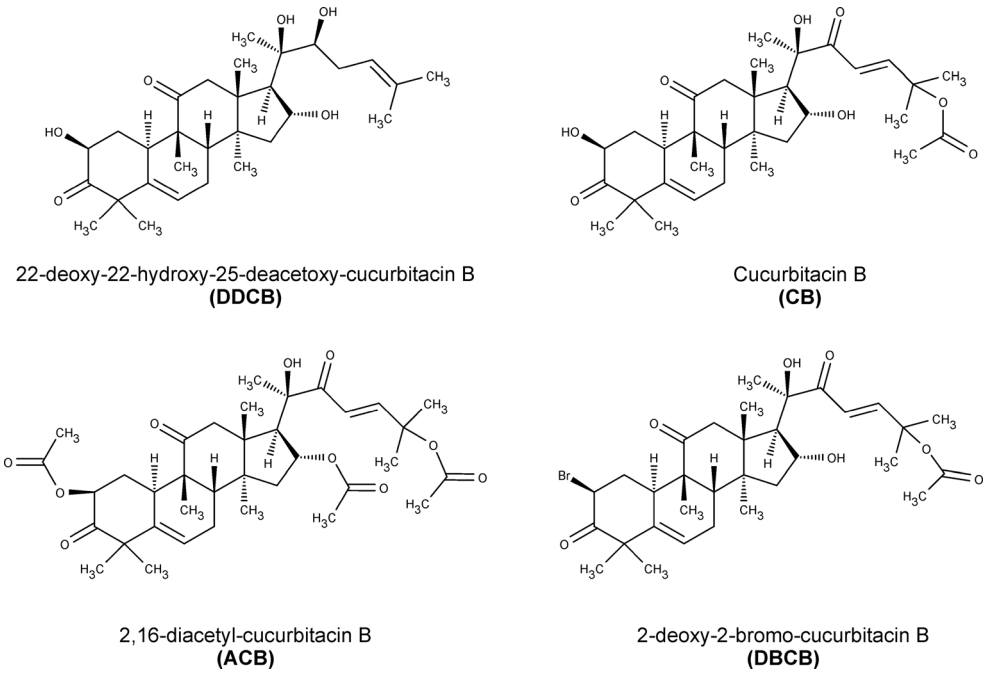

Fig. 1 Chemical structures of the tested cucurbitacins (CUCs)

Cells were harvested and rinsed twice with PBS (pH 7.4), followed by Annexin V-FITC and PI labeling. The stained apoptotic cells were analyzed by flow cytometry as described above.

Caspase-3 assay

Caspase-3 activity was determined using a commercially available kit (Millipore, MA, USA). Briefly, adherent A549 cells (5×10^5/six-well) were treated with different CUCs for 12 h. Cells were harvested and resuspended in 150 μL of chilled cell-lysis buffer, incubated on ice for 10 min, and centrifuged for 5 min (10,000 × *g*). Next, the supernatants (cytosolic extract) were transferred to fresh tubes and put on ice for immediate processing. Cell lysates were tested for caspase activity by adding a labeled caspase substrate (DEVD-pNA), and incubated at 37 °C for 2 h. pNA absorbance was quantified using a Spectra Max M2 (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 405 nm. Fold increase in caspase-3 activity was determined as the ratio between the experimental and the control OD values.

Western blotting analysis

The whole cell lysates containing equal amounts of total proteins were separated on 10 % SDS-PAGE gel and electrotransferred on nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After blocking with 5 % non-fat skimmed milk, the membrane was probed with primary antibodies for phospho-p38 MAPK [Thr180/Tyr182], phospho-NFκB p65 [Ser536], phospho-FAK [Tyr397], FAK, cyclin B1, MMP-2, MMP-9, and E-cadherin (all purchased from Cell Signaling Technology, CST, Danvers, USA). The anti-phospho-JNK/SAPK [Thr183/Tyr185] antibody was obtained from Becton Dickinson (BD, New Jersey, USA), the anti-p38 and IκBα from Santa Cruz Biotechnology (SCB, Dallas, USA), and the phospho-AKT/PKB [pS473] from Invitrogen (Carlsbad, USA). In some assays, loading controls were performed with anti-ERK2 (C-14, SCB), anti-AKT (CST), anti-β-actin or anti-α-tubulin antibodies (both from Sigma-Aldrich). After incubation with the corresponding secondary antibodies conjugated to horseradish peroxidase, protein bands were visualized by using Pierce Enhanced Chemiluminescence ECL substrate (Thermo Scientific, Waltham, USA), according to the protocol. The images were detected acquired using a Bio-Rad ChemiDoc® MP System (Hercules, USA) and digitalized using the Bio-Rad Image Lab® software, version 4.1.

Scratch assay

A549 cells (2×10^5) were seeded into 12-well tissue culture plates with MEM, supplemented with 10 % FBS, and incubated for 24 h. Next, an artificial wound was generated by scratching the confluent cell monolayer with a sterile pipette tip, and fresh medium was added containing different concentrations of the CUCs. Paclitaxel was used as positive control. After 16 h incubation, cells were fixed with 2 % paraformaldehyde (*w*/*v*) for 15 min, stained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min, and photographed in an inverted fluorescence microscope BX-41 (Olympus, Hicksville, NY, USA). Three representative images from each

well of the scratched areas under each treatment were photographed to estimate cell migration. In order to determine the width of the scratch, a zero scratch was fixed immediately after the generation of the artificial wound [19], and the quantification of the percentages of migration and/or proliferation was performed using the free CellC® software [20]. The inhibitions of the cell migration activity by the treatment groups were expressed as percentages of decreasing cells in the wound, relative to control group (0.5 % DMSO). The experiments were performed at least in duplicate.

Invasion assay

The anti-invasive capacity of the different CUCs was evaluated in Transwell® inserts (8 μm pore size polycarbonate membrane, Millipore Corporation, Darmstadt, Germany) coated with Matrigel®. The cells placed in the invasion chambers were rehydrated with MEM, and seeded in 24-well plates. The lower chambers received MEM with no FBS, and were incubated for 24 h to achieve cell confluence. The upper chambers (insert coated with Matrigel®) were then treated with different concentrations of the CUCs and paclitaxel. The basolateral compartments received MEM with 10 % FBS (used here as a chemoattractant) and were incubated for 48 h. Next, non-invading cells were removed with a cotton swab, and cells that invaded the membrane but did not reach the lower compartments were trypsinized, fixed with paraformaldehyde, and stained with DAPI. Images were obtained in an inverted fluorescence microscope BX-41 (Olympus), and the quantification of cell invasion was performed using the free CellC® software [20].

Statistical analysis

The results were expressed as mean ± SD of three or two independent experiments. Statistical analyses were performed by one-way ANOVA followed by a post-hoc test. For determination of IC_{50} values and their 95 % Confidence Intervals (CI), data sets from MTT experiments were analyzed by nonlinear regression and calculated using log (compound) compared with normalized response (variable slope), by GraphPad Prism software.

Results

CUCs inhibit A549 cells growth in vitro

To evaluate the cytotoxic effects of CUCs on A549 cells, they were initially treated with different concentrations of these compounds for 48 and 72 h, and stained with MTT. The IC_{50} values obtained are shown in Table 1. The compounds inhibited A549 cells proliferation, with IC_{50} values ranging from 0.13 to 14.65 μM and 0.04 to 4.93 μM, after 48 h and 72 h of treatment, respectively. The compounds DDCB and ACB were less cytotoxic, showing IC_{50} values up to 10 μM and 2 μM, after 48 h and 72 h of exposure, respectively. On the other hand, CB and DBCB appeared to be the most cytotoxic compounds, with IC_{50} values comparable to those of the anti-cancer drug paclitaxel.

CUCs lead to cell cycle arrest, decreased levels of cyclin B1 protein and increased apoptosis in A549 cells

To better understand the mechanism by which cell proliferation was suppressed by CUCs, we investigated the effects of these compounds on cell cycle distribution of the treated cell population by fluorescence-activated cell sorting (FACS) analysis. The results are summarized in Fig. 2a. The exposure of A549 cells for 24 h to CB and DBCB caused a 2-fold enrichment of cells in the G2/M phase. Similarly, the exposure of A549 cells to 20 μM of DDCB and ACB also resulted in cell accumulation in the same phase. The G2/M phase enrichment was accompanied by a reduction in G1 phase cells, when compared to the untreated control (*$p<0.05$ and **$p<0.01$ vs. the respective control). In accordance with these data, the expression of cyclin B1, an important cell cycle regulator [21], was reduced, especially after exposure to CB, ACB and DBCB (Fig. 2b). Also, the increase of cells in sub-G1 fraction indicated DNA fragmentation and apoptosis. The induction of cell apoptosis was confirmed by Annexin V-FITC staining. As can be seen in Fig. 2c, exposure of A549 cells to DDCB for 12 h increased the percentages of apoptotic cells from 5.18 ± 0.58 % in the untreated controls to 22.15 ± 1.58 % ($p<0.001$ vs. control) and to 47.40 ± 0.48 % ($p<0.0001$ vs. control) in cells treated with 10 and 20 μM of DDCB, respectively. Similar effects were observed for exposure to CB, ACB and DBCB. These results indicate that these compounds inhibit the growth of A549 cells by arresting them in the G2/M cell cycle phase. Thus, treatment of A549 cells with all CUCs not only induced these effects, but also triggered apoptosis.

CUCs induce apoptosis via activated caspase-dependent pathway

Caspase-3 is a well-known executor of the apoptotic pathway, through its ability to cleave several cellular substrates. To evaluate the effects of CUCs on the activation of caspase-3 in A549 cells, the cytosolic extracts of treated and untreated cells were incubated with a caspase3-specific substrate. As

Table 1 Inhibitory effects of cucurbitacins on A549 cell proliferation

CUCs	48 h		72 h		Increase in cytotoxicity
	IC_{50}[a]	95 % confidence interval	IC_{50}	95 % confidence interval	IC_{50} 48 h/IC_{50} 72 h
DDCB	11.41	9.01–14.46	4.93	4.02–6.05	2.3
CB	0.13	0.09–0.19	0.04	0.03–0.07	3.3
ACB	14.65	10.90–19.70	2.64	1.89–3.70	5.5
DBCB	1.33	0.452–3.94	0.12	0.07–0.20	11.1
Paclitaxel	0.211	0.102–0.298	0.188	0.093–0.275	1.12

shown in Fig. 2d, 12 h treatment of A549 cells with the higher concentrations of DDCB, ACB and DBCB, and at both tested concentrations of CB, caused a significant increase in proteolytic activity of caspase-3.

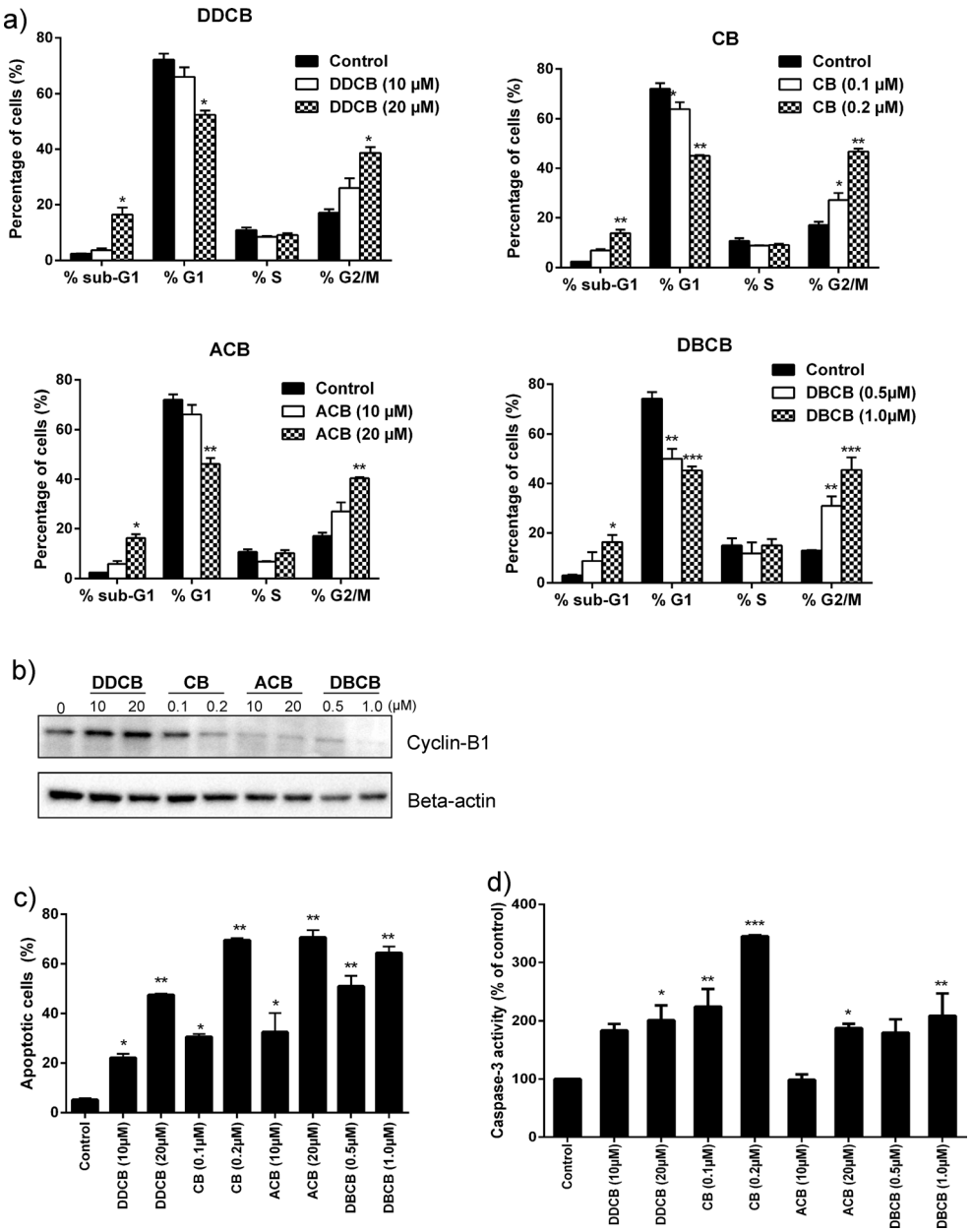

Fig. 2 Effects of CUCs on cell cycle arrest and apoptosis. **a** A549 cells were treated with CUCs and analyzed 24 h later by flow cytometry. The values indicate the percentages of A549 cells in the indicated phases of the cell cycle (sub-G0/G1, G0/G1, S and G2/M). *$p < 0.05$, **$p < 0.01$ and *** $p < 0.001$ as compared with control. **b** A549 cells were treated with CUCs for 12 h, then subjected to Western blotting using Cyclin B1 antibody as indicated. Equal protein loading was confirmed by probing for beta-actin. Representative images of three independent repeated experiments are shown. **c** A549 cells were treated for 12 h with CUCs, stained with Annexin V/PI, and submitted to flow cytometry for analysis of the apoptotic cell proportion. *$p < 0.001$ and **$p < 0.0001$ as compared with control. **d** A549 cells were treated for 12 h with CUCs and their cytosolic fractions were analyzed for changes in caspase-3 activity. *$p < 0.05$, **$p < 0.01$ and *** $p < 0.001$ as compared with the control. Data represent the mean ± standard deviation of three independent experiments

CUCs reduce cell migration of A549 cells

To determine the growth inhibitory effects of CUCs on A549 cell migration, these cells were exposed to different concentrations of the compounds. The highest tested concentration of all CUCs did not affect the growth of cells for 16 h (data not shown). As can be seen in Fig. 3a, significant differences ($p < 0.0001$) were observed when all treated groups were compared to the untreated control (DMSO 0.5 %). The percentages of inhibition caused by CUCs were similar to that of paclitaxel, but significant statistical differences were detected only for DDCB at 15 μM ($p < 0.0001$), DDCB at 30 μM ($p < 0.05$), and ACB at 15 μM ($p < 0.001$), when compared to this drug at 1 μM. It is also important to point out that paclitaxel, DDCB, and ACB presented percentages of concentration-dependent decrease in cells in the wound.

CUCs reduce cell invasion of A549 cells

The suppression of A549 cell invasion by CUCs was assessed by Transwell® coated with Matrigel® As shown in Fig. 3b, after 48 h, most of the CUCs were able to reduce more than 90 % of cell invasion in this model, except for ACB at 15 μM, which was able to reduce cell invasion by approximately 83.2 ± 13.4 %. These results are similar to those of paclitaxel, which showed inhibition of 92.6 ± 11.3 % at 0.1 μM and 88.1 ± 10.6 % at 1 μM. On the other hand, DDCB at 15 μM increased cell invasion by 18.2 ± 12.73 %.

CUCs down regulate phospho-FAK, MMP-2 and MMP-9 in A549 cells

The expression of key regulators of cell migration and invasion, such as matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9), phospho-FAK, and E-cadherin, was verified by Western blotting. The tested CUCs decreased the levels of MMP-2 and MMP-9 on A549 cells, especially at the highest tested concentration of each compound after 48 h of treatment (Fig. 3c). FAK is a cytoplasmic nonreceptor tyrosine kinase stimulated by growth factor receptors or integrins in several types of human cancers [22]. The overexpression and phosphorylation of FAK are common in primary and metastatic cancers and correlated with cell migration, invasion and metastasis, emphasizing FAK as a potential determinant of tumor

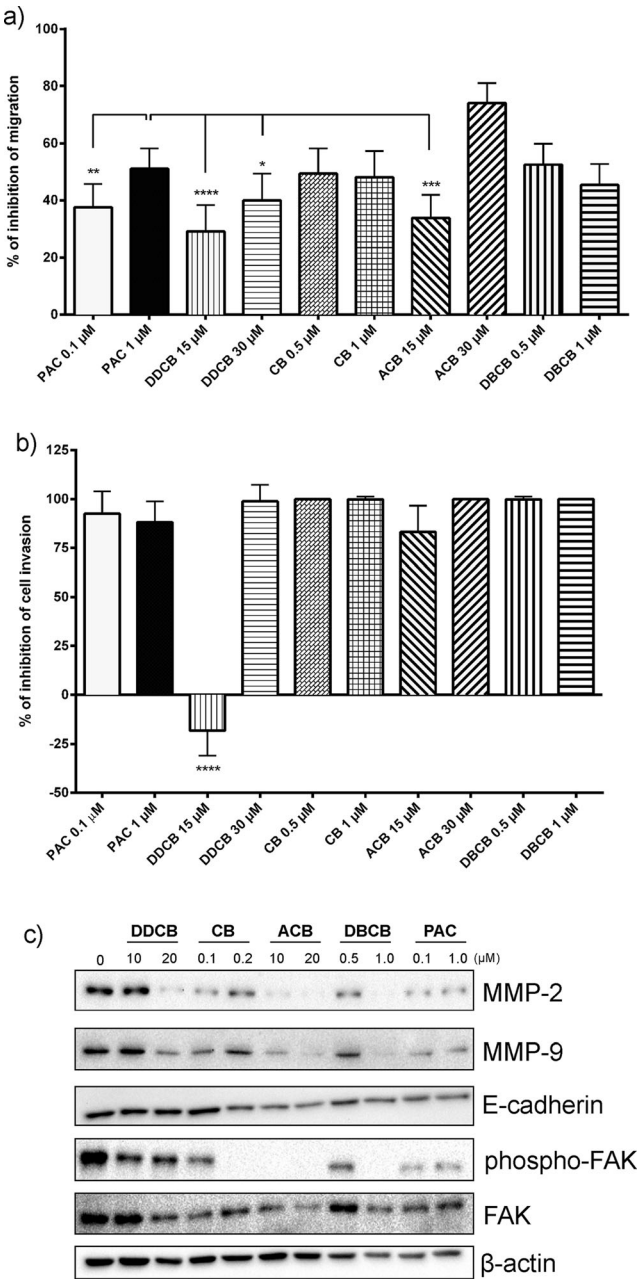

Fig. 3 Effects of CUCs on A549 cell migration and invasion. **a** Inhibitory effects of CUCs and paclitaxel on A549 cell migration after 16 h of incubation. Data are expressed as percentages of cells in the wounded area, relative to control (0.5 % DMSO). Data represent the mean ± SD of two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ indicate statistically significant differences when compared to paclitaxel 1 μM. **b** Inhibitory effects of CUCs and paclitaxel on A549 cell invasion using the Transwell® assay after 48 h of incubation. A549 cells that penetrated through the Matrigel® layer and invaded the lower chambers were stained and photographed under a fluorescent microscope. The data represent the percentages of cells that have invaded the inserts, relative to control (0.5 % DMSO). Data represent the mean ± SD of two independent experiments. **** $p < 0.0001$ indicates statistically significant differences when compared to paclitaxel 1 μM. **c** Effects of CUCs and paclitaxel treatments on MMP-2, MMP-9, E-cadherin and FAK proteins expression. A549 cells were treated with the compounds for 48 h and the samples were assayed by Western blotting. Equal protein loading was confirmed by probing for beta-actin. The most representative images of three independently repeated experiments are shown

development and metastasis [23]. In this study, the phosphorylation of FAK was down-regulated by CB, ACB and DBCB, but not by DDCB. In contrast, the CUCs did not reduce the expression of E-cadherin, an important cell adhesion molecule frequently lost during tumor progression in most cancers. These findings suggest that the inhibition of cell invasion and migration by CUCs could be mediated by a reduction of the proteolytic action of MMPs and the phosphorylation of FAK.

Effects of CUCs on oncogenic signaling in A549 cells

To determine whether or not the PI3K/AKT and MAPK/ERK signaling pathways are functionally involved in the apoptotic and anti-invasive effects of CUCs, A549 cells were treated with these compounds for 24 h, followed by stimulation with TNFα (30 ng/mL) for additional 15 min. TNF activates a broad spectrum of different signaling cascades, thus allowing narrowing down the affected pathways in the same experimental setting. As shown in Fig. 4, the TNFα-mediated activation/phosphorylation of AKT was strongly inhibited when cells were treated with all CUCs. The phosphorylation of ERK was also altered when cells were treated with CB, ACB and DBCB but not with DDCB. The p38 MAP kinase was also down-regulated after 24 h of exposure at the highest tested concentrations of ACB and DBCB. In contrast, the activation state of other key signaling proteins after TNF stimulation, such as JNK and NFκB-p65 as well as the degradation of IκBα, was not significantly altered by treatment with these compounds.

Discussion

Despite the great progress in cancer therapies, the resistance and the absence of effective treatments for some types of lung cancer, especially NSCLC, have led to abundant research efforts to investigate new anti-cancer drugs derived from natural sources. The cucurbitacin family has been a valuable target in this field, revealing a wide array of in vitro and in vivo pharmacological effects, including anti-tumor activity [8, 9, 24, 25] for NSCLC [14, 15, 26–30].

Here, we demonstrate that two natural (DDCB and CB) and two novel semisynthetic derivatives of cucurbitacin B (ACB and DBCB) are potent suppressors of human NSCLC cell growth in vitro through their inhibiting effects on the PI3 kinase and MAPK pathways, which leads to programmed cell death induction, and inhibition of cell migration and cell invasion.

MTT analyses showed significant concentration- and time-dependent inhibition of A549 cell viability in vitro (Table 1). CB and DBCB appeared to be more cytotoxic than DDCB and ACB, with IC_{50} values comparable to those of paclitaxel, which was used as positive control. It is well known that several compounds with anticancer properties exhibit their inhibitory effects on tumor cells by inducing cell cycle arrest and apoptosis. In order to better describe the mechanism of cell death induced by CUCs on A549 cells, a panel of assays on cell cycle distribution and apoptosis detection by flow cytometry was performed. Here A549 cells treated with all CUCs were arrested at the G2/M phase, resulting in a decrease in cell population in G0/G1, particularly when the cells were treated with both tested concentrations of CB and DBCB, and the highest tested concentrations of DDCB and ACB (Fig. 2a). This suggests that these compounds inhibit cell proliferation via blocking the cell cycle at the G2/M phase, similarly to Paclitaxel. Paclitaxel is a member of the taxane class of anticancer agents, which has a well-known mechanism that blocks cell cycle at G2/M phase as we could also demonstrate in our previous study in A549 cells [16]. To confirm this hypothesis, we analyzed the expression content of cyclin B1, which is known as a key molecule involved in cell cycle arrest. The switch from one cell cycle phase to another is

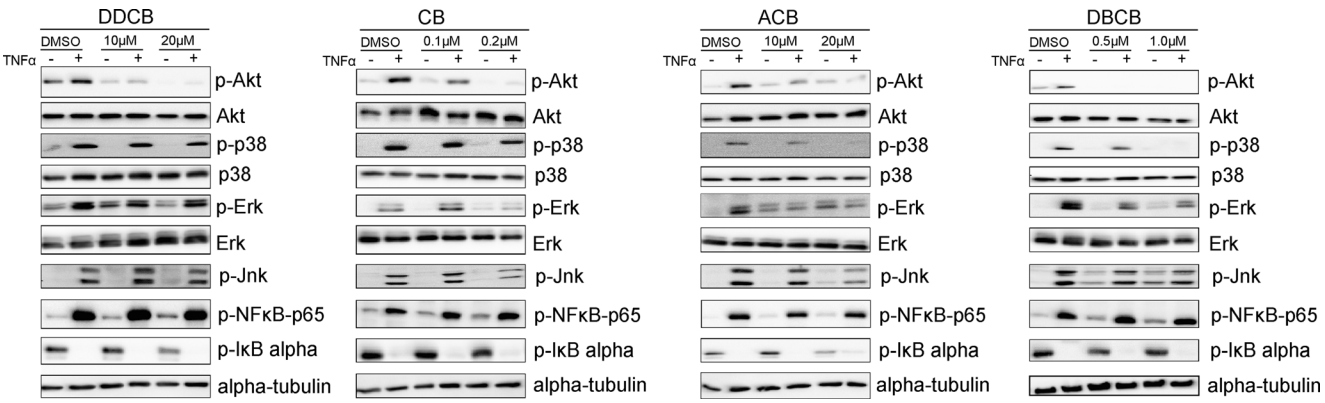

Fig. 4 Effects of CUCs on TNFα-mediated activation of signaling pathways. A549 cells were exposed to different concentrations of each CUC for 24 h, stimulated or not for additional 15 min with 30 ng/mL TNFα and analyzed by Western blotting. Equal protein loading was confirmed by probing for tubulin. The most representative images of three independently repeated experiments are shown

regulated by different cellular proteins, such as cyclin B1, which is required for the progression of the cells into and out of M phase of the cell cycle. For several drugs, the anti-cancer effects are intermediated by cell cycle arrest and involve modulation of the cyclins and cyclin-dependent kinases that control cell cycle progression [21]. In fact, the cyclin B1 protein was decreased in A549 cells treated with CB, ACB and DBCB (Fig. 3c), suggesting that cell cycle arrest was at least partially mediated via suppression of cyclin B1. These findings are consistent with recent reports, which confirm that cucurbitacins induce G2/M arrest and apoptosis in other human cancer cell lines in vitro [15, 26, 28, 31].

The MAPK and PI3K/AKT pathways control many cellular processes including cell proliferation, apoptosis, metastasis, and tumorigenesis [32, 33]. Given the cytotoxic effects of CUCs on A549 cells, we further investigated whether they actually modulate the status of AKT, ERK, JNK, p38 and NFκB-p65 in this cell line. As shown in Fig. 4, the phosphorylation of AKT was strongly inhibited when cells were treated with all CUCs. The phosphorylation of ERK was also altered when cells were treated with CB, ACB and DBCB but not with DDCB. The activity of p38 MAPK was also down-regulated after 24 h exposure to the highest tested concentrations of ACB and DBCB. In contrast, these compounds did not appear to dramatically alter the activity levels of other key signaling proteins, such as JNK and NFκB-p65/IκBα, after TNF stimulation. Evidence indicates that the MAPK pathway is closely connected to the PI3K/AKT pathway [34]. In fact, RAS controls stimulation of both kinase pathways, prompting the phosphorylation of many downstream targets. ERK activation leads to the induction of genes, such as the coding genes for caspase-9 and cyclin B1, regulating cell growth and survival. Moreover, activated AKT induces its anti-apoptotic effect by preventing the release of cytochrome *c*, impeding the start of the G2/M checkpoint, and directly deactivating pro-apoptotic elements such as Bad and procaspase-9 [35]. This evidence may explain, at least in part, the apoptotic effects induced by tested CUCs on A549 cells, as well as their ability to activate caspase-3 (Fig. 2d), the major effector caspase in programmed cell death [36].

The formation of tumor metastasis involves an elaborate multistep cascade, including cell adhesion, migration and proteolysis of the ECM by enzymes called MMPs, which are closely related to invasion and metastasis of numerous tumor cells. Thus, inhibition of these steps is a useful approach to antitumor management [37, 38]. The in vitro Transwell® assay uses the Matrigel® layer to simulate the ECM, enabling degradation of the cell invasive matrix, similarly to what occurs in vivo [39]. Here, the tested CUCs were able to reduce more than 90 % of cell invasion, exceptionally ACB at 15 μM, which was able to reduce cell invasion by approximately 80 %. These results are similar to those obtained with the anti-cancer drug paclitaxel, which was used as positive control. Interestingly, DDCB at 15 μM increased cell invasion by almost 20 % (Fig. 2b). Other studies describe the ability of some cucurbitacins to suppress cell migration and invasion, significantly reducing the metastatic potential during cancer development [40, 41]. Recent reports have also suggested that the inhibition of focal-adhesion kinase (FAK) down-regulated MMP-2 and MMP-9 expression, and it has been demonstrated that there is a positive correlation between the levels of MMPs and tumor cell migration and invasion [42, 43]. It is also known that MAPK (ERK1/2, p38 and JNK) and PI3K/AKT pathways are involved in MMP-9 expression in different cell types [44, 45]. To investigate whether CUCs suppress MMPs signaling pathway in tumor metastasis, we tested the activation of proteins involved by Western blotting. Our data showed that CB, ACB and DBCB strongly inhibited the phosphorylation of FAK in a concentration-dependent manner (Fig. 3c), but it was only slightly inhibited by DDCB. Additionally, the results showed that MMP-2 and MMP-9 expression in A549 cells decreased after treatment with CUCs, especially after exposure to the highest concentrations of each compound, corroborating the results of the Transwell® assays.

Conclusion

In conclusion, we demonstrate, for the first time, that these CUCs can suppress human NSCLC cell growth in vitro through their effects on the PI3 Kinase and MAPK pathways, which lead to apoptosis induction. They can also inhibit A549 cell migration and invasion. These results suggest that two natural (DDCB and CB) and two novel semisynthetic derivatives of cucurbitacin B (ACB and DBCB) are promising compounds with antitumor potential.

Acknowledgments We acknowledge the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, MCTI, Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, MEC, Brazil), Fundação de Apoio à Pesquisa e Inovação do Estado de Santa Catarina (FAPESC, State of Santa Catarina, Brazil), Consejo Nacional de Investigaciones Científicas y Tecnicas (CONICET, Argentina), Agencia Nacional de Promocion Cientifica y Tecnologica (ANPCyT, Argentina) and UBA (Universidad de Buenos Aires, Argentina) for the financial support (grant numbers 472979/2011-6 and 2671/2012-9 from CNPq and PRONEX/FAPESC, respectively) as well as for our research fellowships.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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