Ascleposide, a natural cardenolide, induces anticancer signaling in human castration-resistant prostatic cancer through Na⁺/K⁺-ATPase internalization and tubulin acetylation

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Abstract

Background: Cardiac glycosides, which inhibit Na⁺/K⁺-ATPase, display inotropic effects for the treatment of congestive heart failure and cardiac arrhythmia. Recent studies have suggested signaling downstream of Na⁺/K⁺-ATPase action in the regulation of cell proliferation and apoptosis and have revealed the anticancer activity of cardiac glycosides. The study aims to characterize the anticancer potential of ascleposide, a natural cardenolide, and to uncover its primary target and underlying mechanism against human castration-resistant prostate cancer (CRPC).

Methods: Cell proliferation was examined in CRPC PC-3 and DU-145 cells using sulforhodamine B assay, carboxyfluorescein succinimidyl ester staining assay and clonogenic examination. Flow cytometric analysis was used to detect the distribution of cell cycle phase, mitochondrial membrane potential, intracellular Na⁺ and Ca²⁺ levels, and reactive oxygen species production. Protein expression was examined using Western blot analysis. Endocytosis of Na⁺/K⁺-ATPase was determined using confocal immunofluorescence microscopic examination.

Results: Ascleposide induced an increase of intracellular Na⁺ and a potent antiproliferative effect. It also induced a decrease of G1 phase distribution while an increase in both G2/M and apoptotic sub-G1 phases, and downregulated several cell cycle regulator proteins, including cyclins, Cdk, p21, and p27 Cip/Kip proteins, Rb and c-Myc. Ascleposide decreased the expression of antiapoptotic Bcl-2 members (eg, Bcl-2 and Mcl-1) but upregulated proapoptotic member (eg, Bak), leading to a significant loss of mitochondrial membrane potential and activation of both caspase-9 and caspase-3. Ascleposide also dramatically induced tubulin acetylation, leading to inhibition of the catalytic activity of Na⁺/K⁺-ATPase. Notably, extracellular high K⁺ (16 mM) significantly blunted ascleposide-mediated effects. Furthermore, ascleposide induced a p38 MAPK-dependent endocytosis of Na⁺/K⁺-ATPase and downregulated the protein expression of Na⁺/K⁺-ATPase α1 subunit.
1 | INTRODUCTION

The Na⁺/K⁺-ATPase, an oligomeric protein composed of α subunits, β subunits, and FXYD proteins, is ubiquitously expressed in all animal cells to generate electrochemical ion gradients which are crucial to many cellular functions. There are four isoforms of catalytic α subunits and three isoforms of regulatory β subunits. The α1 and β1 isoforms are extensively expressed, playing a key role for the Na⁺/K⁺-ATPase function in most cells; whereas, other Na⁺/K⁺-ATPase subunit isoforms are expressed tissue specifically. The α2 isoform is expressed predominantly in muscle to regulate contractility. The α2 and α3 isoforms are mainly in the nervous system and are associated with neurological disorders, whereas α4 isoform is expressed in testis. The β2 isoform is expressed mainly in brain and muscle, while the β3 isoform is in various tissues and organs. Notably, the α1, α3, β1, and β2 subunits have been indicated to be associated with cancers, including glioma, breast cancer, hepatocellular carcinoma, non-small-cell lung cancer, prostate cancer and colorectal cancer.

Cardiac glycosides, which inhibit Na⁺/K⁺-ATPase in the myocardium, have well-characterized inotropic effects for the treatment of congestive heart failure and cardiac arrhythmia. Inhibition of Na⁺/K⁺-ATPase induces an increase of Na⁺ in the cytoplasm and consequently Na⁺/K⁺-ATPase action including the regulation of cell proliferation, tubulin acetylation, and cell cycle arrest. Cell apoptosis is ultimately triggered by the activation of caspase cascade attributed to mitochondrial damage through the downregulation of Bcl-2 and Mcl-1 protein expressions while upregulation of Bak protein levels. The data also suggest the potential of ascleposide in anti-CRPC development.

KEYWORDS
ascleposide, endocytosis, Na⁺/K⁺-ATPase α1-subunit, p38 MAPK, tubulin acetylation

Conclusion: Ascleposide displays antiproliferative and apoptotic activities dependent on the inhibition of Na⁺/K⁺-ATPase pumping activity through p38 MAPK-mediated endocytosis of Na⁺/K⁺-ATPase and downregulation of α1 subunit, which in turn cause tubulin acetylation and cell cycle arrest. Cell apoptosis is ultimately triggered by the activation of caspase cascade attributed to mitochondrial damage through the downregulation of Bcl-2 and Mcl-1 protein expressions while upregulation of Bak protein levels. The data also suggest the potential of ascleposide in anti-CRPC development.

2 | MATERIALS AND METHODS

2.1 | Materials

Human prostate adenocarcinoma cell lines, PC-3 and DU-145, were obtained from American Type Culture Collection (Rockville, MD). Roswell Park Memorial Institute Medium (RPMI) 1640 medium, fetal bovine serum (FBS), Pen-Strep Ampho. Solution (10.000U/mL of penicillin, 10 mg/mL of streptomycin, and 0.025 mg/mL of amphotericin B) was purchased from Gibco/BRL Life Technologies (Grand Island, NY). Dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), propidium iodide (PI), sulforhodamine B (SRB), leupeptin, NaF, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide), sodium orthovanadate (Na3VO4), Fluo-3/AM (Fluo-3/Acetoxyethyl ester derivatives) were obtained from Sigma-Aldrich (St. Louis, MO). DCF-DA (2′,7′-dichlorodihydrofluorescein diacetate) was purchased from Molecular Probes (Eugene, OR). CoroNa Green was obtained from Thermo Fisher Scientific Inc (Waltham, MA). Monoclonal antibody of caspase-3 was from Imgenex, Corp (San Diego, CA). Monoclonal antibodies of cleaved caspase-9, acetyl-α-tubulin (Lys40), calpain 2, p21, p-c-JunS65, p-extracellular-signal-regulated kinase (ERK) 1/2T202/Y204, p-c-jun N-terminal kinase (JNK) T183/Y185, p-p38T180/Y182, p-RbS807/811, cyclin D1, and androgen receptor (AR) were from Cell Signaling.
Monoclonal antibodies of Bcl-2, Bak, cyclin-dependent kinase (Cdk) 1, Cdk2, Cdk4, c-Myc, cyclin A, cyclin B, cyclin E, GAPDH, Mcl-1, p27, PARP-1, and HRP-conjugated antimouse and antirabbit IgGs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies of Na+/K+-ATPase α1 and α3 subunits were purchased from ABChem (Cambridge, MA). Monoclonal antibody of Na+/K+-ATPase β1 was purchased from Upstate (Upstate, Lake Placid, NY). Ascleposide (Figure 1A) was isolated from R. formosana and its structure as well as preliminary cytotoxicity in cancer cells were determined in the previous study.21

**FIGURE 1** Intracellular Na⁺ increasing activity and antiproliferative effect of ascleposide in human castration-resistant prostate cancer cells. Chemical structure of ascleposide (A). Cells were treated with or without ascleposide in the absence or presence of 16 mM KCl for 24 hours (B) or 48 hours (C). Intracellular Na⁺ contents were detected using flow cytometric analysis (B) or cell proliferation was determined using sulforhodamine B assay (C). Cells were treated with or without ascleposide for eight days and cell colonies were detected using crystal violet detection assay (D). Cells were incubated with or without 100 nM ascleposide. After treatment, cells were harvested for flow cytometric analysis of carboxyfluorescein succinimidyl ester staining. The proliferation index and the cell populations of a parent or different generations were calculated by Modfit LT Version 3.2 and WinList Version 5.0 software (E). Quantitative data are expressed as mean ± SEM of three independent experiments. **P < .01 compared with the control (Color figure can be viewed at wileyonlinelibrary.com)
2.2 | Cell culture

PC-3, DU-145, and LNCaP cells were cultured in RPMI 1640 medium supplemented with 5% FBS (v/v), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cultures were maintained in a 37°C incubator with 5% CO2. Adherent cultures were passaged using 0.05% trypsin-EDTA after reaching 80% confluence.

2.3 | Measurement of intracellular sodium content

PC-3 and DU-145 cells were treated with vehicle (0.1% dimethyl sulfoxide [DMSO]) or compound and incubated with or without a total concentration of 16 mM KCl for 24 hours. Before the detection of intracellular sodium content, cells were washed with phosphate-buffered saline (PBS) and pretreated with 5 μM CoroNa Green dissolved in HBSS for 45 minutes at 37°C. Cells were then once again washed with PBS and intracellular Na⁺ contents were detected using flow cytometric analysis and CellQuest software (Becton Dickinson, Mountain View, CA). In all, 10,000 events were collected for analysis of each sample.

2.4 | SRB and clonogenic assays

Cells were seeded in 96-well plates in culture medium with 10% FBS. After 24 hours, cells were fixed with 10% trichloroacetic acid to represent the cell population at the time of compound addition (TZ). After incubation of 0.1% DMSO or the compound for 48 hours, cells were fixed with 10% trichloroacetic acid, and SRB at 0.4% (w/v) in 1% acetic acid was added for staining. Unbound SRB was washed with 1% acetic acid and SRB bounded cells were solubilized with 10 mM Tris. Absorbance was examined at 515 nm wavelengths. Growth inhibition (GI[50]) was determined at the compound concentration resulting in 50% reduction of total protein increase in control cells. To examine anchorage-dependent clonogenic effect, cells were seeded in six-well plates. After an 8-day treatment with the compound, cell colonies were rinsed with PBS, stained with 0.4% (w/v) crystal violet/20% methanol and lysed by 50 mM sodium citrate/50% ethanol. The absorbance was read at 595 nm wavelengths.

2.5 | Cell proliferation assay with carboxyfluorescein succinimidyl ester (CFSE) staining

CFSE was dissolved in DMSO and was kept at −20°C until use. The cells were adjusted to 10⁶ cells/mL and treated with CFSE (5 μM). After incubation at 37°C for 10 minutes, labeling was blocked by the RPMI medium with 10% FBS. After centrifugation, cells were seeded in RPMI medium with 10% FCS with or without the compound for 24 and 48 hours at 37°C under 5% CO₂/95% air. The fluorescence intensity was determined by flow cytometry. Cell proliferation was assessed by monitoring the decrease in label intensity in daughter cells. The proliferation index and cell populations of a parent or different generations were calculated by using Modfit LT Version 3.2 and WinList Version 5.0 software (Topsham, ME).

2.6 | Flow cytometric assay with PI staining

Cells were harvested by trypsinization, fixed with 70% (v/v) alcohol at −20°C for 30 minutes and washed with PBS. The cells were centrifuged and re-suspended with 0.5 mL PI solution containing Triton X-100 (0.1% v/v), RNase (100 μg/mL), and PI (80 μg/mL). DNA content was analyzed with the FACScan and CellQuest software.

2.7 | Western blot analysis

After treatment, cells were harvested by trypsinization, centrifuged and lysed in 0.1 mL of lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 50 mM NaF, and 100 μM sodium orthovanadate. Total protein was quantified, mixed with sample buffer and boiled at 90°C for 5 minutes. Equal amount of protein (30 μg) was separated by electrophoresis in 8% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes and was detected with specific antibodies (1:500 to 1:2000 dilutions). The immunoreactive proteins after incubation with appropriately labeled secondary antibody (1:5000 dilution) were detected with an enhanced chemiluminescence detection kit (Amer sham, Buckinghamshire, UK) and images were captured by ChemiDoc MP System (Bio-Rad Laboratories, Hercules, CA).

2.8 | Measurement of mitochondrial membrane potential (ΔΨᵐ)  

JC-1, a mitochondrial dye staining mitochondria in living cells in a membrane potential-dependent fashion, was used to determine ΔΨᵐ. Cells were treated with or without the compound. Thirty minutes before the termination of incubation, cells were incubated with JC-1 (5 μM) at 37°C for 10 minutes. Accumulation of JC-1 was analyzed using flow cytometric analysis with FACScan FL1 and FL2 channel and CellQuest software. In all, 10,000 events were collected for analysis of each sample.

2.9 | Measurement of intracellular calcium levels

Cells were treated with vehicle (0.1% DMSO) or compound and incubated for the indicated time. Before the detection of intracellular calcium levels, cells were washed with PBS, preincubated with 2.5 μM fluo-3/AM for 30 minutes at 37°C and then washed twice with PBS. Intracellular Ca²⁺ was analyzed using flow cytometric analysis with FACScan FL1 channel and CellQuest software. In all, 10,000 events were collected for analysis of each sample.
2.10 Measurement of reactive oxygen species

Cells were treated with vehicle (0.1% DMSO) or compound for the indicated time. For the measurement of reactive oxygen species (ROS) production, cells were treated with 10 μM DCF-DA for 30 minutes at 37°C and then washed twice with PBS. ROS production was analyzed using flow cytometric analysis with FACScan FL1 channel and CellQuest software. In all, 10,000 events were collected for analysis of each sample.

2.11 Immunofluorescence imaging for Na+/K+-ATPase

Cells were allowed to grow on coverslips placed in six-well plates with medium containing 5% (v/v) FBS to reach about 70 to 80% confluence. Cells were then treated with vehicle (0.1% DMSO) or ascleposide in RPMI 1640 medium with or without 16 mM KCl for the indicated time. After the treatment, cells were washed with PBS and fixed by 100% methanol for 10 minutes at −20°C. Cell permeabilization was performed with 0.25% Triton X-100 for 10 minutes at room temperature. Following by PBS washing, cells were blocked with 1% BSA for 1 hour. Cells were incubated with anti-Na+/K+-ATPase α1 primary antibody for 16 hours at 4°C and then washed with PBS. FITC-conjugated secondary antibody (1: 100 dilution) was applied to cells primary antibody for 16 hours at 4°C and then washed with PBS. FITC-conjugated secondary antibody (1: 100 dilution) was applied to cells for 1 hour at room temperature. After PBS washing, cells were stained with DAPI nuclear staining (1: 1000 dilution) for 10 minutes. After PBS wash, coverslips with cells were mounted on microscope slides with DAPI nuclear staining (1: 1000 dilution) for 10 minutes. After PBS wash, coverslips with cells were mounted on microscope slides with DAPI nuclear staining (1: 1000 dilution) for 10 minutes. After PBS wash, coverslips with cells were mounted on microscope slides with DAPI nuclear staining (1: 1000 dilution) for 10 minutes.

2.12 Data analysis

Data were presented as mean ± SEM. Statistical analysis was performed and two-group comparisons were done with Student t-test. P < .05 was considered statistically significant.

3 RESULTS

3.1 Ascleposide potently inhibits cell proliferation and induces cell cycle arrest in CRPC cells

Na+/K+-ATPase, an active electronegic transmembrane ATPase in all animal cells, pumps Na+ out of cells while simultaneously pumping K+ into cells despite against the concentration gradients. Ascleposide induced an increase of intracellular Na+ in both PC-3 and DU-145 cells (Figure 1B). Since the extracellular K+ could provide an additional K+ concentration gradient to overcome the inhibition of Na+/K+-ATPase, the blunted effect by extracellular K+ validated the inhibitory activity of ascleposide against Na+/K+-ATPase (Figure 1B). The cell proliferation assessment was determined using the SRB assay. The data demonstrated that ascleposide inhibits cell proliferation in PC-3 and DU-145 cells with GI50 values of 27.2 ± 1.1 and 65.9 ± 2.4 nM, respectively, showing that ascleposide displayed good antiproliferative activity in both PC-3 and DU-145 cells. Notably, the inhibitory effect was significantly blunted to 55.1 ± 6.3 and 146.6 ± 3.4 nM, respectively, in the presence of high extracellular K+ (Figure 1C). Furthermore, ascleposide-induced antiproliferative effect was further substantiated by using anchorage-dependent clonogenic assay (Figure 1D) and flow cytometric analysis of CFSE staining (Figure 1E). The data showed that ascleposide profoundly inhibited colony formation and cell proliferation in both PC-3 and DU-145 cells. Furthermore, ascleposide also inhibited the cell proliferation of androgen-dependent prostate cancer LNCaP cells using SRB assay and flow cytometric analysis of CFSE staining (Figure S1). The presence of high extracellular K+ also blunted ascleposide-induced inhibitory effect (Figure S1A).

3.2 Ascleposide induces the change of cell cycle distribution and downregulates multiple cell cycle regulators

The Cdk which forms a complex with the associated cyclin is a key player in the regulation of the initiation, progression, and completion of the cell cycle. Cellular damage normally induces an arrest of the cell cycle or apoptosis. Cell cycle arrest most frequently occurs at G1/S or G2/M boundaries. The data showed that ascleposide induced a time- and concentration-dependent decrease of G1 phase distribution while a subsequent increase of that at both G2/M and sub-G1 phases (Figures 2A and 2B). Notably, cyclin D1 was the most susceptible to ascleposide in downregulation of protein levels in a time-dependent fashion; Cdk4 and cyclin A also were downregulated. In contrast, cyclin E, cyclin B, Cdk2, and Cdk1 maintained constant levels to ascleposide action (Figure 3).

c-Myc, a cellular oncprotein in the control of cell proliferation, programmed cell death and differentiation, is implicated to regulate the Cdk4/cyclin D1 complex activity.22 Rb is a tumor suppressor protein in inhibiting excessive cell proliferation through binding to transcription factors E2F family and thereby inhibiting cell cycle progression. Rb can be phosphorylated by both Cdk4/cyclin D1 and Cdk2/Cyclin E complexes, leading to the dissociation of E2F and facilitating cell cycle progression.23 As expected, ascleposide induced a significant downregulation of c-Myc protein and inhibited the phosphorylation of Rb (Figure 3), which were responsible for the inhibition of cell cycle progression and thereby blocking the cell proliferation. Of note, ascleposide also downregulated the protein expressions of both p21 and p27 Cip/Kip proteins (Figure 3). This is different from the general function of Cip/Kip proteins as Cdk inhibitors that are upregulated upon the cell cycle arrest. The possible function of p21 and p27 unrelated to Cdk inhibition will be discussed below.
FIGURE 2  Effect of ascleposide on cell cycle distribution. PC-3 cells were incubated in the absence or presence of ascleposide at the indicated concentration (A) or 100 nM (B) for the indicated time. The cells were harvested for propidium iodide staining to analyze the distribution of cell populations in cell cycle phases using FACScan flow cytometric analysis. Data are expressed as mean ± SEM of three independent determinations. *$P < .05$, **$P < .01$, and ***$P < .001$ compared with the control.
FIGURE 2  Continued
Ascleposide also induced a concentration- and time-dependent increase of the sub-G1 population in LNCaP cells indicative of cell apoptosis (Figure S2A). Notably, ascleposide significantly downregulated the protein expression of AR and its downstream target, c-Myc. These effects were almost completely abolished by the presence of extracellular high K+ (Figure S2B).

### 3.3 Ascleposide regulates protein expression of Bcl-2 family members and mitochondrial stress

The mitochondria are involved in cell apoptosis through various signaling pathways in which the loss of integrity of the outer membrane resulted from the regulation of Bcl-2 family of protein plays the most critical role. The Bcl-2 family consists of three sub-families, including antiapoptotic Bcl-2-like proteins (eg, Bcl-2, Bcl-xL, and Mcl-1), proapoptotic Bax-like proteins (eg, Bax and Bak) and proapoptotic Bcl-2 homology 3 (BH3)-only proteins (eg, Bid and Bad).24 The data demonstrated that ascleposide induced a significant loss of mitochondrial membrane potential indicating the induction of mitochondrial stress. Notably, the application of extracellular K+ significantly rescued the loss of mitochondrial membrane potential suggesting the inhibition of Na+/K+-ATPase by ascleposide induced the mitochondrial stress (Figure 4A). Furthermore, ascleposide induced the downregulation of antiapoptotic Bcl-2 members (eg, Bcl-2 and Mcl-1) but the upregulation of proapoptotic member (eg,
FIGURE 4  Effects of ascleposide on mitochondrial membrane potential and expression of several proteins. PC-3 cells were incubated in the absence or presence of the indicated condition for 24 hours (A) or 48 hours (B and C). The detection of mitochondrial membrane potential was performed using flow cytometric analysis of JC-1 staining (A). The detection of protein expression was using Western blot analysis (B and C). The immunoreactive proteins were detected and images were captured by ChemiDoc™ MP System (Bio-Rad Laboratories). Data were quantified using computerized image analysis system Image Lab™ Software (Bio-Rad Laboratories). Data are expressed as mean ± SEM of three independent determinations. *P < .05, **P < .01, and ***P < .001 compared with the control [Color figure can be viewed at wileyonlinelibrary.com]
Bak), suggesting the altered expression levels of Bcl-2 family protein contributed to mitochondrial stress (Figure 4B). Besides, ascleposide-mediated impact on Bcl-2 family members was rescued by the presence of extracellular high K⁺ (Figure S3). Furthermore, both the initiator (eg, caspase-9) and effector caspases (eg, caspase-3) upon the activation of mitochondrial apoptotic pathway was examined showing that ascleposide profoundly activated these caspases and the cleavage of PARP-1, a caspase-3 substrate (Figure 4C).

### 3.4 Disturbance of cell cycle regulation and tubulin acetylation are attributed to inhibition of Na⁺/K⁺-ATPase pumping activity

Inhibition of Na⁺/K⁺-ATPase pumping activity is the major pharmacological effect to cardiac glycoside action; however, it has been suggested the existence of pumping activity-independent effects.²⁵,²⁶ The data demonstrated that extracellular high K⁺ significantly rescued the inhibition of cell cycle regulators induced by ascleposide (Figure 5A) indicating pumping activity-dependent effect on the regulation of these regulators.

Tubulin acetylation, a posttranslational modification, involves the reversible addition of an acetyl group at the ε-amino group of Lys40.²⁷ It has been documented that acetylated tubulin efficiently associates with plasma membrane Na⁺/K⁺-ATPase and inhibits the catalytic activity of the enzyme.²⁸ Our data showed that ascleposide induced an increase of α-tubulin acetylation, suggesting the inhibition of Na⁺/K⁺-ATPase activity (Figure 5B). Notably, this effect was significantly blunted by the supplement of extracellular high K⁺ (Figure 5B) indicating the pumping activity-dependent effect.

### 3.5 Ascleposide downregulates α1 subunit protein levels and induces internalization of Na⁺/K⁺-ATPase

The effect of ascleposide on protein expression levels of several Na⁺/K⁺-ATPase subunits has been determined. The data showed that Na⁺/K⁺-ATPase α1 subunit was one of the major isotypes and was downregulated by ascleposide in a concentration-dependent fashion (Figure 6A). It has been suggested that cardiac glycosides, when interacting with Na⁺/K⁺-ATPase, are able to induce endocytosis of this pump.²⁹ By using fluorescence imaging, the data demonstrated that ascleposide caused profound endocytosis of Na⁺/K⁺-ATPase (Figure 6B). Mitogen-activated protein kinases (MAPKs) including p38 kinase, Erk, and JNK, have been suggested to regulate the endocytosis of Na⁺/K⁺-ATPase.³⁰-³² Ascleposide induced a time-dependent increase of p38 phosphorylation at Thr180/Tyr182, but not phosphorylation of both Erk and JNK (Figure S4). Furthermore, SB203580 (a specific inhibitor of p38 MAPK pathway) completely abolished ascleposide-induced endocytosis of Na⁺/K⁺-ATPase (Figure 6C) suggesting a p38 kinase-dependent endocytosis of the pump.
DISCUSSION

Several decades ago, Na⁺/K⁺-ATPase was identified as a molecular target of cardiac glycosides, which selectively inhibit the enzyme. The primary function of this membrane pump is to keep a low intracellular Na⁺/K⁺ ratio through pumping three Na⁺ ions outside along with two K⁺ ions inside the cell against concentration gradients. Recently, Na⁺/K⁺-ATPase appears to be an attractive cellular target on fighting against cancers. A growing body of evidence shows that cardiac glycosides serve as anticancer agents on inhibiting cell proliferation as well as inducing apoptosis in various cancers.\(^{11,13,15}\) Recently, Zeino et al\(^ {33}\) have reported the study using quantitative structure-activity relationship analysis and molecular docking into Na⁺/K⁺-ATPase suggesting a possible differential expression of the pump in multidrug-resistant and sensitive cancer cells. The tested cardiac glycosides show high activities against both resistant and sensitive cancer cells, suggesting the high anticancer potential of cardiac glycosides. Ascleposide, a natural cardenolide, induced an increase of intracellular Na⁺ that was rescuable by the supplementation of extracellular high K⁺ suggesting the inhibitory activity of ascleposide against Na⁺/K⁺-ATPase.

The data in this study demonstrated the nanomolar efficacies of ascleposide on inhibiting proliferation in both PC-3 and DU-145 cells through governing cyclin D1/Cdk4/Rb axis in cell cycle regulation. Both p21 and p27 Cip/Kip proteins are recognized as cyclin-dependent kinase inhibitors capable of inhibiting cyclin/Cdk complexes. Both p21 and p27 proteins are generally upregulated in response to the induction of cell cycle arrest. Notably, ascleposide caused a downregulation other than upregulation of these Cip/Kip proteins during an antiproliferative as well as apoptotic stress. Alternative roles of both p21 and p27 proteins have been suggested.

**FIGURE 6** Effect of ascleposide on α₁ subunit protein levels and internalization of Na⁺/K⁺-ATPase. (A) PC-3 cells were incubated in the absence or presence of ascleposide for 24 hours. Cells were lysed for the detection of indicated protein expression by Western blot analysis. The immunoreactive proteins were detected and images were captured by ChemiDoc MP System (Bio-Rad Laboratories). Data were quantified using computerized image analysis system Image Lab Software (Bio-Rad Laboratories). Data are expressed as mean ± SEM of three independent determinations. **P < .01 and ***P < .001 compared with the control. (B and C) PC-3 cells were treated with or without 100 nM ascleposide in the absence or presence of 5 μM SB203580. After the treatment, cells were fixed, permeabilized and immunofluorescence labeled with anti-Na⁺/K⁺-ATPase α₁ primary antibody and FITC-conjugated secondary antibody (green fluorescence). The nucleus was detected using DAPI staining (blue fluorescence). The images were captured by a confocal microscope Zeiss LSM 880 (Carl Zeiss) [Color figure can be viewed at wileyonlinelibrary.com]
accordingly. Rosato et al. have reported that flavopiridol blocks sodium butyrate (a histone deacetylase inhibitor)-mediated p21 induction, inducing mitochondrial damage and caspase-3 activation in leukemic cells. Suzuki et al. have shown that p21 is able to interact with procaspase-3 on mitochondria to resist Fa-s-mediated cell death in hepatocellular carcinoma cells. Furthermore, p27 overexpression has been suggested to induce resistance to drug-mediated apoptosis in leukemic cells. Given accumulating evidence supporting that both p21 and p27 play roles in resisting apoptotic response in cancer cells, the downregulation of both p21 and p27 proteins might, at least partly, explain ascleposide-induced anticancer activity in CRPC cells.

Mitochondrial outer membrane permeabilization (MOMP) is frequently the decisive step between survival and death in lots of stress signal transduction pathways which converge on mitochondria. Pro- and antiapoptotic members of Bcl-2 family are key players on determining the MOMP. Inhibition of MOMP is critical for cell survival and, therefore, strategic induction of MOMP in tumor cells is a goal of anticancer chemotherapy. Ascleposide induced a profound loss of mitochondrial membrane potential resulted from an inhibition of Na+/K+-ATPase pumping activity. Uptregulation of proapoptotic Bcl-2 family members (eg, Bak) and downregulation of antiapoptotic members (eg, Bcl-2 and Mcl-1) were responsible to the mitochondrial damage. Several lines of evidence suggest that Bcl-2 sequesters BH3 domain-only molecules including Bad, Bim, and NOXA1 in stable mitochondrial complexes, inhibiting the activation of Bak and Bax. Furthermore, Hockings et al. have reported that Mcl-1 preferentially sequesters Bak in resistance to anticancer treatments, including BH3 mimetics. Our data showed that ascleposide significantly downregulated Bcl-2 and Mcl-1, while upregulated Bak protein expression, suggesting the convergent of the Bcl-2 family members to mitochondrial damage. Of note, several events have been suggested to induce the upregulation of Bak, including p53, intracellular calcium levels, and oxidative stress. The role of p53 in this study was disregarded because of the use of p53-null PC-3 cells. Besides, ascleposide did not induce the production of ROS (data not shown). However, it induced a profound increase of intracellular Ca2+ levels monitored by using Fluo-3 AM calcium indicator and the detection of 40- and 55-kDa autocatalytic forms of m-calpain (Figure S5). The data suggest that the increase of intracellular Ca2+ levels may be probably responsible for ascleposide action.

Recent studies have shown that l-glutamate induces dissociation of acetylated tubulin/Na+,K+-ATPase complex, leading to an increase of the enzyme activity. The sodium ionophore monensin displays a similar effect to l-glutamate. These studies support the function of acetylated tubulin on reversibly forming a complex with Na+/K+-ATPase, leading to the inhibition of the enzyme activity. This inhibitory effect has been shown to play a crucial role of physiological importance in a wide variety of cell types. The cytoplasmic domain 5 of Na+/K+-ATPase has been identified to interact with acetylated tubulin, supporting that Na+/K+-ATPase may serve as an anchorage site for the interaction between microtubule and plasma membrane. Our data showed that ascleposide induced an increase of tubulin acetylation and this effect was blunted by the external high K+. These data supported the inhibition of Na+/K+-ATPase activity to ascleposide action and addressed the crucial role of acetylated tubulin on the regulation of Na+/K+-ATPase activity.

Ascleposide induced the endocytosis of Na+/K+-ATPase in this study. The activities of Erk, JNK and p38 MAPK were determined accordingly because these MAPKs have been addressed to regulate the endocytosis of Na+/K+-ATPase. Khundmiri et al. have reported that parathyroid hormone inhibits Na+/K+-ATPase activity through Erk dependent signaling pathways that are associated with an increase of serine phosphorylation of the α1 subunit and a subsequent endocytosis. In contrast, high CO2 (hypercapnia) has been suggested to induce JNK-dependent phosphorylation of LMO7b, a scaffolding protein, and to facilitate the endocytosis of Na+/K+-ATPase in alveolar epithelial cells. However, ascleposide did not modify the activity of both Erk and JNK. Notably, activation of p38 kinase has been evident in regulating the endocytosis of several cell surface proteins including Na+/K+-ATPase. In consistent with the notion, ascleposide induced a time-dependent phosphorylation and activation of p38 MAPK. Furthermore, the specific inhibitor of p38 MAPK significantly blunted the endocytosis of Na+/K+-ATPase to ascleposide action suggesting the p38 kinase-dependent mechanism.

5 | CONCLUSION

The data suggest that ascleposide induces anticancer effects in CRPC in a sequential manner. Ascleposide displays antiproliferative and apoptotic activities dependent on the inhibition of Na+/K+-ATPase pumping activity through p38 MAPK-mediated endocytosis of Na+/K+-ATPase and downregulation of α1-subunit, which in turn cause tubulin acetylation and cell cycle arrest. Cell apoptosis is ultimately triggered by the activation of caspase cascade attributed to mitochondrial damage through the downregulation of Bcl-2 and Mcl-1 protein expressions while upregulation of Bak protein levels. The data also suggest the potential of ascleposide in anti-CRPC development.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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