Cleistanthin A inhibits the invasion and metastasis of human melanoma cells by inhibiting the expression of matrix metallopeptidase-2 and -9

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Abstract. It has been demonstrated that numerous types of metastatic cancer overexpress vacuolar-type H+ (V)-ATPases. It may be possible to inhibit the growth and metastasis of human cancer cells by inhibiting V-ATPases. It was previously reported that diphyllin, a novel V-ATPase inhibitor, can inhibit the migration and invasion of SGC7901 human gastric cancer cells; however, the effects of cleistanthin A (CA), a diphyllin glycoside, on melanoma cells has not been demonstrated. The present study aimed to investigate the effect of CA as a V-ATPase inhibitor and its effects on the invasion and metastasis of A375 cells. The results of an MTT assay in the present study indicated that the growth inhibition of A375 cells by CA was induced in a dose- and time-dependent manner; however, A375 cell viability was not significantly affected by low concentrations (0.03, 0.1 and 0.3 µM) after 24 h. Similar results were obtained by viable cell counting with trypan blue. Therefore, these concentrations of CA were selected for the treatment of A375 cells in further experiments. It was demonstrated that CA inhibited the expression of V-ATPases in a dose-dependent manner and decreased the internal pH level of A375 cells. Alterations to the lysosomal pH were associated with the CA concentration. Furthermore, CA treatment induced a significant decrease in cell migration and invasion, as demonstrated with wound-healing and Transwell assays. Gelatin zymography and western blot analysis demonstrated that the expression levels of matrix metalloproteidase (MMP)-2 and -9 decreased following CA treatment. Therefore, CA can be characterized as a novel V-ATPase inhibitor for the treatment of melanoma that may inhibit invasion and metastasis by downregulating the expression of MMP-2 and -9.

Introduction

Cutaneous melanoma is the deadliest form of skin cancer, which arises from melanocytes and their precursors (1). Despite major efforts to identify novel therapeutic tools to treat metastatic melanoma, durable regression remains a rare event in patients with advanced disease, and no significant benefit in survival time has been achieved with targeted inhibitors (2).

A previous study revealed that an acidic tumor microenvironment is critical to cancer progression and metastasis (3). The majority of tumors develop in a pathophysiological microenvironment that is characterized by low oxygen tension (pO2), elevated interstitial fluid pressure, low glucose concentration, high lactate concentration, low extracellular pH and/or energy deprivation (4,5). This hostile microenvironment activates a number of transcription factors, including hypoxia-inducible factor-1, leading to the upregulated expression of a large number of gene products that promote malignant progression and metastatic dissemination (6,7). In addition, cell surface vacuolar-type H+ (V)-ATPase activity has been postulated to create an acidic extracellular microenvironment, which is required for the activation of proteases critical for tumor cell invasion (8).

V-ATPases are ubiquitously expressed ATP-dependent proton pumps that may regulate the pH in endomembrane systems (9). They are overexpressed in a number of types of metastatic cancer and are positively associated with invasion and metastasis (10). It has been demonstrated that pharmacologic or genetic reductions of V-ATPase activity significantly reduce the migration of invasive tumor cells in vitro (9). For example, the V-ATPase inhibitor archazolid abrogated tumor dissemination in a syngeneic mouse 4T1 breast tumor metastasis model (9). Consistent with this, the inhibition of V-ATPase with concanamycin or short hairpin RNA targeting the V1E subunit reduced matrix metalloproteidase (MMP)-9 activity (11). Bafilomycin and concanamycin were the first discovered V-ATP inhibitors (12); however, as they also inhibit...
mitochondrial ATP enzymes and affect the function of mitochondria in normal cells, they lead to cell death (13) and so are difficult to apply in clinical practice. Recently, cleistanthin A (CA), a natural compound, was demonstrated to inhibit the activity of V-ATPase in HepG2 cells and to neutralize the pH of lysosomes at nanomolar concentrations (14); to the best of our knowledge, no previous studies have assessed how it may affect tumor cell motility and migration.

The present study aimed to investigate whether CA may inhibit the V-ATPases of A375 cells and to explore the potential underlying mechanisms. It was revealed that CA inhibits the invasion and migration of A375 cells in vitro.

Materials and methods

Chemicals and materials. RPMI-1640 and fetal bovine serum (FBS) were acquired from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Penicillin and streptomycin were obtained from Thermo Fisher Scientific, Inc. Matrigel was purchased from Beijing Unique Biotechnology Co., Ltd. (Beijing, China). A V-ATPase-specific kit was supplied by Shanghai GenePharma Co., Ltd. (Shanghai, China). The pH sensitive fluorescent probe 20,70-bis-(2-carboxyethyl)-5-carboxyfluorescein (BCECF), was acquired from the Beyotime Institute of Biotechnology (Haimen, China). The pH LysoTracker Red lysosomal fluorescent probe was purchased from Invitrogen (Thermo Fisher Scientific, Inc.) and the nuclear dye Hoechst 33258 (5 µg/ml) was purchased from the Beyotime Institute of Biotechnology (Haimen, China). Bafilomycin A1 was obtained from Shanghai Genepharma Co., Ltd. CA and bafilomycin A1 were dissolved in dimethylsulfoxide (DMSO) and stored at -4°C until analysis.

Cell culture. A375 human melanoma cells were kindly provided by the Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). Cells were cultured in RPMI-1640 supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) in humidified air with 5% CO2 at 37°C throughout the study.

Proliferation assay. Cell proliferation was determined with an MTT colorimetric assay and trypan blue viable cell counting. Each experiment was repeated ≥3 times. For the MTT colorimetric assay, A375 cells (1x104/well) were plated in 96-well plates in 200 µl of medium and treated with various concentrations of CA (0, 0.001, 0.01, 0.03, 0.1, 0.3 and 1 µM) dissolved in 100% DMSO, with final DMSO concentrations <0.1% in RPMI-1640 supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) in humidified air with 5% CO2 at 37°C throughout the study.

V-ATPase activity. V-ATPase activity was evaluated using the V-ATPase-specific kit (Genmed Sciences, Inc., Arlington, MA, USA), according to the manufacturer’s protocol. Briefly, 8x104 cells untreated or treated with CA or bafilomycin A1 (0.1 µM) were incubated for 24 h. The supernatant was discarded and the cells were washed with reagent A 2-3 times, finally discarding a clean fluid. Each sample had 60 µl Genmed lysis liquid (reagent B) added to it and the cells were scraped down into the Eppendorf tube. The tube was incubated on ice for 30-50 min in the ice to lyse cells and centrifuged at 4°C, 10 min at 13,201 g. The supernatant contains the cell lysate and its protein concentration was determined with a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Inc.). V-ATPase activity was detected using a microplate reader.

Determination of intracellular pH values. The internal pH (pHi) value was evaluated in the cell monolayer using the pH sensitive fluorescent probe BCECF. A standard curve was established using a RF-5301PC fluorescence spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Cells were treated with CA (0, 0.03, 0.1 and 0.3 µM) for 24 h prior to the application of BCECF as previously described (15). The fluorescence intensity at 490 nm was recorded, and the pHi value was determined according to the pHi standard curve.

Wound-healing assay. The in vitro wound-healing assay was used to observe the migration of A375 cells following CA treatment. Cells (1x105 cells/ml) were seeded into a 6-well plate and incubated for 24 h. The center of the cell monolayer was then scraped with a sterile micropipette tip to create a straight gap of constant width. The wells were washed with PBS and the cells were exposed to various concentrations of CA (0, 0.03, 0.1 and 0.3 µM) or bafilomycin A1 (0.1 µM) (as the positive control). Wound closure was imaged at 0 and 24 h using an inverted light microscope (magnification, x200).

Matrigel invasion assay. For assessment of the motility of A375 melanoma cells treated with CA and bafilomycin A1, an invasion assay was performed in a 24-well Transwell plate with 8 µm pore filter inserts (Corning Incorporated, Corning, NY, USA) coated with 50 µl Matrigel. The cells were then seeded at a density of 1x104 cells per chamber in 200 µl RPMI-1640 with CA (0, 0.03, 0.1 and 0.3 µM) or bafilomycin A1 (0.1 µM), added to chamber inserts in wells filled with 0.6 ml of RPMI-1640 supplemented with 20% FBS as the chemoattractant and incubated for 24 h. The cells and Matrigel on the upper side of inserts were completely removed by swabbing. The transmigrated cells on the lower side of the membrane and well bottom were fixed for 30 min with formaldehyde and stained for 5 min with 0.1% of crystal violet at room temperature. The number of cells was observed using an inverted microscope (magnification, x200).

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**Immunofluorescence staining analysis.** Prior to seeding cells, aseptic glasses were placed on 6-well plates. A total of 1.2x10⁴ cells in the logarithmic growth phase were seeded on 6-well plates with pre-paved glasses and treated with CA (0, 0.03, 0.1 and 0.3 µM) or bafilomycin A1 (0.1 µM) for 24 h. Then incubated 100 nM lysosomal fluorescent probe (Invitrogen; Thermo Fisher Scientific, Inc.), LysoTracker with CA and bafilomycin A1 (0.1 µM) for 3 h. Cells were then washed with PBS, fixed with 4% formaldehyde for 15 min at room temperature and washed again with PBS for 2-3 times, then they were stained with DAPI (10 µg/ml) for 5 min at room temperature. Then discarded the dye solution, washed again with PBS for 2-3 times, and pasted the glasses with quenching inhibitor. Finally, a DMRXA2 Leica laser confocal microscope (magnification, ×63; oil mirror) was used to observe the cells.

**Gelatin zymography.** The protein content of supernatants from cells treated with 0, 0.03, 0.1 and 0.3 µM CA or bafilomycin A1 (0.1 µM) for 24 h was determined using a BCA assay following centrifugation at 13,201 x g at 4°C. Equal amounts of protein (25 µg) for each sample were mixed with 5X loading buffer [50 mmol/l Tris-HCl (pH 6.8), 10% glycerol, 2% SDS and 0.1% bromophenol blue] and loaded onto a 10% SDS-PAGE containing 0.1% gelatin. Following electrophoresis, gels were rinsed twice in 0.25% Triton X-100 for 40 min at room temperature and then incubated at 37°C in developing buffer (50 mmol/l Tris, pH 7.5-7.6, 10 mmol/l CaCl₂, 0.02% NaN₃, and 150 mmol/l NaCl) for 42 h. The gels were then stained with 0.05% Coomassie Brilliant Blue R-250 in 30% methanol and 10% glacial acetic acid for 3 h, and subsequently destained in 30% methanol and 10% glacial acetic acid for 30 min, 20% methanol and 10% glacial acetic acid for 1 h and 10% methanol and 5% glacial acetic acid for 2 h. Each staining step was performed at room temperature. Images were obtained using the Gel Image Formation system (LI-COR Biosciences, Lincoln, NE, USA) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Pro-MMP-9 and MMP-9 expression was detected using an ECL detection kit (Beyotime Institute of Biotechnology).

Data analysis. All experiments were conducted at least 3 times in duplicates/triplicates. Results are presented as the mean ± standard deviation. One-way analysis of variance with a post hoc Tukey's test was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

CA treatment inhibits the proliferation of A375 cells. The effect of treatment with CA on cell viability was determined with an MTT assay. Cells were in various concentrations (0, 0.001, 0.01, 0.03, 0.1, 0.3 or 1 µM) of CA. Growth inhibition was induced in a dose- and time-dependent manner (Fig. 1A and B). As presented in Fig. 1A, cell viability was not significantly affected by low concentrations at 24 h compared with high-concentration treatment. Similar results were obtained from viable cell counting with trypan blue staining with CA concentrations of 0.03, 0.1 and 0.3 µM (Fig. 1C). Therefore, a concentration range of 0.03, 0.1 and 0.3 µM CA was selected for the subsequent experiments.

CA inhibits the activity of V-ATPases, increases the acidity of the cytoplasm and increases the alkalinity of the lysosome in A375 cells. To determine whether CA inhibits the expression of V-ATPase, A375 cells were treated with DMSO, CA (0.03, 0.1 and 0.3 µM) or bafilomycin A1 for 24 h and were analyzed for V-ATPase activity using a V-ATPase-specific kit.

Following a 24-h CA treatment at various concentrations, the expression level of V-ATPase was altered when compared with the control group (Fig. 2A). The expression levels of V-ATPase in the low concentration group were significantly less compared with the control group (P<0.001). A dose-dependent association was observed between the CA concentration and the expression level of V-ATPase.

Following the inhibition of V-ATPase, hydrogen ions in the cytoplasm can no longer be transported from the cell. Therefore, the present study investigated the pH of A375 cells treated with CA. The standard curve of fluorescence intensity vs. the pH value of A375 cells is presented in Fig. 1C.
model was established with the equation: \[ Y = 0.1625X^{3.1947}. \]

The pH values of A375 cells prior to and following CA treatment with various concentrations for 24 h were calculated based on this formula. As included in Fig. 2C, it was revealed that the pH values in A375 cells treated with CA at concentrations of 0.1 and 0.3 µM for 24 h were significantly lower compared with that in the control group (P<0.05 and P<0.01 respectively).

It was determined whether CA can alkalize the lysosomal pH using the LysoTracker Red lysosomal fluorescent probe. The lysosome is usually acidic, which induces the production of red fluorescence; when the lysosomal pH increases, the intensity of red fluorescence decreases. The present study determined the lysosomal pH of A375 cells following treatment with CA and bafilomycin A1. It was revealed that the red fluorescence intensity was decreased in a dose-dependent manner (Fig. 2D).

CA inhibits cell migration. The wound-healing assay was used to investigate the migration of A375 cells cultured with the investigated concentrations (0, 0.03, 0.1, and 0.3 µM) of CA and the positive control drug (bafilomycin A1). The results revealed that CA significantly reduced the migration of A375 cells in a dose-dependent manner at 24 h (Fig. 3A and B).

CA inhibits cell invasion. A significant difference was observed in the motility of A375 cells treated with CA in the Transwell assay. Following a 24-h treatment with CA, the invasion ability...
Figure 3. Effect of CA on the cell migration and invasion of A375 cells. (A) A375 cells were wounded and then treated with CA (0, 0.03, 0.1 and 0.3 µM) or bafilomycin A1 for 24 h. At 0 and 24 h, phase-contrast images of the wounds were captured (magnification, x200). (B) Histogram of cell mobility. Data are presented as the percentage change (means ± SD) and represent three independent experiments. (C) Cell invasion at 24 h following treatment with CA was evaluated by a Matrigel invasion assay. Representative microscopic images following CA treatment are included (magnification, x200). (D) Comparison of the rate of A375 cell invasion. Data are expressed as the percentage change (means ± SD) and represent three independent experiments. *P<0.05 and ***P<0.001 vs. the control. CA, cleistanthin A; SD, standard deviation; Con, control.

Figure 4. Effects of CA on the activity and expression of MMP-2 and MMP-9 in A375 cells. A375 cells were treated with CA or bafilomycin A1. The (A) activity and (B) expression of MMP-2, and the (C) activity and (D) expression of MMP-9 were determined with gelatin zymography and western blot analysis. Data represents 3 replicates and 3 repeats. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001. CA, cleistanthin A; MMP, matrix metallopeptidase; Con, control.
Fig. 4. With cell migration (subfamily involved in pericellular proteolysis associated MMP-14), an activator of proMMP-2, was the first MMP to the extracellular matrix (proteases that contribute to cancer cell invasion by degrading multi-factorial processes, regulated by complex mechanisms, respectively.

Gelatin zymography was used to analyze the activity of secreted MMP-2 and -9 in A375 cells treated with or without CA. Fig. 4A demonstrated that the activity of MMP-2 in the 0.03, 0.1 and 0.3 µM CA groups was significantly lower compared with the control group in a dose-dependent manner (P<0.05, P<0.01, P<0.001, respectively). The protein expression level of MMP-2 was also reduced with CA treatment (Fig. 4B). A similar result was obtained for MMP-9 (Fig. 4C and D).

Discussion

CA, a natural compound, may exhibit anti-proliferative activity in vitro against MCF-7, HeLa, HepG2, HCT-116 and U251 cancer cell lines (14). In the present study the toxic effect of CA on A375 cells was preliminary investigated. It was revealed that the growth inhibition of A375 cells was induced in a dose- and time-dependent manner (Fig. 1A and B); however, the viability of A375 human melanoma cells was not significantly affected at low concentrations at 24 h, compared with high-concentration treatment. CA also inhibited cell invasion and metastasis in a dose-dependent manner at 24 h. Based on these results, CA may have potential as an anticancer agent.

It was previously reported that blocking the V-ATPases can inhibit the growth and metastasis of human cancer cells (10,17). It has also been demonstrated that diphyllin inhibits V-AtPase, and therefore, lysosomal acidification in osteoclasts, which leads to the abrogation of bone resorption (18). The present study suggested that CA inhibits the expression of V-ATPases on A375 cells at concentrations of 0.03, 0.1 or 0.3 µM. The administration of CA may inhibit the concentration of V-ATPases in the cell membrane, affecting their role in transporting H+ out of the cell, as V-ATPase is a proton pump targeting to the subunit ATP6L of proton pump. Cancer Res 65: 771-779, 2010.

In conclusion, the present study revealed that CA is a novel inhibitor of V-ATPases that induced a decrease in A375 cell migration and invasion via the inhibition of the expression of MMP-2 and -9. Recently, it was reported that the transcription of MT1-MMP was regulated by the Wnt signaling pathway (28,29). It is necessary to determine whether CA inhibits the activation of MMPs via the Wnt signaling pathway further study.

References


