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Research article

Anticancer activity and potential mechanisms of 1C, a ginseng saponin derivative, on prostate cancer cells

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ABSTRACT

Background: AD-2 (20(*R*)-dammarane-3b, 12b, 20, 25-tetrol; 25-OH-PPD) is a ginsenoside and isolated from *Panax ginseng*, showing anticancer activity against extensive human cancer cell lines. In this study, effects and mechanisms of 1C ((20*R*)-3b-*O*-(L-alanyl)-dammarane-12b, 20, 25-triol), a modified version of AD-2, were evaluated for its development as a novel anticancer drug.
Methods: MTT assay was performed to evaluate cell cytotoxic activity. Cell cycle and levels of reactive oxygen species (ROS) were determined using flow cytometry analysis. Western blotting was employed to analyze signaling pathways.
Results: 1C concentration-dependently reduces prostate cancer cell viability without affecting normal human gastric epithelial cell line-1 viability. In LNCaP prostate cancer cells, 1C triggered apoptosis via Bcl-2 family-mediated mitochondria pathway, downregulated expression of mouse double minute 2, upregulated expression of p53 and stimulated ROS production. ROS scavenger, N-acetylcysteine, can attenuate 1C-induced apoptosis. 1C also inhibited the proliferation of LNCaP cells through inhibition on Wnt/β-catenin signaling pathway.
Conclusion: 1C shows obvious anticancer activity based on inducing cell apoptosis by Bcl-2 family-mediated mitochondria pathway and ROS production, inhibiting Wnt/β-catenin signaling pathway. These findings demonstrate that 1C may provide leads as a potential agent for cancer therapy.

1. Introduction

Prostate cancer is a great threat to human health. In the United States, prostate cancer is one of the most frequently diagnosed cancers and the third leading cause of cancer death in men [1]. Anti-androgen drugs can treat prostate cancers, but a lot of patients are more likely to develop androgen-independent tumors; these tumors are generally more aggressive, more resistant to currently used chemotherapeutic agents, and more metastasize than other tumor types [2–4]. Thus, novel therapeutic agents are needed to be developed to improve the treatment outcomes of prostate cancer.

Natural products obtained from medicinal herbs provide a rich source for developing novel anticancer agents and offer abundant and safe parent structures for synthetic drugs. We have recently been interested in evaluating the anticancer activity of modified compounds isolated from *Panax ginseng*. Ginseng is consumed in many countries, particularly in China and other Asian countries, to treat and prevent many diseases, such as cancer [5]. Individuals consume ginseng to reduce risks of cancers [6], including oral cavity, stomach, lung, pancreas, liver, ovary, and colon [7]. *P. ginseng* (Korean ginseng), *P. quinquefolius* (American ginseng), and other associated plants, including *P. notoginseng/pseudoginseng* (*P. notoginseng*, Buck FH Chen), are used to cure some diseases [8]. Ginseng is a widely used medicinal herb in the United States [9]. Although ginsenosides (saponins and triterpene glycosides) are only a part of the complex mixture of compounds present in these

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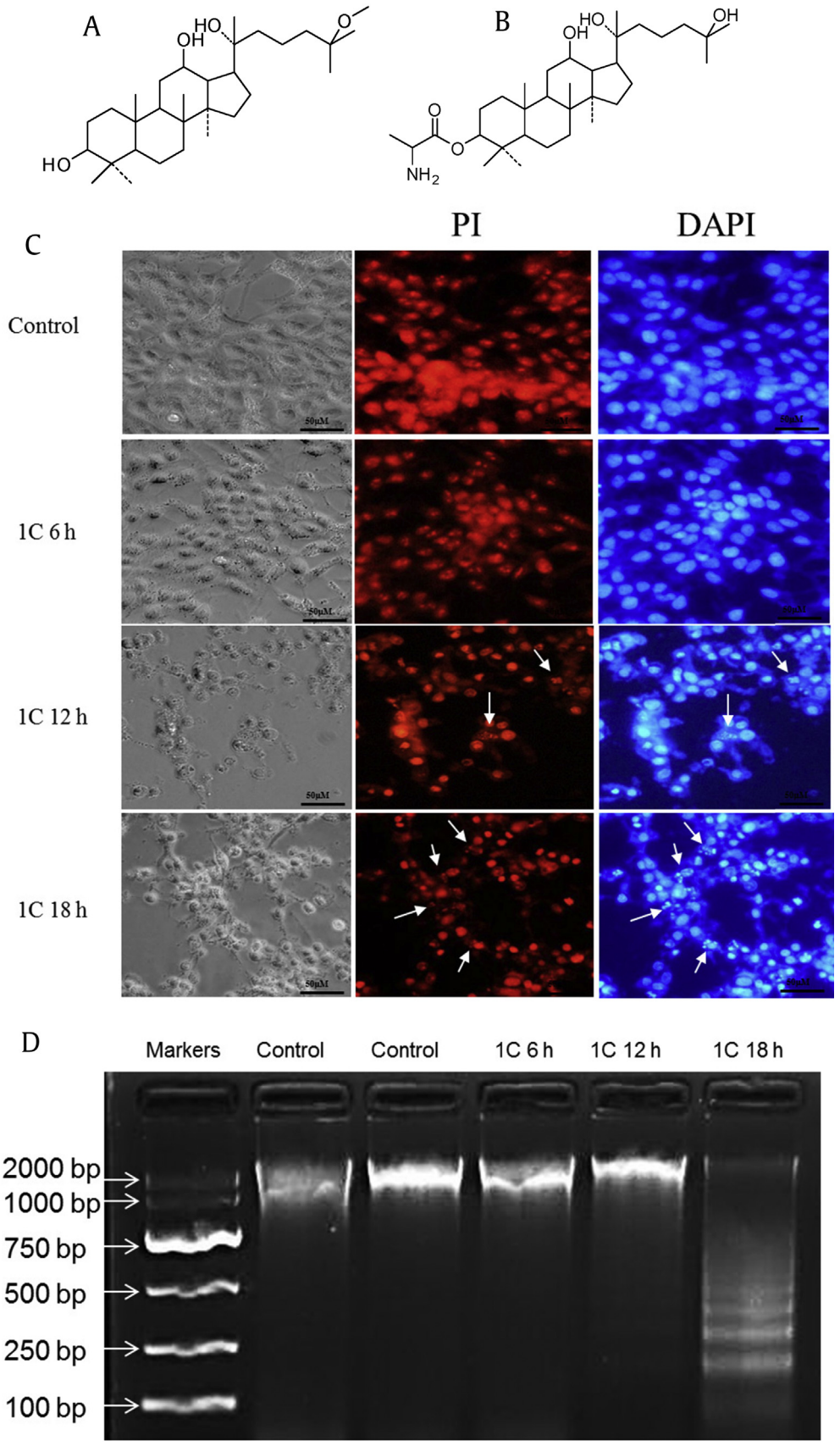


Fig. 1. 1C induced LNCaP cells apoptosis. Structures of (A) 1C and (B) AD-2. (C) The cells of nuclear morphology treated with 1C (25μM) for 0 h, 6 h, 12 h, and 18 h, as observed by a fluorescence microscope following staining with PI and DAPI. (D) DNA fragmentation analysis. DNA were separated in 2% agarose gel and visualized under ultraviolet light after staining with ethidium bromide. (E) Cell cycle analysis by flow cytometry. a (1C, 0h), b (1C, 6h), c (1C, 12h), d (1C, 18h). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (F) The expression levels of apoptosis-related proteins, inducing caspase-9, cleaved-caspase-3, and PARP were examined by Western blotting assay.

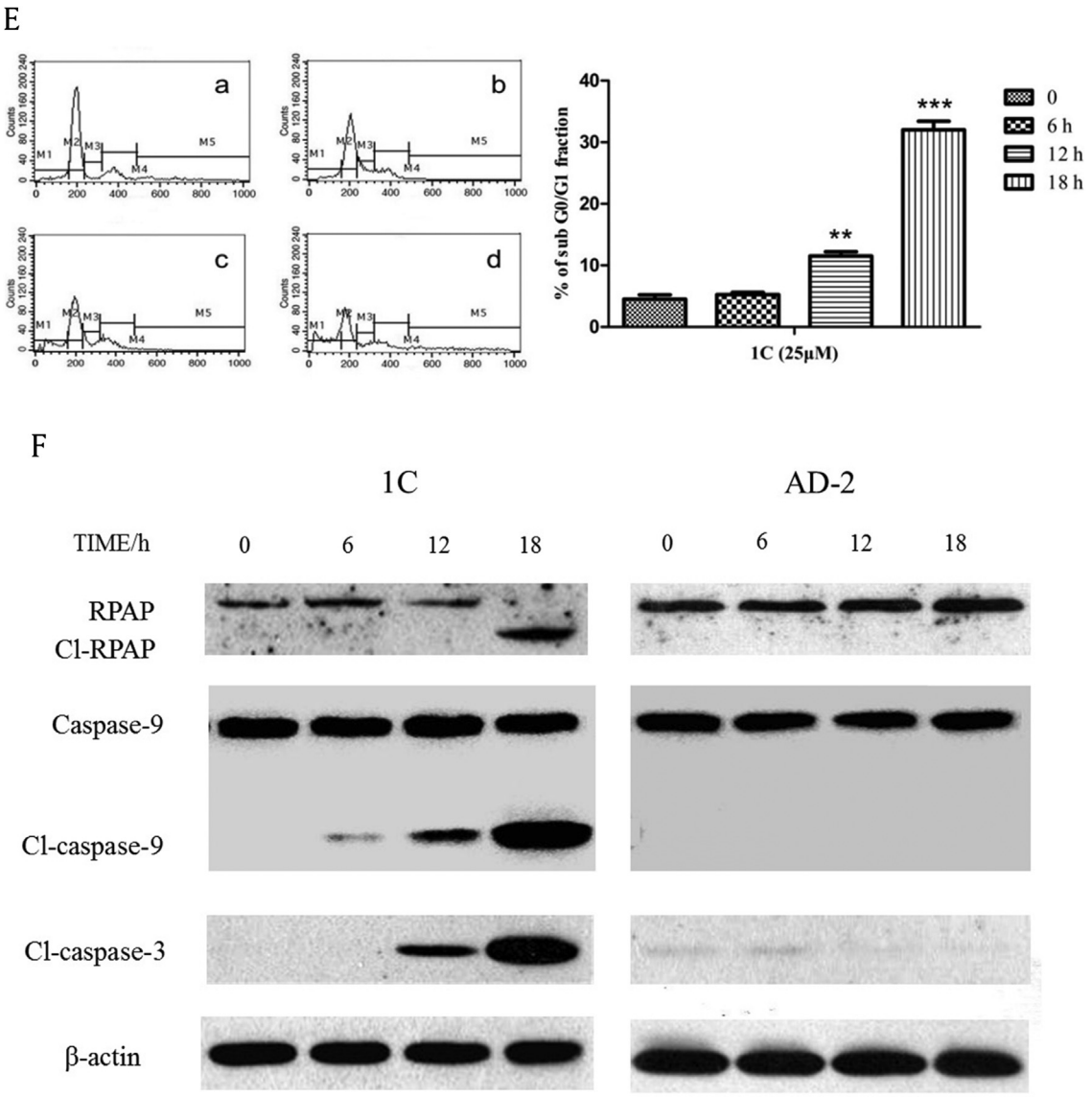


Fig. 1. (*continued*)

plants, these substances exhibit a wide range of pharmacological activities. Some ginsenosides exhibit anticancer properties via reducing DNA synthesis, angiogenesis, transformation, DNA damage, host susceptibility to mutation, and increasing immunosurveillance and apoptosis [10–13]. Ginsenosides also effectively improve the outcomes of traditional chemotherapeutic agents to prevent damages on normal tissues [5,10,14–16].

AD-2 (20(*R*)-dammarane-3b, 12b, 20, 25-tetrol; 25-OH-PPD), the structure is shown in Fig. 1A, is isolated from *P. ginseng*. The effects of this compound on breast and pancreatic cancers were examined in previous reports [17–19]. It is well known that appropriate modification of natural products' structure can improve its performance, which is one of the effective ways to explore the development of new drugs. Therefore, to improve the antitumor activity of AD-2, we introduced an extra amino group to AD-2 [20]. Finally, we obtained 1C and found that 1C exhibited better antitumor activity on MCF-7, A549, LoVo, HCT-116, HT-29, and U-87 cell lines than AD-2 via MTT assay [20], and the chemical structure is shown in Fig. 1B; however, the effect of 1C on prostate cancers was not reported. Our present study aimed to evaluate the anticancer activity of 1C *in vitro* and preliminarily investigated active mechanism and determined whether 1C can be developed as a promising therapeutic agent against prostate cancers. This study is a part of our continuous effort to search for effective modified products that can be used as anticancer agents.

2. Materials and method

2.1. Chemical and reagents

Rg3(β-D-Glucopyranoside,(3β,12β)-12,20-dihydroxydammar-24-en-3-yl-2-O-β-D-glucopyranosyl) (> 98%), AD-2 (> 95%), and 1C (> 95%) were obtained as previously described [20,21]. Mitomycin C (Mito C; > 98%) was bought from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum, Dulbecco's Modified Eagle's medium (DMEM) medium, and penicillin–streptomycin were purchased from Thermo Fisher Scientific Co., Ltd (St. Wyman, MA, USA). MTT reagent was acquired from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell cultures

Human LNCaP, PC3, 22RV1, DU-145, C4-2B, and GES-1 cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All prostate cancer cell lines and human normal cell-GES-1 were cultured in DMEM medium, containing 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C in a humid atmosphere (5% CO_2–95% air). All cells were harvested by brief incubation in 0.25% (w/v) EDTA-phosphate buffered saline (PBS).

2.3. Cell viability assay

Cell proliferation inhibition caused by Rg3, AD-2, 1C, and Mito C was examined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay. LNCaP, PC3, 22RV1, DU145, C4-2B and GES-1, were cultured in 96-well plates (5000 cells/well). Control cells were exposed to 0.1% dimethyl sulfoxide (DMSO). Test cells were treated with different concentrations (10μM, 20μM, 30μM, 40μM, and 50μM) of Rg3, AD-2, 1C, and Mito C for 24 h. The cells were subsequently incubated with 10 μL MTT (5 mg/mL) for 4 h, and the plates were then read by a plate reader (Bio-Rad iMARK, Berkeley, California, USA) at 495 nm to determine the percentage of surviving cells. Three replicates were performed for each treatment.

2.4. Determination of morphological changes of cells

LNCaP cells were seeded at a concentration of 1×10^5 cells/well in a six-well plate. After 24 h of incubation, the cells were then treated with 1C for 0 h, 6 h, 12 h, and 18 h. Control cells were exposed to 0.1% DMSO. The cells were fixed with 4% formaldehyde for 1 h at 4°C. After fixation, the cells were treated with 5μM DAPI (4′,6-diamidino-2-phenylindole) and propidium iodide (50 μg/mL PI, 50 μg/mL RNase, 0.1% sodium citrate, and 0.1% Triton X-100, pH 8.0) at 37°C in the dark for 10 min [19]; then, the cells were observed using a fluorescence microscope.

2.5. Detection of DNA fragmentation

LNCaP cells were collected after they were treated with 1C and 0.1% DMSO for 0 h, 6 h, 12 h, and 18h. Genomic DNA was extracted from the cells using the Animal cell genomic DNA isolation kit (Ding Guo Biotech Ltd., Beijing). The extracted DNA was subjected to electrophoresis using 2% agarose gel. After electrophoresis, the gel was stained with ethidium bromide and visualized under UV exposure using Gel Doc XR + (Bio-Rad Laboratories, Inc., USA).

2.6. Cell cycle analysis by flow cytometry

The cells were trypsinized, harvested, and washed with PBS; 75% ethanol was used to fix the treated and untreated cells for overnight at −20°C. PI solution (50μg/mL PI, 50μg/mL RNase, 0.1% sodium citrate, and 0.1% Triton X-100, pH 8.0) was employed to stain the fixed cells after washing with PBS for 30 min in the dark. FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) was performed to analyze cycle distribution. Apoptotic cells with hypodiploid DNA content were measured by quantifying the subG1 peak in the cell cycle pattern.

2.7. Measurement of intracellular reactive oxygen species' levels

Intracellular levels of reactive oxygen species (ROS) were examined using 2′,7′-dichlorofluorescein diacetate (DCFH-DA). LNCaP cells (2×10^5 cells/well) were seeded in six-well plates and incubated for 24 h. The cells exposed to 1C for 12 h and 18 h at a concentration of 25μM. DCFH-DA was added to the cells, which were incubated for 30 min at 37°C. A Cytomics FC500 flow cytometer (Beckman Coulter, California, USA) was employed to measure fluorescence intensity at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.8. Western blot analysis

RIPA lysis buffer was employed to extract total cellular proteins, and the protein concentration was determined using the bicinchoninic acid. SDS–PAGE was performed in 10% gel with equal loading amount of protein per lane. After electrophoresis, the resolved protein bands were transferred to a polyvinylidene difluoride (PVDF) membrane, and the membrane was blocked with 5% bovine serum albumin (BSA) in TBST buffer for 1 h. After blocking, the membrane was incubated with a 1:1000 dilution of primary antibody against mouse double minute 2 (MDM2; Bioworld Technology, Inc., Minnesota, USA), P53 (Proteintech Group, Inc., Chicago, USA), Cl-caspase-3 (Cell Singaling Technology, Inc., Danvers, MA, USA), Cl-caspase-9 (Cell Singaling Technology Inc), PARP (Cell Singaling Technology, Inc), Bcl-2 (Santa Cruz Biotechnology, Inc), Cytochrome C (Proteintech Group, Inc), Bax (Proteintech Group, Inc), CCND1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), β-catenin (Proteintech Group, Inc), TCF-4 (Proteintech Group, Inc), C-Myc (Proteintech Group, Inc) or β-actin (Ding-Guo Biotech Ltd., Beijing) in 5% BSA and at 4°C for overnight. Next, the PVDF membrane was washed with TBST containing 0.1% Tween-20 and was then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h. Positive bands were visualized on an X-ray film using an enhanced chemiluminescence system (Kodak).

2.9. Statistical analysis

All the data are shown as mean ± SD (standard error of mean). The two-way analysis of variance was employed to determine the significance of differences and then Student–Newman–Keuls multiple comparison test as *post hoc*. Differences with $p < 0.05$ were considered statistically significant. Inhibitory concentration IC_{20}, IC_{50}, and IC_{80} values were calculated using SPSS version 16.0.

3. Results

3.1. 1C decreases prostate cancer growth

The cytotoxic activity of 1C, AD-2, Rg3, and Mito C for prostate cancer cells was evaluated using the MTT assay. Five prostate cancer cells were treated with different concentrations of 1C, AD-2, Rg3, and Mito C (0μM, 10μM, 20μM, 30μM, 40μM, and 50μM) for 24 h. The results indicated that 1C, AD-2, and Mito C obviously inhibited the growths of prostate cancer cells. At 20μM and 30μM, 1C reduced the growth of five prostate cancer cells by 80% and was more effective than AD-2 and0 Mito C. Antigrowth effects of 1C, AD-2, and Mito C on five prostate cancer cells were all evident at 40μM and 50μM. The effects of Rg3 were minimal at all doses on the five prostate cancer cells (Fig. 2). Then, the IC_{20}, IC_{50}, and IC_{80} values of four compounds were calculated using the SPSS version 16.0 statistical analysis software. We found that the IC_{20}, IC_{50}, and IC_{80} values of 1C were much lower than those of AD-2, Rg3, and Mito C for the five prostate cancer cells (Table 1). On GES-1 (human normal cells), 1C showed 3-fold and 10-fold lesser growth inhibition than AD-2 and Mito C, respectively (Table 1).

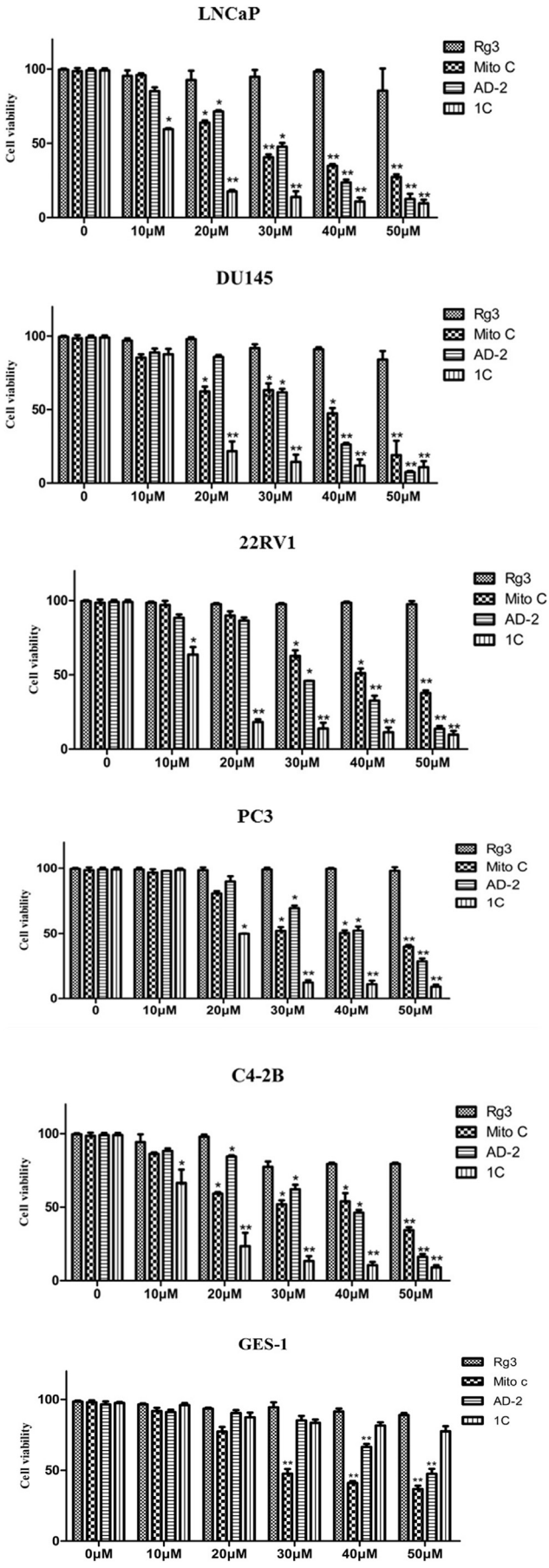


Table 1
IC_{20}, IC_{50}, and IC_{80} values of 1C, AD-2, Rg3, and Mito C in 22RV1, C4-2B, DU145, LNCaP, PC3, and GES-1. Values are the mean of triplicate experiments

Cell line	Inhibitory concentration	1C	AD-2	Rg3	Mito C
22RV1	IC_{20}	6.267	17.429	> 100	25.179
	IC_{50}	12.922	28.795	> 100	40.781
	IC_{80}	26.561	47.575	> 100	66.052
C4-2B	IC_{20}	1.682	13.532	> 100	15.985
	IC_{50}	8.287	25.07	> 100	26.749
	IC_{80}	40.838	46.446	> 100	44.763
DU145	IC_{20}	5.715	18.354	> 100	12.536
	IC_{50}	14.637	32.743	> 100	30.468
	IC_{80}	37.487	58.413	> 100	74.645
LNCaP	IC_{20}	8.34	18.586	> 100	13.877
	IC_{50}	17.942	30.536	> 100	28.771
	IC_{80}	32.212	50.17	> 100	59.647
PC3	IC_{20}	14.173	25.197	20.419	28.792
	IC_{50}	21.647	36.463	38.797	50.042
	IC_{80}	33.063	52.767	73.715	88.23
GES-1	IC_{20}	33.078	19.721	> 500	9.042
	IC_{50}	197.803	70.213	> 500	27.258
	IC_{80}	>500	249.973	> 500	82.176

IC_{20}, IC_{50}, and IC_{80} are the concentrations that inhibit growth by 20%, 50%, and 80%, respectively, relative to the control
IC, inhibitory concentration

3.2. 1C induces prostate cancer apoptosis

To examine whether 1C induces apoptosis in LNCaP cell, we used DAPI/PI staining to observe morphological changes of cell nucleus and DNA ladder method to detect DNA fragmentation. As Fig. 1C, D revealed that the LNCaP cells treated with 1C displayed condensed chromatin nucleus and DNA fragmentation, which are characteristics of cell apoptosis.

The cell cycle was examined using flow cytometric analysis. The data revealed that the cells in the SubG1 phase increased to 5.64%, 12.46% and 31.26% after treated with 1C for 6, 12, 18h respectively, compared to 4.99% in non-treated group (Fig. 1E).

Subsequently, the expression levels of cleaved caspase-9, 3 and cleaved PARP were evaluated via western blotting method in LNCaP cells. Fig. 1F exhibited that the expression levels of cleaved caspase-9, cleaved caspase-3 and cleaved PARP were activated after treated with 1C, but the expression levels of those proteins did not change after treated with AD-2 in LNCaP cells.

3.3. 1C induces apoptosis through mitochondrial pathway

To reveal the molecular mechanism of 1C, we detected the expression levels of Bax, Bcl-2, P53, and MDM2 using Western blotting. The data exhibited that the expression levels of Bax and P53 were obviously increased and those of Bcl-2 and MDM2 were evidently decreased in LNCaP cells after treated with 1C; however, AD-2 did not obviously alter those proteins (Fig. 3A). The results suggested that 1C treatment increased the ratio of Bax/Bcl-2, which was in favor of the disruption of mitochondrial function and the occurrence of apoptosis. Thus, we further analyzed the content of Bax and cytochrome C in the extract from either cytosol or mitochondria after LNCaP cells were treated with 1C. As showed in Fig. 3B, the level of Bax was decreased in cytosol, accompanied by a corresponding increase in the mitochondrial fraction, while the release of mitochondrial cytochrome C (Cyt c) was observed after LNCaP cells were treated with 1C; however, all

Fig. 2. Effects of Rg3, Mito C, AD-2, and 1C on the growths of LNCaP, DU145, 22RV1, PC3, C4-2B, and GES-1. Data are expressed as mean ± SDs of triplicate experiments performed independently. $^{*}p < 0.05$, $^{**}p < 0.01$.

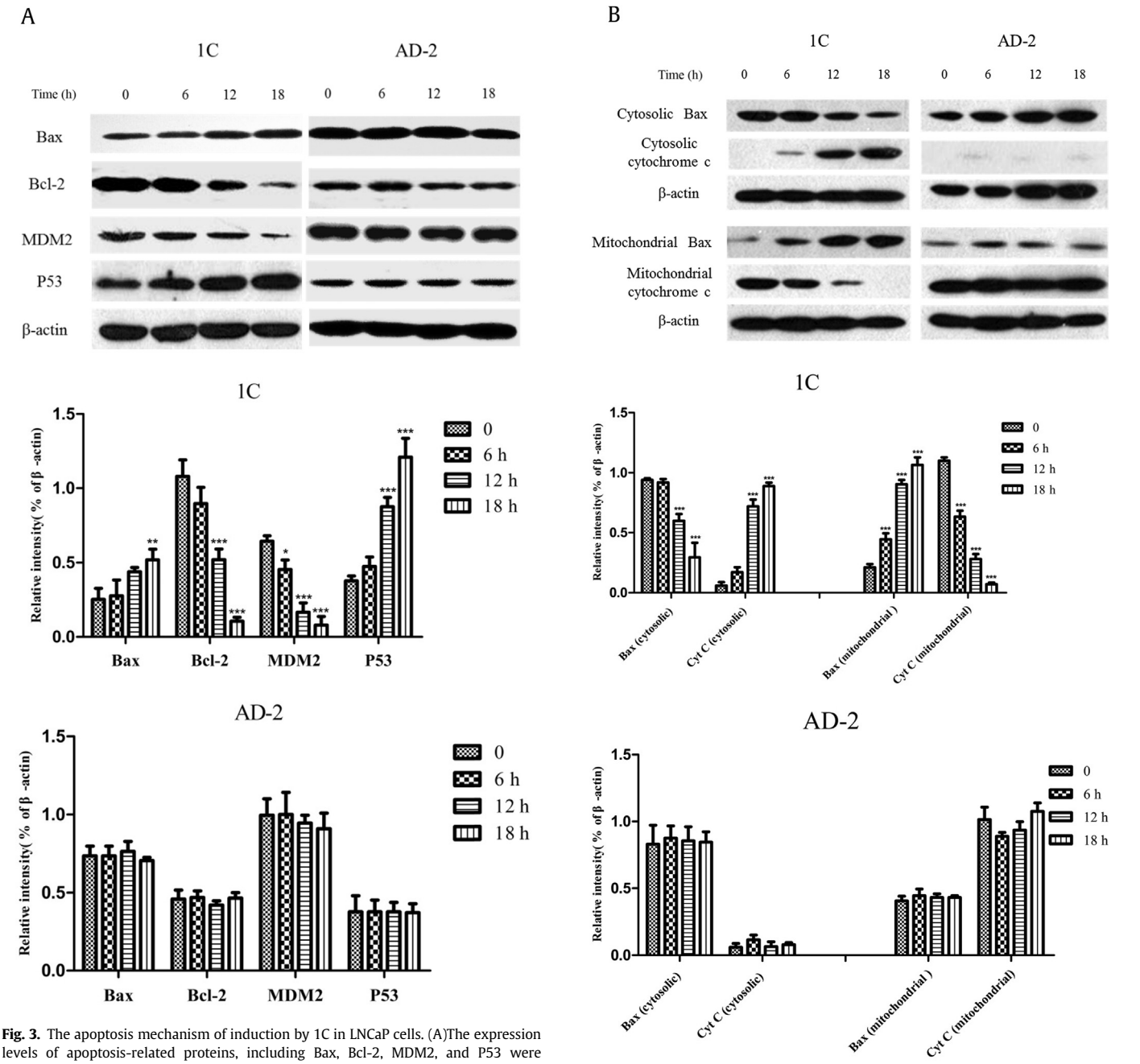


Fig. 3. The apoptosis mechanism of induction by 1C in LNCaP cells. (A)The expression levels of apoptosis-related proteins, including Bax, Bcl-2, MDM2, and P53 were examined by Western blotting assay. (B) The translocation of Bax and release of mitochondrial cytochrome C (Cyt C) were examined by Western blotting assay in LNCaP cells. Data are expressed as means ± SDs of triplicate experiments performed independently. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

of these changes could not be detected in LNCaP cells exposed to AD-2.

3.4. 1C triggers ROS generation, which is involved in 5-induced apoptosis

DCFH-DA, the oxidation-sensitive fluorescent dye, was employed to examine the change of intracellular ROS. As shown in Fig. 4A, DCFH-DA-base assay revealed that intracellular ROS levels were increased after LNCaP cells were exposed to 1C. Cells treated with 1C (25μM) for 12 h and 18h revealed much higher ROS production than untreated cells.

Fig. 3. (*continued*)

Intracellular ROS levels were elevated after the cells were treated with 1C. Therefore, we postulate that 1C-induced apoptosis might be associated with the generation of ROS. To investigate whether ROS was related with 1C-induced apoptosis, cells were exposed to 1C in the presence or absence of N-acetylcysteine (NAC), an ROS scavenger. Fig. 4A revealed that pretreatment with NAC reduced the generation of ROS induced by 1C. We investigated the effect of ROS on the expression levels of proteins associated with apoptosis in LNCaP cells after treated with 1C. Fig. 4B showed that 1C observably increased the levels of Cl-caspase-9, 3 and Cl-PARP, and these proteins were decreased via pretreating the cells with 5mM NAC prior to 1C treatment. The results revealed that ROS plays a crucial role in 1C-induced cell apoptosis in LNCaP cells.

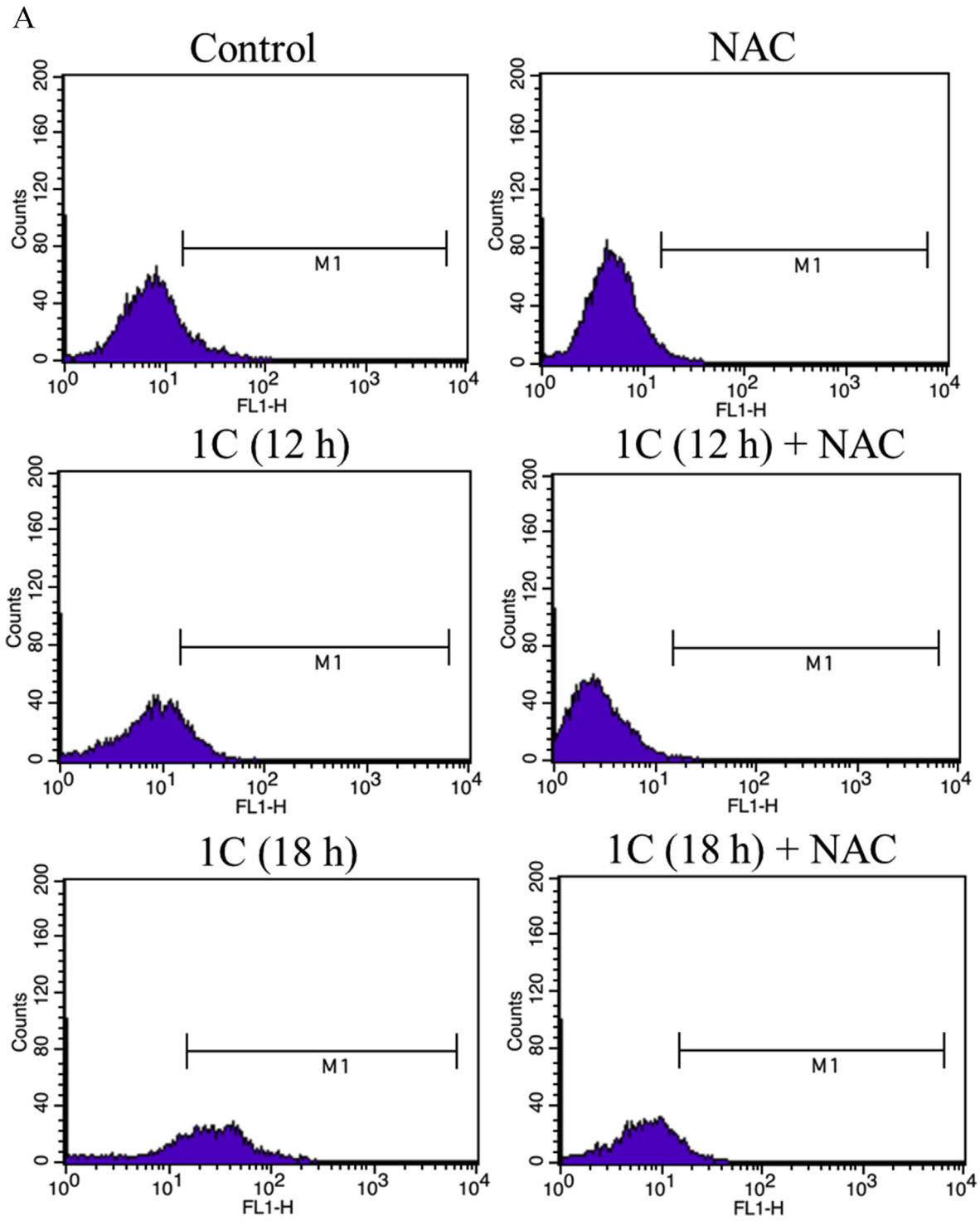
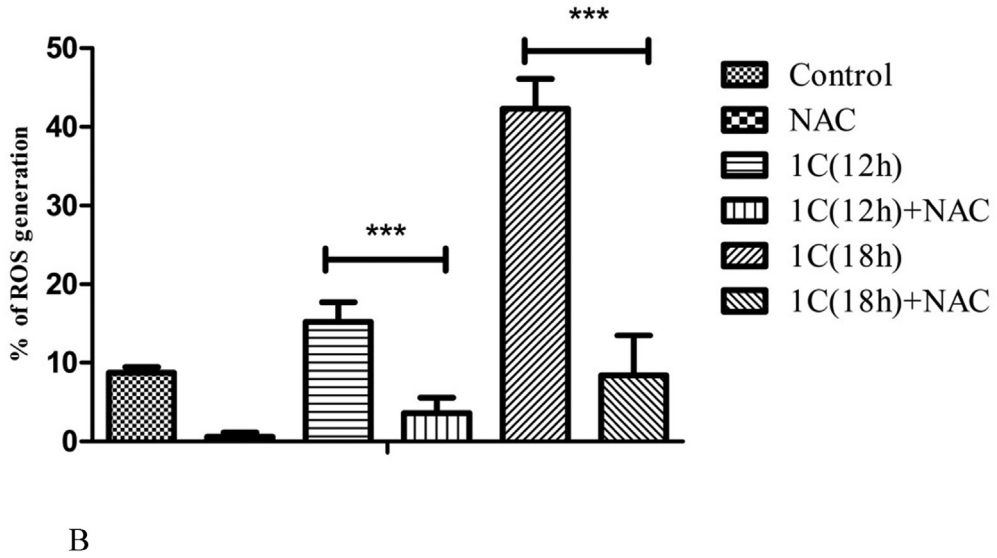


Fig. 4. 1C induces ROS production in LNCaP cells and induced apoptosis is attenuated by ROS scavenger N-acetylcysteine (NAC). (A) Intracellular ROS was examined by flow cytometry-based ROS levels assays. Representative plots of flow cytometry-based ROS levels are shown. Group data analysis of the percentage of ROS cells is shown. All assays were performed in triplicate. (B) Pretreatment with NAC cells and untreated cells were exposed to various times of 1C (25μM), followed by Western blot analysis.

3.5. 1C inhibited proliferation of LNCaP cells via modulation of Wnt/β-catenin signaling pathway.

The expression levels of proteins related with Wnt/β-catenin pathway were detected using Western blotting after the cells were treated with 1C. As shown in Fig. 5, 1C significantly reduced the expression levels of β-catenin, a key component of Wnt/β-catenin pathway, but the protein was slightly altered in AD-2 group. Further analyzing the downstream proteins of β-catenin, the data showed that the expression levels of TCF-4, CCND1, and



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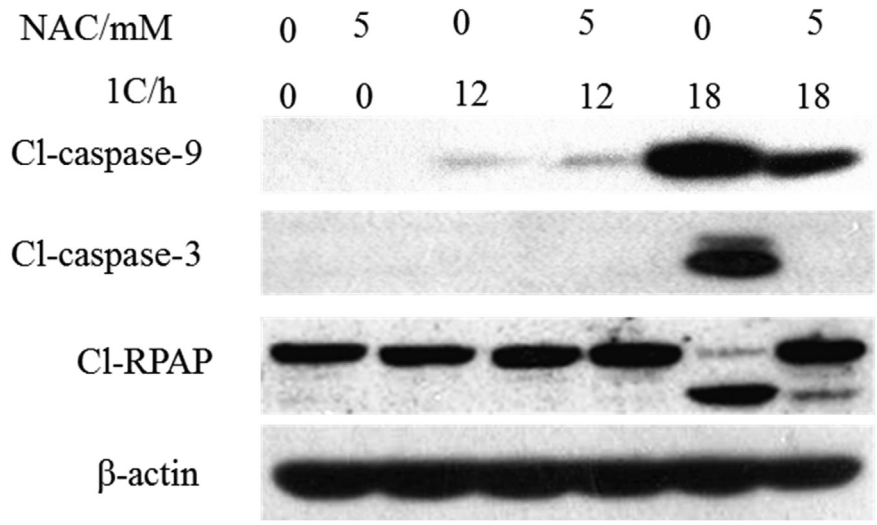

Fig. 4. (*continued*)

C-Myc were obviously decreased after LNCaP cells were exposed to 1C. By contrast, the levels of TCF-4, C-Myc, and CCND1 were slightly altered in AD-2 group.

4. Discussion

By evaluating the cytotoxic activity of the synthesized AD-2 derivatives, 1C showed that IC_{50} was lower than other AD-2 derivatives[20]. In this study, 1C strongly suppressed the prostate cancer cell lines growth by MTT assay. 1C induced apoptosis via mitochondrial apoptotic pathway in LNCaP cells. The interaction of MDM2 and P53 was interrupted after LNCaP cells were treated with 1C. Further results suggested that 1C-induced apoptosis can be partly inhibited after the cells were pretreated with NAC. Meanwhile, 1C effectively inhibited Wnt/β-catenin signaling pathway. Compared with its parent compound AD-2, we found that 1C possessed more potential for inhibiting cancer cells proliferation.

The balance of cell proliferation and cell death significantly influence the cancer cells growth, and apoptosis plays an important role in continuous cell death in tumors. Inhibition of tumor growth always self-limits the cancer proliferation via the mechanism of apoptosis [22]. Recently, drugs of inducing apoptosis have been employed to cure cancer. The mitochondrial pathway and the death receptor pathway are two main pathways to induce cell apoptosis. The collapse of $\Delta\Psi m$, a crucial incident of activation-induced cell apoptosis, mainly triggers the mitochondrial apoptotic pathway [23]. Bcl-2 family contains the anti-apoptosis (Bcl-2, Bcl-XL) and the pro-apoptosis proteins (Bax, Bad, Bak), which regulate the intrinsic apoptotic pathway via balancing the change of $\Delta\Psi m$. The anti-apoptosis proteins can maintain $\Delta\Psi m$ by keeping the mitochondrial membrane integrity, and the pro-apoptosis proteins can induce $\Delta\Psi m$ collapse [24]. In our study,

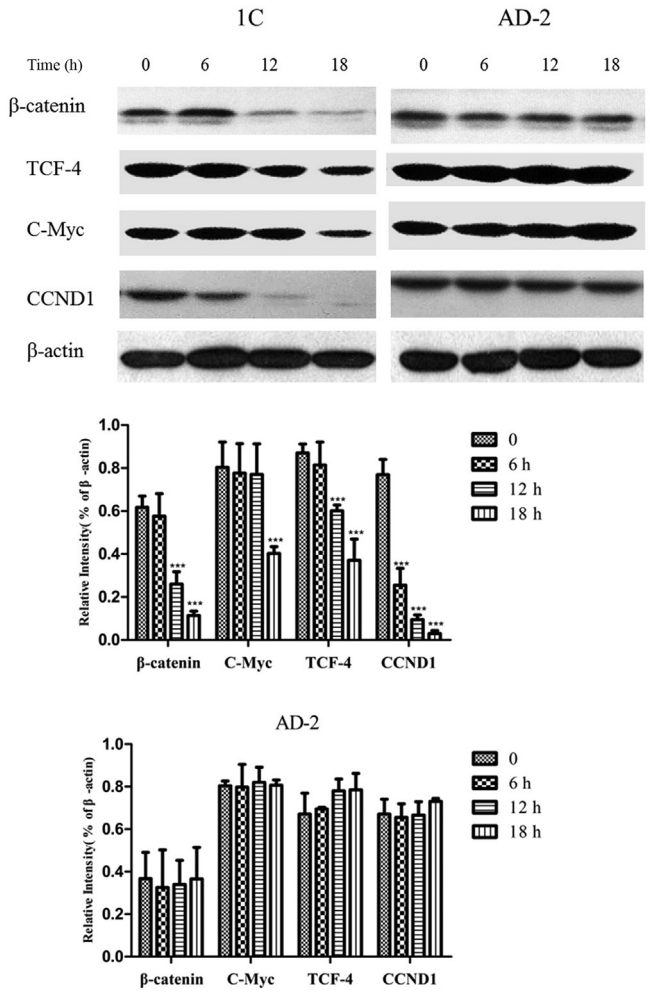

Fig. 5. 1C inhibited Wnt/β-catenin signaling pathway. The expression levels of Wnt/β-catenin signaling pathway involving proliferation were examined by Western blotting assay. Data are expressed as means ± SDs of triplicate experiments performed independently. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

the expression level of Bcl-2 was downregulated and the expression level of Bax was upregulated after the cells were exposed to 1C. Bax was translocated to mitochondrial membranes from cytosol and cytochrome C was released into the cytosol from mitochondrial membranes after the $\Delta\Psi m$ collapse; furthermore, the functional apoptosome was assembled by the interaction of Apaf-1, pro-caspase-9, and Cytochrome C in cell cytosol, and the downstream executioner caspase-3 was subsequently activated. Activated caspases are well known as biochemical markers of apoptosis and play a major role in DNA fragmentation and chromatin condensation [25]. In this study, we observed that cells treated with 1C exhibited these morphological characteristic of apoptosis. The expression levels of cleaved caspase-9, cleaved caspase-3, and cleaved PARP were upregulated as apoptotic cells increased. By contrast, AD-2 did not exhibit similar profile. All the results suggested that 1C manifested higher efficacy on inhibiting cancer cells proliferation by inducing cell apoptosis.

MDM2 is recognized as E3 ubiquitin ligase protein. MDM2 is a human protein encoded by the *MDM2* gene [26,27]. This protein is a crucial suppressor of the anticancer factor p53. The MDM2 protein functions as an E3 ubiquitin ligase, which recognizes the N-terminal trans-activation domain of p53; this protein also acts as a suppressor of p53 transcriptional activation. In this study, 1C significantly downregulated the expression level of MDM2 protein and upregulated the p53 expression; however, similar results were not observed in AD-2 group. These results indicated that 1C exhibited the novel ability of downregulating the expression of MDM2 because it contained an amino acid at C-3-OH compared with AD-2. Although our findings may be insufficient to explain this result, we ascertained that the downregulation of the MDM2 oncoprotein could be partially responsible for the obvious cytotoxic effect of 1C.

The role of ROS remains controversial because ROS is a signal mediator of cell death [28]. Increased oxidative stress is involved in the apoptotic response induced by some anticancer agents [29,30]. Our results demonstrated that 1C can increase the intracellular ROS level. Furthermore, we observed that the inhibition of the increasing ROS levels by NAC decreased the intracellular ROS level and the apoptosis induced by 1C. Western blot analysis also revealed that NAC pretreatment inhibited caspase-9, 8, 3, and PARP activation stimulated by 1C in LNCaP cells. Our findings indicated that 1C-induced apoptosis is partly involved in the accumulation of ROS in human cancer cells.

In addition to apoptosis, induction contributed to inhibition of cancer cell growth. Wnt/β-catenin signaling has been recognized as an important factor in the development of tumorigenesis and resistance to anticancer therapies [31]. Wnts are secreted cysteine-rich glycoproteins implicated in embryonic development and tissue homeostasis in adults. Dysregulation of the Wnt signaling can result in many kinds of cancer, including prostate cancer. The stabilization of the transcriptional coactivator β-catenin is recognized a feature of Wnt signaling pathway. β-catenin influences the expression of many genes associated with cancer and functions as an essential component of cadherin cell adhesion complexes. β-catenin regulates the expression level of genes via binding to the members of the T-cell-specific transcription factor/lymphoid enhancer-binding factor 1 family. β-catenin is also related with androgen receptor, an important regulator of prostate growth that stimulates prostate cancer progression. The Wnt/β-catenin signaling can be inhibited by secreted Wnt antagonists, and a lot of these antagonists are downregulated in cancer. The activation of the Wnt/β-catenin pathway affects prostate cell differentiation, proliferation, and epithelial-to-mesenchymal transition, which may regulate the invasive behavior of tumor cells [32]. The levels of proteins associated with Wnt/β-catenin signaling (β-catenin, TCF-4, CCND1, and C-Myc) were decreased by 1C treatment in LNCaP cells, but the expression of these proteins was slightly affected by AD-2. These results indicated that 1C exhibited stronger resistance to the proliferation of prostate cancer than AD-2 by blocking the Wnt/β-catenin pathway.

In conclusion, Fig. 6 shows that 1C exhibited an anticancer effect against prostate cancer *in vitro*. The mechanism may induce apoptosis via mitochondrial pathway, production of ROS, and inhibit proliferation. By contrast, AD-2 slightly altered the levels of proteins associated with apoptosis and proliferation. Altogether, the current findings suggest that 1C may be a potential candidate as a new therapy for prostate cancer. In fact, the active component and the molecular target of 1C are still mysterious, and we plan to conduct further studies to elucidate novel abilities of structurally modified 1C by applying computer analog technology and other related methods.

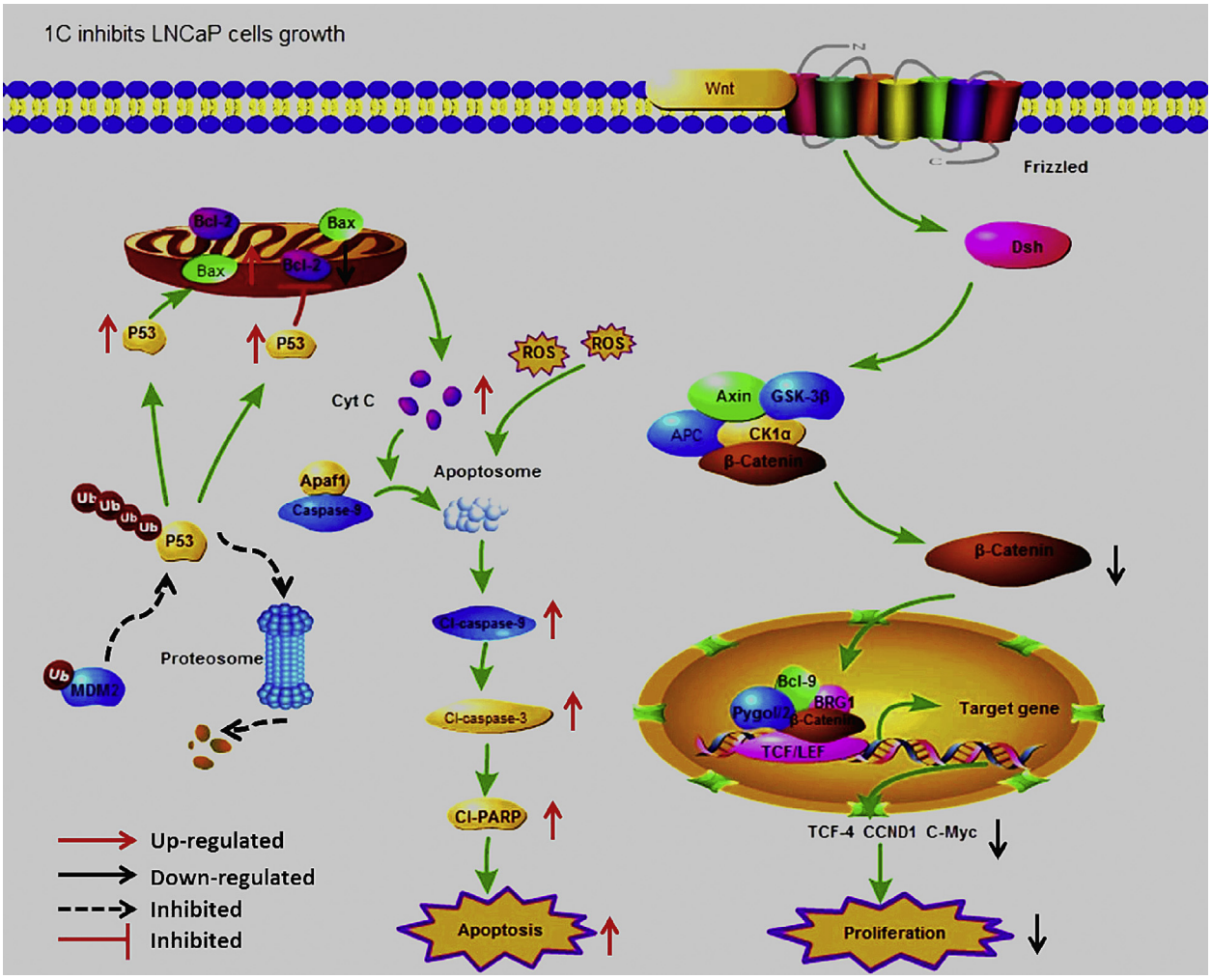

Fig. 6. Proposed mechanisms of action. Cartoon, showing the effects of 1C on various proteins, demonstrates how the compound could exert its anticancer effects.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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