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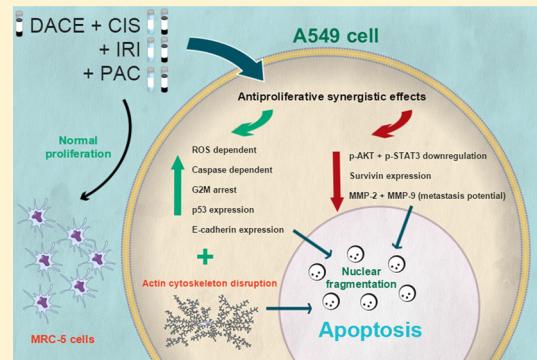
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ABSTRACT: Nonsmall cell lung cancer (NSCLC) represents an important cause of mortality worldwide due to its aggressiveness and growing resistance to currently available therapy. Cucurbitacins have emerged as novel potential anticancer agents showing strong antiproliferative effects and can be promising candidates for combined treatments with clinically used anticancer agents. This study investigates the synergistic antiproliferative effects of a new semisynthetic derivative of cucurbitacin B (DACE) with three chemotherapy drugs: cisplatin (CIS), irinotecan (IRI), and paclitaxel (PAC) on A549 cells. The most effective combinations were selected for studies of the mechanism of action. Using an *in silico* tool, DACE seems to act by a different mechanism of action when compared with that of different classes of drugs already used in clinical settings. DACE also showed potent synergic effects with drugs, and the most potent combinations induced G2/M cell cycle arrest by modulating survivin and p53 expression, disruption of F-actin cytoskeleton, and cell death by apoptosis. These treatments completely inhibited the clonogenic potential and did not reduce the proliferation of nontumoral lung cells (MRC-5). DACE also showed relevant antimigratory and anti-invasive effects, and combined treatments modulated cell migration signaling pathways evolved with metastasis progression. The effects of DACE associated with drugs was potentiated by the oxidant agent L-buthionine-sulfoximine (BSO), and attenuated by *N*-acetylcysteine (NAC), an antioxidant agent. The antiproliferative effects induced by combined treatments were attenuated by a pan-caspase inhibitor, indicating that the effects of these treatments are dependent on caspase activity. Our data highlight the therapeutic potential of DACE used in combination with known chemotherapy drugs and offer important insights for the development of more effective and selective therapies against lung cancer.

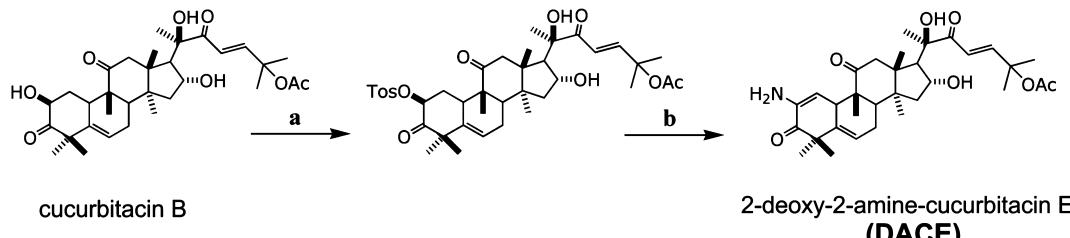


INTRODUCTION

Among the different types of cancer, lung cancer is a leading cause of cancer-related mortality worldwide, with a poor 5-year survival rate. Nonsmall cell lung cancer (NSCLC) is the cause of nearly 85% of all lung cancers, comprising squamous cell carcinoma, large cell carcinoma, adenocarcinoma, and bronchioloalveolar carcinoma. In recent years, there has been important progress in the understanding of the biological characteristics of NSCLC, and these studies support the development of new therapeutic strategies.¹ A review of cancer statistics estimated that lung cancer will be the leading cause of new diagnostics and cancer-related mortality in 2015.² Therefore, many patients present recurrent disease due to drug resistance,³ which highlights the importance of new anticancer agents that can replace and/or supplement the therapeutic options currently available.

Cucurbitacins are a group of tetracyclic triterpenoid molecules, which showed potent antiproliferative activity against many different human cancer cell lines. For this reason, there is growing interest in their use as new anticancer agents. Cucurbitacin B is one of the most abundant and widely studied members of this group.^{4,5} Recently, a novel cucurbitacin B semisynthetic derivative (DACE) (Figure 1) was investigated by our research group and showed potent antiproliferative activity against nonsmall cell lung cancer *in vitro* and *in vivo* a mouse model of lung cancer.⁶ Other cucurbitacins with potential antiproliferative effects have also been described by our research group.^{6–10} As described by Silva et al.,⁶ DACE presents advantages from the chemical synthesis perspective when compared with the precursor cucurbitacin B. The

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Reagents and conditions: a) 4-toluenesulfonyl chloride, DABCO, DCM, 0°C; b) NaN₃, DMF, 70°C

Figure 1. Reagents, conditions, and synthesis of DACE.⁶ Semisynthesis process of a new derivative compound of cucurbitacin B (DACE). DACE was obtained after the conversion of the precursor cucurbitacin B into a tosylated intermediate by reaction with *p*-toluenesulfonyl chloride and DABCO in dichloromethane. The next procedure to obtain the DACE was the nucleophilic substitution with NaN₃ in dimethylformamide. This reaction scheme has been described and published recently by our colleagues and collaborators of this research.⁶

semisynthesis process is simple, short, and might represent a new route to generate new anticancer compounds. In addition, the *in vitro* and *in vivo* results obtained with DACE⁶ validated the pharmacophore model of a previous QSAR study published by our research group.⁹

The potential synergism between new compounds and chemotherapy drugs represents an interesting strategy for the treatment of different types of cancer. The combination of antiproliferative agents can potentiate the therapeutic effects, reduce the dose, and consequently, the toxicity, and minimize or delay cases of drug resistance. These effects are primarily obtained when drugs with different mechanisms of action are combined.^{11–21} For example, the synergistic effects of cucurbitacin B with chemotherapeutics agents for breast cancer^{18,20} and medulloblastoma,¹⁹ among other various tumor types,^{11–14} have been described.

In this study, we investigate the antiproliferative effect of DACE in different cancer cell lines. After this screening, A549 cells were selected to investigate the synergistic antiproliferative effects of DACE combined with CIS, IRI, or PAC on the nonsmall cell lung cancer cell line (A549). The possible mechanism of action suggested for the most effective synergic combinations was also investigated, using a systematic collection of assays.

MATERIALS AND METHODS

Cell Lines. Human nonsmall-cell lung cancer (A549), human rhabdomyosarcoma cells (RD), and prostate cancer cells (LNCap) were obtained from the American Type Culture Collection (Manassas, VA, EUA). Human fetal lung fibroblast cells (MRC-5) were obtained from European Collection of Cell Cultures (Porton Down, WI, England). Human ileocecal adenocarcinoma cells (HCT8) were obtained from Adolfo Lutz Institute (São Paulo, SP, Brazil). A549 and RD cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum. LNCap and HCT8 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. MRC-5 cells were cultured in MEM supplemented with 10% fetal bovine serum, 1 mM of glutamine, and 1% of nonessential amino acids. Both cell lines were cultured in a humidified incubator with 5% CO₂ atmosphere at 37 °C and were routinely screened for the presence of potential contaminants, including mycoplasma.

Drugs and Inhibitors. DACE was synthesized from cucurbitacin B.⁶ CIS, IRI, PAC, and camptotecin were purchased from Sigma-Aldrich (St. Louis, MO, USA). DACE and drugs were diluted in DMSO, stored at -20 °C, and prepared extemporarily in MEM.

Cell Proliferation Assay. The antiproliferative effects were determined by the sulforhodamine B assay, based on the measurement of cellular protein content as previously described.²² Cells were treated with different concentrations of DACE, CIS, IRI, and PAC, for 48 h. The inhibitory concentration (IC₅₀) was defined as the concentration

that inhibited cell proliferation by 50% when compared to that of the untreated controls.

ChemGPS-NP *in Silico* Analyses. A PCA-based model called ChemGPS-NP (<http://chemgps.bmc.uu.se>) has recently been shown to be useful for differentiating biological activities and mapping chemical compounds for the prediction of anticancer mode of action.^{23,24} It is basically a tool for navigation in the biologically relevant chemical space. Compounds with unknown modes of action are positioned on this “map” using the projections of eight principal components (PC; dimensions) derived from 35 molecular descriptors of physicochemical properties of a reference set of compounds with known modes of action. The resulting cluster pattern can provide valuable information on the structural and biological properties of the compounds.

In our study, descriptors were calculated for DACE using the software DRAGON Professional (Taleta srl, Italy), based on structure information provided as simplified molecular-input line-entry specification (SMILES). The compound was then mapped onto ChemGPS-NP together with the NCI (National Cancer Institute) reference set of known anticancer agents (http://dtp.nci.nih.gov/docs/cancer/searches/standard_mechanism.html). Compounds in the reference anticancer data sets were classified according to the following mode of action classes: DNA/RNA antimetabolites, alkylating agents, topoisomerase inhibitors, and tubulin agents. Principal component analysis and PCA score prediction were performed using SIMCA P+ 11.5 software (Umetrics AB, Malmo, Sweden). All data were centered and scaled to unit variance.

Synergism Analyses. A549 cells were treated with each drug alone or in combination with DACE, at fixed ratios, equivalent to the respective IC₅₀ values (i.e. at IC₅₀ × 0.25, × 0.5, × 1, × 2, and × 4) for 48 h. Cell proliferation was then determined by the sulforhodamine B assay, as previously described.²² The degrees of interaction between DACE and CIS, IRI, or PAC were calculated through the combination index (CI) equation, based on the median-effect principle of the mass-action law, using the software CalcuSyn (version 2.1, Biosoft). According to the CI theorem, CI values <1, = 1, and >1 indicate synergism, additive effect, and antagonism, as described.²⁵

Influence of Intracellular Reactive Oxygen Species on Cell Proliferation. The influence of ROS (reactive oxygen species) on the antiproliferative effects induced by different combinations of drugs and DACE was investigated by the sulforhodamine B assay, in the presence of *N*-acetylcysteine (NAC) (Sigma, St. Louis, USA) or *L*-buthionine-sulfoximine (BSO) (Sigma, St. Louis, USA), antioxidant and oxidant agents, respectively. Briefly, cells were treated with different concentrations of DACE, CIS, IRI, and PAC, alone and their combinations, in the absence and presence of NAC (500 μM) or BSO (10 μM), for 48 h. Next, cell proliferation was determined as described for the standard sulforhodamine B assay.²²

Clonogenic Assay. This assay was performed according to the protocol previously described.²⁶ A549 cells (5 × 10² cells/well) were seeded in six well plates and incubated at 37 °C for 24 h. Thereafter, the treatments with both DACE and their combinations with drugs were performed for 48 h. The medium was removed, and each well

received fresh medium supplemented with 10% FBS for 10 days. After this period, the colonies were stained with crystal violet and counted using a stereomicroscope (Olympus Company, Center Valley, USA).

Cell Cycle Analyses by Flow Cytometry. A549 cells were treated with each drug alone or in combination with DACE, for 48 h, and their effects on the cell cycle were evaluated by propidium iodide staining, as previously described.²⁷ Flow cytometry analyses were performed on a FACS Canto II cytometer (BD Becton Dickinson GmbH, Heidelberg, Germany), and the events were acquired for each group. The percentages of cells in each phase of the cell cycle were determined using the software Flowing 2.5.0 (University of Turku, Finland).

Apoptosis Analyses by Flow Cytometry. A549 cells were treated with each drug alone or in combination with DACE, for 24 h, and then subjected to the Annexin V-FITC Apoptosis Detection Kit (Sigma, St. Louis, USA). Data were obtained with a FACS Canto II cytometer, and analyses were performed using Flowing 2.5.0 software. Camptotecin (10 μ M) (Sigma, St. Louis, USA) was used as the positive control for apoptosis, and two freezing (-80° C)/defrosting (56° C) cycles were used as the necrosis control.

Influence of Caspase Inhibition on Cell Proliferation. The influence of caspase inhibition on the effects of DACE and their combinations with drugs was evaluated by a sulforhodamine B assay. The cells were previously treated for 1 h with a Z-VAD-FMK pan-caspase inhibitor (50 μ M) (Merck Millipore, Billerica, MA, EUA) and with different concentrations of DACE, CIS, IRI, and PAC, alone and their combinations, in the absence and presence of a Z-VAD-FMK pan-caspase inhibitor (50 μ M). Next, cell proliferation was determined as described for the sulforhodamine B assay.²²

Cytoskeletal and Nuclei Staining. A549 cells were plated in eight-well slide chambers and treated with each drug alone or in combination, for 24 h. After treatment, cells were incubated with TRITC-labeled-phalloidin (Invitrogen, Carlsbad, USA) for F-actin staining and with Hoechst staining (Invitrogen, Carlsbad, USA) to detect nuclei. Fluorescence microscopic images were obtained with a BX-41 microscope (Olympus Company, Center Valley, USA).

Scratch Assay. A549 cell monolayers were scraped in a straight line to create a scratch with a sterilized pipet tip. Scratched monolayers were then treated with DACE at 0.5 and 1.0 μ M, and 0.5% DMSO and PAC (at 0.1 and 1 μ M) as controls, and incubated for 16 h. Images were obtained with a BX-41 fluorescence microscope, and cell migration inhibition quantification was performed using the CellC software (Cell C, Berlin, Germany), as previously described.²⁸

Invasion Assay. The anti-invasive capacity was evaluated by cell migration through Transwell inserts (8 μ m pore size polycarbonate membrane Millipore Corporation, Darmstadt, Germany) coated with Matrigel. A549 cells were seeded in the apical compartment, and the basolateral chamber received serum-free MEM. Afterward, the upper chambers were treated with DACE (at 0.5 and 1 μ M), DMSO (0.5%), and PAC (at 0.1 and 1 μ M) as controls. The basolateral compartment received MEM containing 10% FBS. The experiment was incubated for 48 h. Finally, cells were fixed with paraformaldehyde and stained with DAPI for 15 min. Images were obtained with a BX-41 fluorescence microscope, and cell invasion inhibition quantification was performed using the CellC software as previously described.²⁸

Western Blotting. A549 cells were treated with each drug alone or in combination with DACE, for 48 h. Cell lysates were obtained after lysis with RIPA buffer containing proteinases and phosphatases inhibitors (25 mM Tris-HCl buffer (pH 8.0), 137 mM NaCl, 10% glycerol, 0.1% SDS, 0.5% DOC, 1% NP40, 2 mM EDTA (pH 8.0), 5 mg/mL leupeptin, 5 mg/mL aprotinin, 0.2 mM pepstatin, 1 mM sodium vanadate, and 5 mM benzamidine) (Sigma, St. Louis, USA), cleared by centrifugation, and equal amounts of total protein lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on nitrocellulose membranes (Millipore Corporation, Darmstadt, Germany). After blocking with 5% milk solution, the membranes were incubated overnight with the following primary antibodies: phospho-cofilin1; p53; phospho-FAK; survivin; E-cadherin; MMP2; MMP9; phospho-AKT; and phospho-STAT3, all purchased from Cell Signaling Technology

(Danvers, USA). After incubation with the corresponding secondary antibodies conjugated to horseradish peroxidase, protein bands were revealed by Pierce ECL substrate (Thermo Scientific, San Jose, USA), according to the manufacturer's instructions. Gel images were obtained with the Molecular Imager Gel Doc XR System (Bio-Rad, California, USA). Beta-actin antibody (Sigma-Aldrich, St. Louis, MO, USA) was used as loading control. The total band densities were measured against the local background and normalized to the density of the appropriate Beta-actin loading control bands.

Statistical Analyses. The results were expressed as the mean \pm SD of three independent experiments. GraphPad Prism 5 Software (GraphPad, San Diego, USA) was used to calculate the IC₅₀ values and their 95% confidence intervals through a nonlinear fit-curve (log of compound concentration versus normalized response-variable slope). Statistical analyses were performed by one-way analysis of variance (ANOVA followed by Tukey's posthoc test). *P* values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

DACE is a novel molecule, and recently, its mechanism of action was reported for the first time by our colleagues.⁶ Since the antiproliferative effects of DACE were described only in A549 cells, we decided to screen it against four different tumor cell lines from distinct origins. Initially, we explored the effects of DACE on the proliferation of different cancer cell lines, using the sulforhodamine B assay. As shown in Table 1, DACE

Table 1. Cell Proliferation Inhibition of DACE on Different Cancer Cell Lines

cell line	IC ₅₀ (μ M) ^a	95% confidence interval
A549	0.24	0.16–0.31
LNCaP	0.36	0.29–0.43
RD	1.2	0.90–1.58
HCT-8	2.1	1.27–3.62

^aIC₅₀: Concentration that inhibited cell proliferation by 50% when compared to that of untreated cells.

showed antiproliferative effects against four different cancer cell lines. According to the ATCC cell bank, these cells differ in origin and tissue disease, morphological characteristics, gene expression, and specific receptors. Moreover, our colleagues⁶ also described that the treatment with DACE showed potential antiproliferative effects on A549 cells with overexpression of AKT, EGFR, v-RAF, and k-RAS genes. Taken together, these data indicate that the treatment with DACE is not specific for the A549 cell line. Since DACE was more effective in inhibiting A549 cells proliferation, we decided to study the synergism of this compound combined with drugs in these cells. As shown in Table 2, DACE and drugs demonstrated potent antiproliferative effects.

Most protocols applied to the treatment of cancer include combination regimens of drugs with different mechanisms of

Table 2. Cell Proliferation Inhibition of DACE and Chemotherapy Drugs on A549 Cells

compound	IC ₅₀ (μ M) ^a	95% confidence Interval
DACE	0.24	0.16–0.31
CIS	17.91	15.2–21.4
IRI	8.53	5.6–10.3
PAC	0.23	0.14–0.35

^aIC₅₀: Concentration that inhibited cell proliferation by 50% when compared to that of untreated cells.

action. The main advantages of this practice are that it enhances the effectiveness of the treatment, prevents the recurrence of resistant tumor cells, reduces the effective dose and systemic toxicity, and improves the pharmacodynamics and pharmacokinetics of the drug. Lung cancer, in particular, usually presents metastatic evolution; therefore, it is likely that a single therapeutic target is insufficient to generate an effective clinical response, for example, drugs that inhibit the epidermal growth factor receptor (EGFR), a signaling pathway that is commonly deregulated in patients with lung cancer. Several EGFR inhibitors have been approved for the treatment of lung cancer; however, the efficacy of these drugs as monotherapy is limited. This suggests that monotherapies may be insufficient to achieve satisfactory clinical results, indicating that combined treatments using drugs with different mechanisms could be useful.^{3,29}

The ChemGPS-NP (Chemical Global Positioning System for Natural Products) is an *in silico* tool that has been used successfully to categorize and predict the mechanisms of new and previously unstudied compounds.^{30–32} It is based on principal component analysis (PCA) of physicochemical properties of known drugs. The approach of this model is based on the cornerstone of medicinal chemistry, i.e., that structurally similar molecules frequently present similar biological activities. As shown in Figure 2, when DACE scores

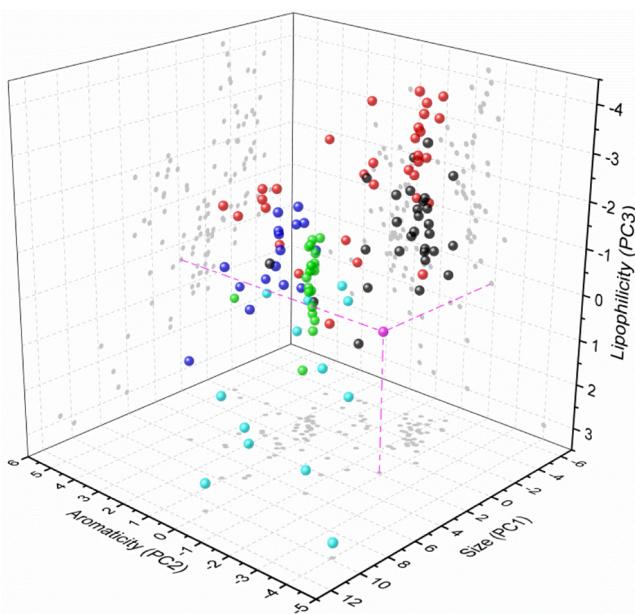


Figure 2. ChemGPS-NP analysis of DACE (pink) together with the NCI reference data set of anticancer drugs. Score plot of three principal components (PC1, size; PC2, aromaticity; PC3, lipophilicity) of DACE mapped onto the ChemGPS-NP model for the prediction of the anticancer mode of action. The NCI reference set of anticancer drugs included alkylating agents (black), RNA/DNA antimetabolites (red), tubulin inhibitors (light blue), topoisomerase I (green), and topoisomerase II inhibitors (dark blue).

were interpolated together with a reference set of anticancer drugs, this compound did not overlap with any of the well-known mode of action clusters. This result suggests that DACE may possess a distinct mode of action when compared with these drugs. In fact, this compound has previously been investigated by our group, and was found to induce cell apoptosis by interfering with EGFR activation and its

downstream signaling.⁶ Thus, as DACE appears to present a different mode of action from those of some approved anticancer drugs, the evaluation of synergistic effects of this compound with selected anticancer drugs is highly desirable.

The next step was to evaluate the synergism between DACE and three drugs: CIS, IRI, and PAC. As shown in Table 3,

Table 3. Synergistic Antiproliferative Effects of the Most Effective Combinations of DACE with Selected Chemotherapy Drugs on A549 Cells

tested combinations	combination ratio	CI ^a values	effect
DACE (0.125 μM) + CIS (9 μM)	0.5× IC ₅₀	0.251	strong synergism
DACE (0.125 μM) + IRI (4.25 μM)	0.5× IC ₅₀	0.680	synergism
DACE (0.125 μM) + PAC (0.125 μM)	0.5× IC ₅₀	0.202	strong synergism

^aCI: combinatorial index, based on the analysis of concentration-response of combinations of each drug with DACE, which indicates the degree of interaction between compounds in terms of synergism, additive effect, or antagonism.

DACE showed potential synergistic effects with all studied drugs; however, the degree of synergism observed, as well as the concentration range of the combinations, were different for each treatment regimen. At half of the IC₅₀ value, DACE and drugs, individually and combined, were selected for studies of the antiproliferative mechanism.

Figure 3A, B, and C shows the synergic effects of DACE and CIS, IRI, or PAC combinations on A549 proliferation, respectively. At 0.5× IC₅₀ treatments, the cell proliferation values of A549 cells treated for 48 h with DACE, CIS, and DACE plus CIS were 76.4%, 73.3%, and 49%, respectively. When the cells were treated with DACE, IRI, and DACE plus IRI, cell proliferation values were 76.4%, 47.6%, and 31.7%, respectively. When the cells were treated with DACE, PAC, and DACE plus PAC, cell proliferation values were 76.4%, 42.3%, and 32%, respectively. Several studies have reported the synergistic effects of cucurbitacin B and different chemotherapeutics agents, such as gemcitabine on pancreatic cancer cells,¹¹ and with CIS on laryngeal cancer cells¹² and cutaneous squamous cell carcinoma cells.¹³ These findings, together with our promising preliminary results, led us to further investigate the mechanism of action of DACE in combination with the selected drugs.

Previous studies have reported the influence of oxidative damage as mediators of antiproliferative effects exerted by cucurbitacins on different cell lines.^{33,34} To evaluate the influence of free radical generation on the effects induced by DACE alone or combined with drugs, the experiments were carried out in the presence of an antioxidant agent (NAC 500 μM) and an oxidative damage potentiating agent (BSO 10 μM). When cells were treated with DACE combined with drugs in the presence of NAC (Figure 3D), the antiproliferative effects of the combinations were significantly reduced ($p < 0.0001$). Concerning the treatment of A549 cells with DACE alone and in combination with drugs, supplemented with BSO, the results showed a significant increase ($p < 0.0001$) in the antiproliferative effects of all treatments (Figure 3E). BSO is an agent that potentiates oxidative damage through the inhibition of glutathione synthesis. Another study has demonstrated a similar effect for cucurbitacin B on different lung cancer cell

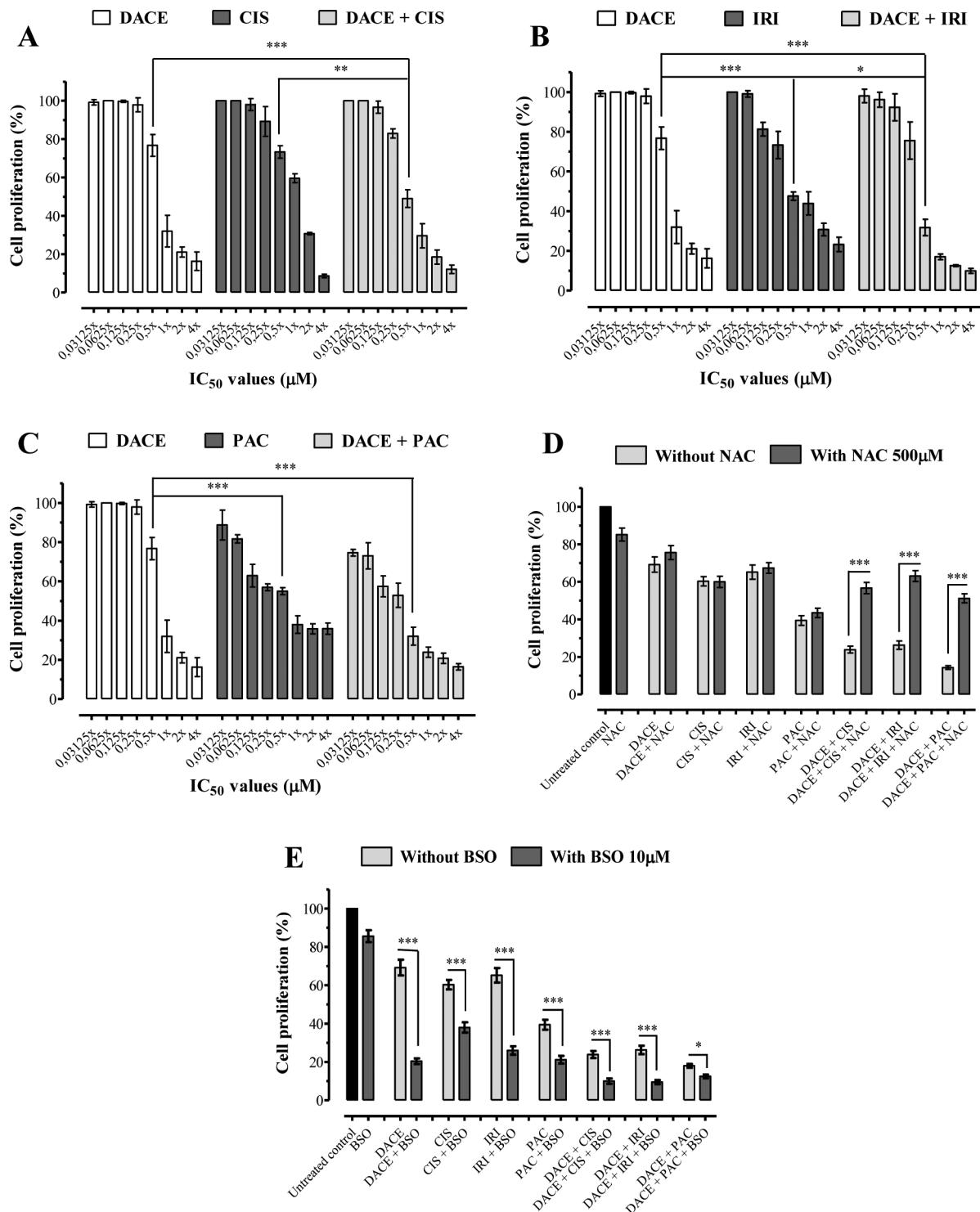


Figure 3. DACE combined with CIS, IRI, and PAC showed potential synergistic cell growth inhibition and dependent manner of ROS generation on A549 cells. (A,B,C) A549 cells were incubated with different concentrations of DACE, drugs, and combinations of DACE with drugs for 48 h and cell viability evaluated by sulforhodamine B assay. Comparative graphics demonstrating the effects of DACE, drugs, and their combinations on A549 cell proliferation. (D,E) A549 cells were treated with DACE, drugs, and their combinations for 48 h in the presence or absence of an antioxidant agent (NAC 500 μ M) or an oxidative agent (BSO 10 μ M). After treatments, cell proliferation was determined by the sulforhodamine B assay. The treatment concentrations were DACE (0.125 μ M), cisplatin (9 μ M), irinotecan (4.25 μ M), and paclitaxel (0.125 μ M), individually and combined. Data represent the mean \pm standard deviation of three independent experiments. Asterisks (****) represent $p < 0.001$; (**) $p < 0.01$; (*) $p < 0.05$ compared to DACE isolated vs combined with each drug by Tukey's test.

lines, wherein the simultaneous treatment with cucurbitacin B and BSO or NAC exacerbated or attenuated their effects, respectively.³³ Guo and colleagues³⁴ also showed that

cucurbitacin B causes G2/M cell cycle arrest in a ROS-dependent manner on human lung and breast cancer cells. The results obtained suggest that DACE combined with the selected

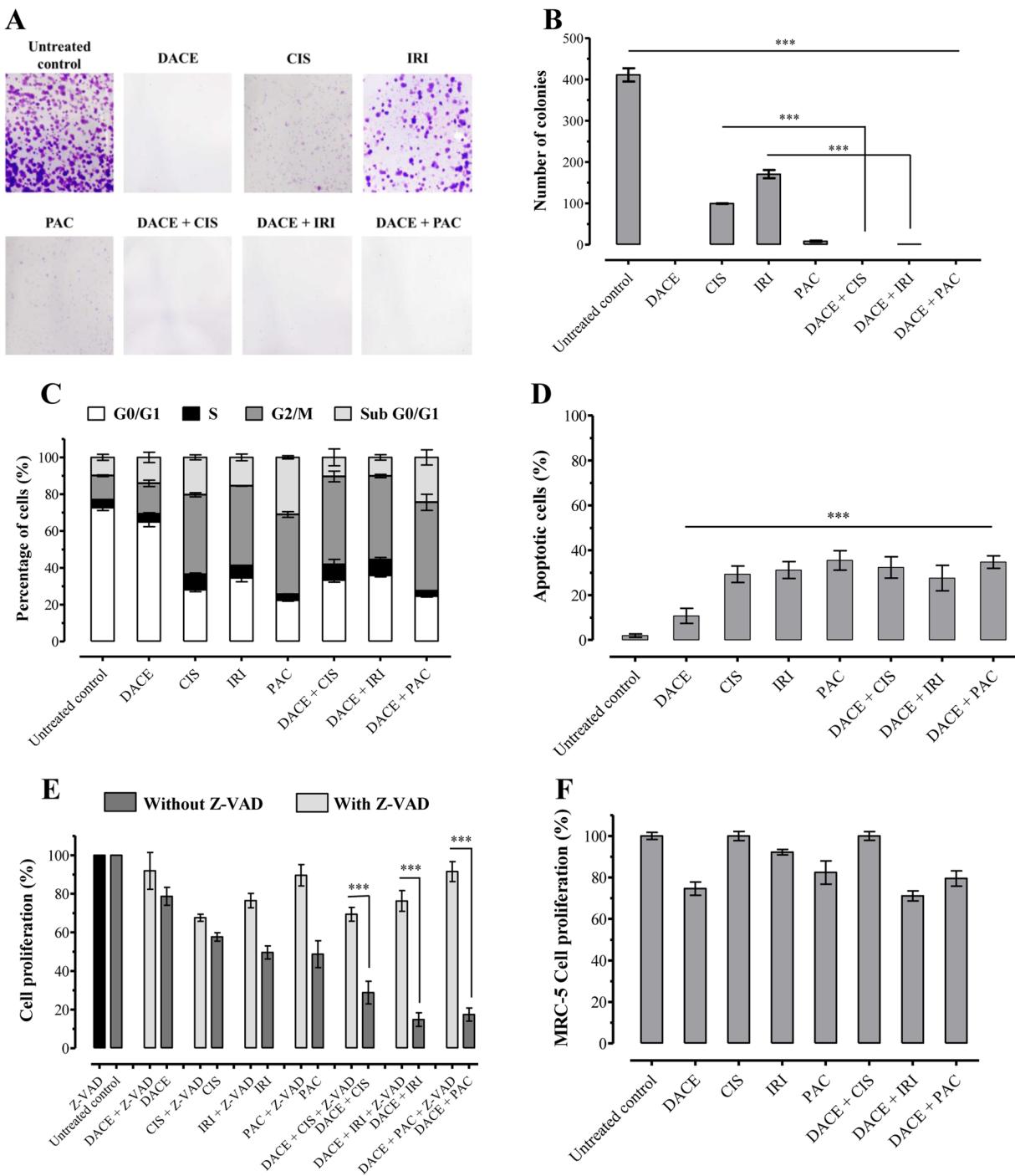


Figure 4. DACE combined with drugs completely reduced clonogenic survival, induces G2/M cell cycle arrest, and apoptosis on A549 cells. Combined treatments did not harm the proliferative potential of nontumoral lung cells (MRC-5). (A,B) A549 cells were treated for 24 h with DACE, drugs, and their combinations. Cells were then washed with warm PBS, given fresh medium, and allowed to grow for 10 days. Colonies of A549 cells were measured by staining colonies using crystal violet. (C) Effects of DACE individually and combined with drugs on cell cycle distribution of A549 cells after 48 h of treatment with propidium iodide staining. The values indicate the percentage of A549 cells in each phase of the cell cycle. (D) Percentage of apoptotic cells after 24 h of treatment with DACE and drugs analyzed by the Annexin V/PI assay. (E) A549 cells were treated with DACE, drugs, and their combinations for 48 h in the presence or absence of a pan-caspase inhibitor (Z-VAD-FMK). After treatments, cell proliferation was determined by the sulforhodamine B assay. (F) MRC-5 cells were treated with DACE, drugs, and their combinations for 48 h at the same concentrations for all experiments. After treatment, cell proliferation was determined by the sulforhodamine B assay. The treatment concentrations were DACE ($0.125 \mu\text{M}$), cisplatin ($9 \mu\text{M}$), irinotecan ($4.25 \mu\text{M}$), and paclitaxel ($0.125 \mu\text{M}$), individually and combined. Data represent the mean \pm standard deviation of three independent experiments. Asterisks (***) represent $p < 0.001$; (**) $p < 0.01$; (*) $p < 0.05$ comparing the groups by Tukey's test. Scale bars are $20 \mu\text{m}$; the magnification was $400\times$.

drugs induced antiproliferative effects on A549 cells, in part, by increasing intracellular ROS production.

The clonogenic potential of A549 cells was also evaluated in this work, and the results are shown in Figure 4A and B. After 2

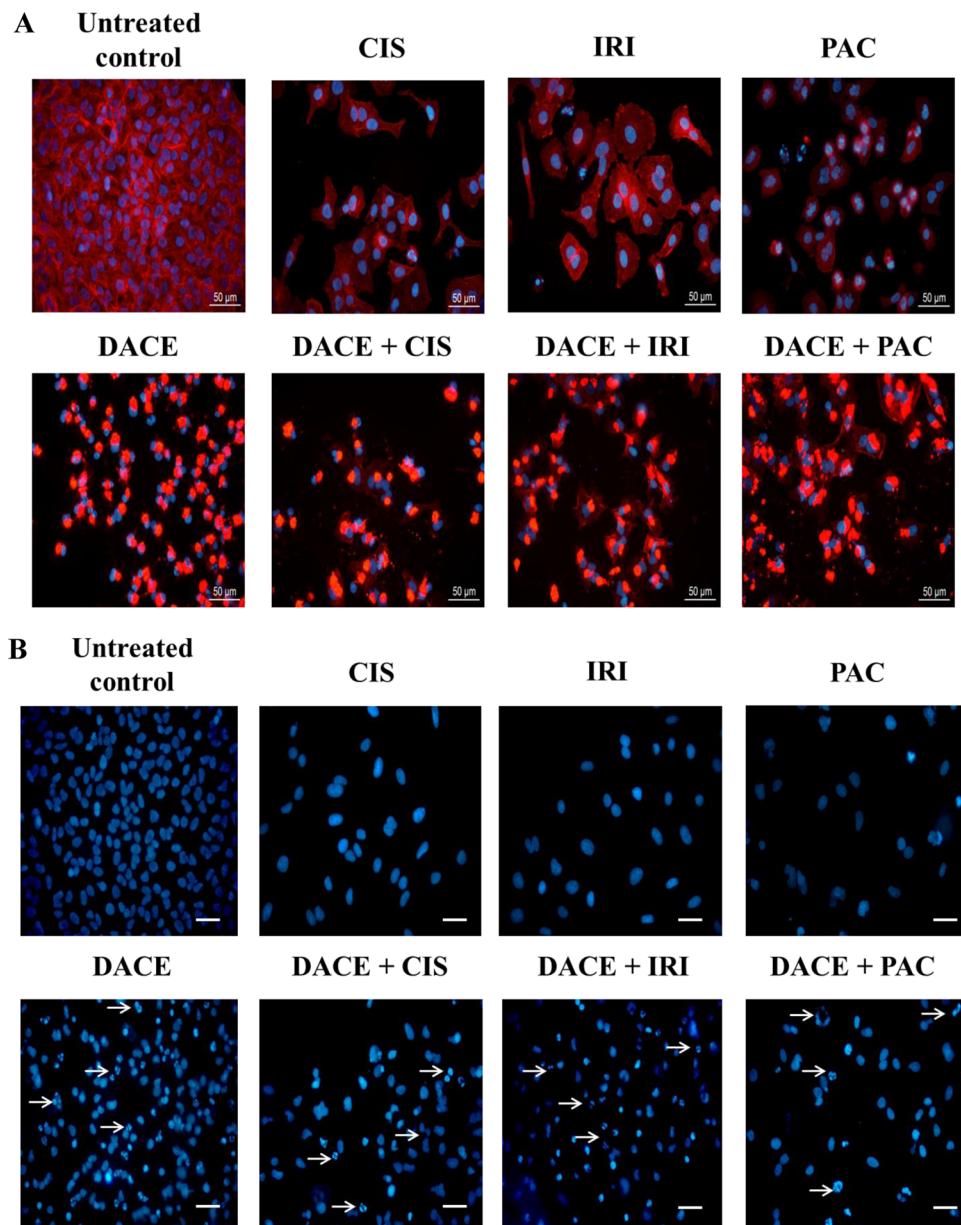


Figure 5. DACE combined with drugs induces morphological alterations of F-actin on cytoskeleton and nuclear morphological alterations. (A) A549 cells were treated with DACE, drugs, and their combinations, for 48 h. Actin filaments were labeled with TRITC-labeled-phalloidin staining (red), nuclei labeled with Hoechst staining (blue), and representative images obtained by immunofluorescence microscopic analysis. (B) A549 cells were treated with DACE, drugs, and their combinations, for 48 h and nuclei labeled with Hoechst staining (blue). White arrows indicate nuclear morphological alterations characteristics of cell death by apoptosis, as nuclear fragmentation. Immunofluorescence microscopic pictures of A549 cells treated. The treatment concentrations were DACE ($0.125 \mu\text{M}$), cisplatin ($9 \mu\text{M}$), irinotecan ($4.25 \mu\text{M}$), and paclitaxel ($0.125 \mu\text{M}$), individually and combined. Data are representative of three independent experiments. Scale bars are $50 \mu\text{m}$; the magnification was $400\times$.

days of treatment and more than 8 days without treatments, cell colonies were stained and quantified in order to evaluate whether the previous treatment with DACE and drugs alone, or with the selected combinations, allowed cells to resume their growth potential and acquire resistance to the respective treatments. After the treatment with drugs alone, cells restarted proliferation, suggesting a possible resistance to these drugs. In the group of untreated cells, 411 ± 16 colonies were counted, and when cells were treated with CIS, IRI, and PAC, the numbers of cell colonies were 99 ± 1 , 170 ± 10 , and 8 ± 1.6 , respectively, after 8 days of incubation in the absence of drugs. Surprisingly, combinations of DACE with all drugs completely inhibited the resumption of cell proliferation, suggesting that

these combinations are less likely to be affected by resistance. The potential of curcubitacin B to partially inhibit the clonogenic growth of pancreas tumor cells, in a concentration dependent-manner, has been reported previously.¹¹ Previous studies also described that H460 lung cancer cells are also resistant after treatment with high concentrations of cisplatin.³⁵ A549 cells also demonstrated resistance after treatment with topotecan alone, which is a similar drug with irinotecan,³⁶ and after treatment with paclitaxel in high concentrations.³⁷ In this context, particularly, the present results for DACE in combination with CIS, IRI, and PAC may be significant.

Most chemotherapy regimens currently used to treat cancer exert their effects by modulating the cell cycle and inducing cell

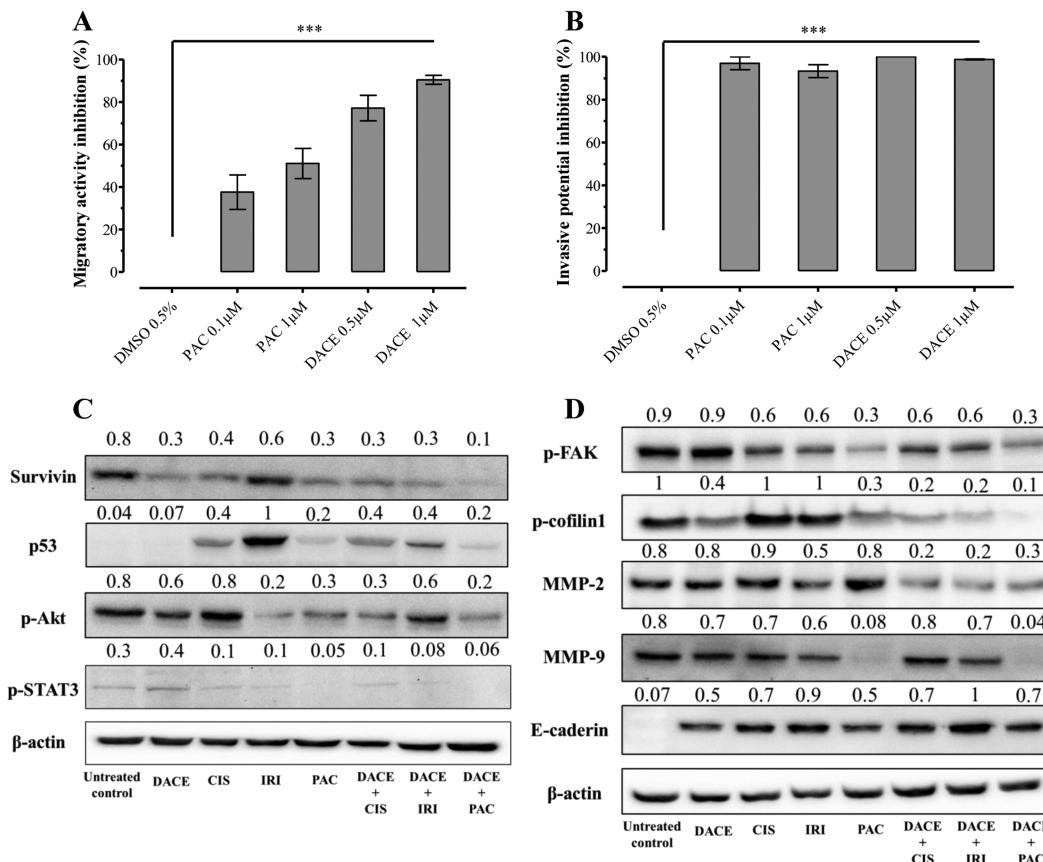


Figure 6. DACE is a potential inhibitor of cell migration and cell invasion and combinations of DACE with drugs modulates proliferation, cell death, and migration pathways on A549 cells. (A) A549 cells were treated with PAC (0.1 and 1 μ M) or DACE (0.5 and 1 μ M) for 16 h. The antimigratory activity was observed in response to an artificial injury. Images were obtained on an inverted fluorescence microscope, and quantification of the inhibition percentages was performed using CellC software. (B) A549 cells were grown in Transwell inserts and received the same treatments as the scratch assay, for 48 h. Afterward, cells were fixed and stained with DAPI, photographed with an inverted fluorescence microscope, and quantification of inhibition percentages performed using CellC software. (C,D) A549 cells were treated with DACE, drugs, and their combinations, for 48 h. Afterward, protein extraction was performed, and the Western blotting technique was used to evaluate protein expression. B-Actin has been shown for equal loading. All Western blots were performed three times to validate the results, and each Western blotting is representative in this experiment. The treatment concentrations were DACE (0.125 μ M), cisplatin (9 μ M), irinotecan (4.25 μ M), and paclitaxel (0.125 μ M), individually and combined. Data are representative of three independent experiments. Asterisks (****) represent $p < 0.001$; (**) $p < 0.01$; (*) $p < 0.05$ comparing the groups by Tukey's test.

death by apoptosis. These cell processes were also evaluated in the present study through flow cytometry protocols. It is already known that cucurbitacin B inhibits the proliferation of different cancer cells by inducing G2/M cell cycle arrest.^{11–13,18,38–42} In comparison with the untreated control, a significant increase from 13.1 to 16.6%, to 43.2%; to 43.3% and to 43.4% ($p < 0.0001$) cells in G2/M phase was detected after the treatments with DACE, CIS, IRI, and PAC, respectively (Figure 3C). When DACE was combined with CIS, IRI, or PAC, they also significantly increased the proportion of G2/M phase cells ($p < 0.0001$) when compared to that of the untreated controls, and the percentages of cells in this phase were of 47.8%, 45.5%, and 48.1%, respectively. However, there are no statistical differences between the combined treatments and DACE or the drugs alone ($p > 0.05$). Notably, the combination of DACE with PAC increased the proportion of cells in Sub-G0/G1 phase, when compared to that of DACE alone and the untreated control ($p < 0.01$), with cell percentages of 24.4%, 14.1%, and 9.9% in the SubG0/G1 phase, respectively.

Figure 4D shows the effects of drugs on DACE apoptosis induction. It was found that DACE and drugs, individually or

combined, induced apoptosis on A549 cells when compared to that of the untreated controls. Percentages of apoptotic cells after treatments with DACE alone or combined with CIS, IRI, and PAC were 10.7%, 32.3%, 27.6%, and 34.7%, respectively. However, combinations of drugs with DACE only significantly increased the proportion of apoptotic cells when compared with that of DACE alone or the untreated control, not when compared with the respective drug alone. Thus, at the concentration ranges used, the effects of combined treatments regarding the G2/M cell cycle arrest and the apoptosis induction appear to be mainly due to the effects of the drugs individually.

In this view, we hypothesized that the higher antiproliferative activity of the combinations was indeed related to different mechanisms. Another prominent feature of cucurbitacins is the induction of rapid morphological alterations on tumor cells.^{43,44} Our data showed that the treatments with the clinically used drugs alone did not induce changes in cytoskeletal morphology; however, treatments with DACE alone and in combination with drugs altered the organization of actin filaments on the cytoskeleton (Figure 5A). Combined treatments induced aggregation of F-actin and possibly the disruption of the

microtubule network. A previous study reported that cucurbitacin B caused morphological changes in the cytoskeleton of leukemic cells by inducing F-actin polymerization.⁴³ An additional study described the melanoma cell inhibitory effects of cucurbitacin I in interfering with the dynamic organization of actin filaments.⁴⁴ Similar results were reported by our research group recently.^{6,10} Our data demonstrate that the combined treatments prominently alter the cytoskeletal network of lung cancer cells, inducing rapid aggregation of the F-actin network. This effect may contribute to the antiproliferative action of these treatments because the maintenance of homeostasis of the cytoskeleton is an important factor in the mitosis process.

Additionally, DACE alone and combined with CIS, IRI, and PAC induced apoptosis features such as nuclear fragmentation (Figure 5B). These results indicate that the process of cell death induced by the combined treatments is at least in part due to apoptosis as revealed in the cell cycle arrest results. However, Zhang and colleagues⁴⁵ also reported that cucurbitacins I and B act as inducers of cell death by the autophagy pathway. Park and co-workers⁴⁶ also demonstrated that the generation of ROS by sodium selenite induced apoptosis and autophagy simultaneously in A549 cells. However, the main cell death mechanism described for cucurbitacins is apoptosis induction.^{6,10,40,41} The antiproliferative effects of the combined treatments also were dependent on caspase activation, as shown in the Figure 4E. The combined treatments of DACE with CIS, IRI, or PAC reduced cell proliferation to 28.8%, 14.8%, and 17.4%, respectively. When A549 cells were exposed to the same treatments, in the presence of a caspase inhibitor (Z-VAD), the effect was significantly attenuated ($p < 0.0001$), and the cell proliferation was 69.4%, 76.3%, and 91.5%, respectively. These results suggest that the combinations of DACE with CIS, IRI, and PAC induce antiproliferative effects, caspase-dependent apoptosis, and disruption of cytoskeletal network.

To check whether DACE and drugs, individually or in combination, result in improved selectivity, their effect was evaluated on MRC-5 cell proliferation. It has been a constant challenge to find ways to improve the selectivity of cucurbitacins toward tumor cells by structural changes in the cucurbitan skeleton.⁴⁷ Treatment with DACE and CIS did not reduce MRC5 cell proliferation when compared to the untreated controls (Figure 4F). Combined treatment with DACE and IRI or PAC revealed cell proliferation values of 70% and 80%, respectively. These results showed that the combinations caused minimal effects on nontumor lung cells and reinforce the benefits of synergic treatments.

Cell adhesion molecules, named cadherins, have been highlighted as important regulators of tumor growth and metastasis. The loss of E-cadherin-mediated cell adhesion is a hallmark of the transition from a normal epithelium to the poorly differentiated type of carcinoma.⁴⁸ In our study, we evaluated the modulation of E-cadherin through Western blotting (Figure 6D). The results showed that the treatments with DACE and drugs, alone and combined, maintained the expression of E-cadherin on A549 cells. This data suggest that the selected combinations reduced the possibility of cancer cells to migrate to other tissues and cause metastasis. In comparison with the untreated control, DACE alone (0.5 and 1.0 μ M) inhibited 77.2% and 90.5% of A549 cell migration, respectively, after 16 h of treatment (Figure 6A). PAC (0.1 and 1.0 μ M) was used as a control and inhibited 37.6% and 51.1% of cell migration, respectively. Similarly, PAC (0.1 and 1 μ M) and

DACE (0.5 and 1 μ M) inhibited A549 cell invasion potential almost completely (Figure 6B). Other studies have described the ability of other cucurbitacins to suppress cell migration and invasion, significantly reducing the metastatic potential during cancer evolution.^{49,50}

The combined treatments investigated in this study also modulate the signaling pathways linked to cell migration processes. As shown in Figure 6D, DACE plus PAC reduced the phosphorylation of FAK, which is a kinase of focal adhesion, associated with cancer cell migration. Metalloproteinases proteins (MMP) are thought to play a major role on cell behaviors, such as cell migration (adhesion/dispersion), differentiation, and angiogenesis, and they are also essential to metastasis progression.⁵¹ All combinations of the selected drugs with DACE reduced MMP-2 expression, but only the combination of DACE with PAC reduced MMP-9 expression (Figure 6D). The modulation of these proteins partially explains the inhibitory effects of DACE on the cell migration and invasion assays (Figures 6A,B).

The actin cytoskeleton organization of cells is regulated by various proteins, such as cofilins, a type of actin-binding protein. This function is essential to the cell motility process, facilitating the evolution to metastasis. Overexpression of cofilin-1 has been associated with aggressiveness in several types of cancer, including nonsmall cell lung cancer.⁵² Our data also showed that treatment with DACE, in combination with the selected drugs, reduced the phosphorylation of cofilin-1 (Figure 6D). Nakashima and colleagues⁵³ also reported that cucurbitacin E inhibited cofilin phosphorylation in human leukemia cells. Zhang and colleagues also showed that cucurbitacin B reduced cofilin phosphorylation and induced actin-cofilin aggregation in melanoma cells.⁵⁴ These researchers also demonstrated that the pretreatment with NAC suppressed actin aggregation induced by cucurbitacin B. In our study, treatments with DACE, individually and combined with drugs, also induced cofilin-1 aggregation in A549 cells (data not shown) and correlated with data described by these authors. Taken together, these results suggest the combined treatments inhibit A549 cell migration and invasion by modulating metalloproteinases, E-cadherin, and p-cofilin-1 expression, and disrupting actin cytoskeleton.

Other proteins involved in cell proliferation and apoptosis were also analyzed by Western blotting. As shown in Figure 6C, all combinations of DACE with the selected drugs reduced survivin expression, especially the combination with PAC. The combinations also increased p53 expression. Survivin is an antiapoptotic protein, and p53 protein acts during the suppression of tumor growth. These effects could explain the alterations observed during cell cycle progression, in which the treatments with DACE in combination with drugs induced G2/M phase arrest (Figure 4C). It was also reported that cucurbitacin E increased p53 expression in the human bladder cancer cell line.⁴¹ The Akt and Stat-3 pathways are key regulators of cell survival and proliferation, and their overexpression is commonly found in tumors.⁵⁵ Figure 6C shows that these signaling pathways were also modulated by the tested treatments and that the synergic antiproliferative effects may be, at least in part, mediated via downregulation of STAT3 and Akt signaling pathways. Combinations of DACE plus CIS or plus PAC reduced the phosphorylation of Akt, and all combinations downregulated p-STAT3 expression. Our colleagues⁶ showed that the treatment with DACE at 0.5 and 1 μ M reduced the p-STAT3 expression in A549 cells. However, in the present study,

DACE at 0.125 μM did not reduce p-STAT3 expression. Therefore, the reduction of the p-STAT3 expression mediated by DACE is probably concentration-dependent. Other studies also demonstrated that other cucurbitacins showed antiproliferative effects by inhibiting the STAT3^{6,11,41} and Akt pathways.^{6,10}

The main challenge for the cancer treatment remains the acquired resistance by tumor cells. This difficulty can be attenuated with combined therapies, which act by different pharmacological pathways, consequently increasing the efficacy and decreasing the resistance. Overexpression of AKT and STAT3 pathways promotes cell proliferation in an unlimited way⁵⁵ as well as misregulates metalloproteinases MMP-2 and MMP-9 that are related to metastasis.⁵¹ In the present study, the combined treatments showed interesting antiproliferative effects mainly due to the downregulation of those pathways, which were dependent on ROS generation.

CONCLUSIONS

DACE showed important antiproliferative effects which were potentiated, when combined with chemotherapeutics drugs. To our knowledge, this is the first report to elucidate the mechanism of action of this new semisynthetic cucurbitacin B derivative combined with different chemotherapeutic drugs used to treat cancer. Our data indicated that the combinations of DACE with the selected drugs reduced the risk of resistance on A549 cells, and they did not affect the cell proliferation of nontumoral lung cells (MRC-5). Combined treatments induced antiproliferative effects by G2M cell cycle arrest and downregulation of the STAT3 and Akt signaling pathways. Combined treatments also induced F-actin aggregation on cytoskeletal, nuclear fragmentation, and cell death by apoptosis. DACE also showed relevant inhibitory effects on migration and invasion of lung cancer cells. Combined treatments reduced the phosphorylation of cofilin-1 and maintained the expression of E-cadherin on A549 cells reducing the risk of metastasis development. These effects can be explained by DACE whose likely mechanism of action is different from most other classes of anticancer drugs, according to the results obtained with the ChemGPS-NP *in silico* model. It is important to note that the treatment with DACE combined with chemotherapics presented a mechanism of action similar to that of its precursor cucurbitacin B. Despite this, the findings of this study offer important insights for the development of more effective and selective therapies against cancer, especially nonsmall cell lung cancer.

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Notes

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ABBREVIATIONS

CIS, cisplatin; IRI, irinotecan; PAC, paclitaxel; DACE, 2-deoxy-2-amino-cucurbitacin E; DMSO, dimethyl sulfoxide; NSCLC, nonsmall cell lung cancer; NAC, N-acetylcysteine; BSO, L-buthionine-sulfoximine; FACS, fluorescence activated cell sorting system; ROS, reactive oxygen species; DAPI, 4',6-diamidino-2-phenylindole; STAT3, signal transducers and activators of transcription 3; PI, propidium iodide; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine

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