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COX-2 Inhibitor Nimesulide Analogs are Aromatase Suppressors in Breast Cancer Cells

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6-Hydroximino-4-aza-A-homo-cholest-3-one and related analogue as a potent inducer of apoptosis in cancer cells

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Introduction

The synthesis of some aza homosteroid compounds with unusual and interesting structures has been reported recently [1–4]. These compounds exhibit valuable biological activities such as cytotoxicity and antibacteria. Study of aza homosteroids indicates that the presence of the characteristic group (NH–CO) in the aza homosteroid molecule has been demonstrated to be important in lowering toxicity and improving anti tumor activity of the compounds in cancer treatment [5].

Recently we designed and synthesized several new steroidal lactams with the introduction of N atom on A or D ring. Our results have shown that these compounds displayed a distinct cytotoxicity against different cancer cell types [6,7]. In this study, we report that 6 hydroximino 4 aza A homo cholest 3 one (**1**) and 6 hydroxyl 4 aza A homo cholest 3 one (**2**), new steroidal lactams were synthesized recently [8], exerted potent cytotoxic activity against several cancer cells including HT 29 (colorectal adenocarcinoma), GNE 2 (nasopharyngeal carcinoma), SPC A (lung carcinoma), Tu 686 (laryngocarcinoma) and PC 3 (prostate adenocarcinoma) cancer cells. Further investigation revealed that the compounds were able to induce cancer cell apoptosis and inhibited tumor growth in athymic mice.

Materials and methods

Drug preparations

The tested compounds (Fig. 1) were synthesized by previously described methods [8]. Stock solutions of the tested compounds were made immediately before use.

Biological assays

Cell culture and assay for cell viability

GNE 2 (nasopharyngeal carcinoma), SPC A (lung carcinoma) and Tu 686 (laryngic carcinoma) cell lines were obtained by Guangxi Medical University (China); HT 29 (colorectal adenocarcinoma) and PC 3 (prostate adenocarcinoma) cancer cells were obtained by ATCC, Manassas, VA. Cells were grown in RPMI 1640 supplemented with 10% cosmic calf serum (Hyclone) and antibiotics in a humidified atmosphere of 5% CO₂ at 37 °C. The viability of these cells was determined using the colorimetric CellTiter 96 aqueous Cell Proliferation Assay (MTT) according to the instructions provided by the manufacturer (Promega, Madison, WI). Briefly, cells (1.3×10^4 cells per well) were seeded in 96 wells plates. One day after seeding, the cells were treated with or without different concentration of each compound and reincubated for 72 h. After the cells were washed with sterile phosphate buffer saline (PBS), 190 μ L of RPMI 1640 and 10 μ L of the tetrazolium dye (MTT) (5 mg/mL) solution were added to each well, and the cells were incubated for an additional 4 h. The medium was discarded;

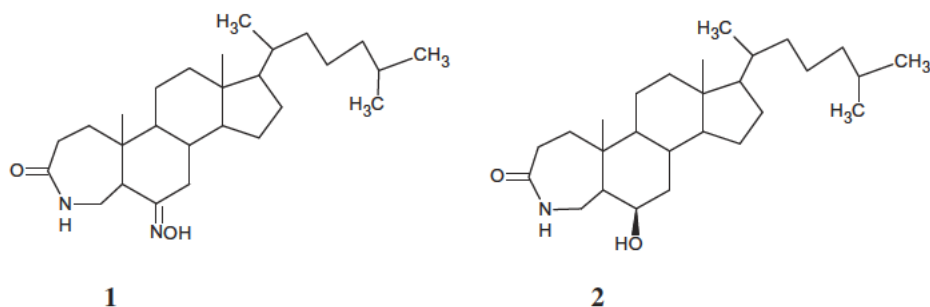


Fig. 1. Chemical structures of 6-hydroximino-4-aza-A-homo-cholest-3-one and 6-hydroxyl-4-aza-A-homo-cholest-3-one.

Table 1
In vitro anti-tumor activities (IC_{50} in $\mu\text{mol/L}$) of the compounds.

Compound	Carcinoma cell lines				
	GNE 2	SPC-A	Tu 686	PC-3	HT-29
1	12.1	74.5	24.9	14.5	10.6
2	15.8	35.3	31.2	18.3	15.5

200 μL of DMSO was added to dissolve the purple formazan crystals formed. The absorbance (A) at 492 nm was measured using a Biocell ELISA analysis spectrometer.

Annexin V assay

Annexin V assay was performed using an Annexin V FITC/propidium iodide apoptosis detection kit (BD Biosciences, San Jose, CA). Briefly, cells treated with or without 10 $\mu\text{g/mL}$ of compound 1 or 2 for 0, 12 and 24 h or different concentrations of compound 1 or 2 for 12 h were scraped and centrifuged at $1000\times g$ for 10 min at 4°C , and washed with ice cold PBS, and then resuspended in $1\times$ binding buffer provided by the manufacturer at a concentration of $1\times 10^6/\text{mL}$. FITC Annexin V (5 μL) and propidium iodide (5 μL) were added to 100 μL of the cell suspension and the cells were incubated at room temperature for 15 min in the dark. After incubation, 400 μL of $1\times$ binding buffer was added to the cell suspension and the cells then were analyzed by two color cytometry using a FACScan™ (Becton Dickinson, Franklin Lake, NJ).

Immunofluorescent microscopy

Cells were grown to 60–70% confluence on a culture slide (BD Falcon, Bedford, MA). After treatment with 20 $\mu\text{g/mL}$ of compound 1 for various times, the cells were rinsed twice with PBS, and fixed with freshly prepared 3.7% formaldehyde at 37°C for 15 min. The fixed cells were rinsed twice with PBS before incubated in 1 mL PBS containing 0.2% Triton X 100 and 1 μL of 1 $\mu\text{g/mL}$ DAPI for 10 min on ice. The cells were incubated with 5% goat serum for 30 min and then a monoclonal antibody to human cytochrome c (BD Biosciences, San Jose, CA) in PBS containing 1% goat serum for 2 h at room temperature. After washing, the cells were incubated with a secondary antibody conjugated with Cy3 (BD Pharmingen, San Diego, CA) for 1 h in dark. The cells were washed three times with PBS and covered with anti fade mounting medium. Cell images were captured with an LSM 510 Zeiss confocal microscope (Carl Zeiss, Inc. Thornwood, NY).

Western blot analysis

After treatment, cells were washed twice with ice cold phosphate buffered saline (PBS) and collected with a scraper. The cell pellet was resuspended in the extraction buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES KOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM MgCl_2 , 1 mM dithiothreitol (DTT)

and protease inhibitors. After 30 min incubation on ice, cells were homogenized with a glass dounce and a B pestle (40 strokes). Cell homogenates were spun at $14,000g$ for 15 min and the cell extracts (100 μg per sample) were fractionated on SDS 10% polyacrylamide gels and transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat milk in PBS containing 0.02% sodium azide and 0.2% (v/v) Tween 20, and incubated with a monoclonal antibody to human cytochrome c (Santa Cruz, Biotechnology, Santa Cruz, CA) for 1 h at room temperature. The membranes were then washed with PBS containing 0.2% (v/v) Tween 20 and incubated with specific secondary antibodies conjugated with horseradish peroxidase (Cell Signaling, Billerica, MA) for 1 h at room temperature. After washing, these proteins were detected by a chemiluminescence method according to the manufacturer's specification (Pierce, Rockford, IL).

Caspase activity

Cells were treated with 20 $\mu\text{g/mL}$ of compound 1 for 12 and 24 h. The cells were trypsinized and washed twice with cold PBS. The washed cells (1×10^6 cells) were incubated in 100 μL of PBS containing 1 μL FITC VAD FMK at room temperature in dark for 15–25 min. After washing once in PBS, the cells were resuspended in 400 μL of PBS and analyzed by flow cytometry using a FACScan™ (Becton Dickinson, Franklin Lake, NJ).

In vivo evaluation of therapeutic effectiveness

PC 3 cells (1×10^6) were injected subcutaneously into the anterior flank of each mouse in six NCRNU M nude mice (Taconic, Hudson, NY) at 6 weeks old. Compound 1 was dissolved in DMSO to make a 10 mg/mL stock solution, which was diluted with PBS containing 1% Tween 80 to reach a desired concentration for injection. Tumor volume was assessed by measuring length width height with a caliper. After tumors grew to a volume of 50 mm^3 , a half of the tumor bearing mice were intraperitoneally injected 10 mg/kg body weight of compound 1 at the lower abdomen for five consecutive days. Rest of tumor bearing mice were injected with an equal amount of PBS containing 1% Tween 80 and the same volume of DMSO as used for compound 1 at the identical location. After termination of the experiment, tumors were excised, photographed and weighed. All animal studies were conducted in accordance with the guidelines of the National Institute of Health for the Care and Use of Animals, and the protocol approved by the IACUC of Cleveland State University.

Results and discussion

Antiproliferative activity of the compounds

To evaluate the antiproliferative activity of the compound 1 and 2, the IC_{50} values were determined in GNE 2, SPC A, Tu 686, HT 29 and PC 3 cancer cells by using a MTT assay according to the

manufacturer's instructions. MTT [3 (4, 5 dimethylthiazol 2 yl) 2, 5 diphenyl tetrazolium bromide] is a compound that can be taken up by viable cells and reduced by a mitochondrial dehydrogenase forming a formazan product in living cells. The absorbance of the formazan product at 492 nm is in linear proportion to cell numbers. The results were summarized as IC_{50} values in $\mu\text{mol/L}$ in Table 1. Apparently the compound 1 and 2 displayed a distinct antiproliferative function on these cancer cells.

Compounds 1 and 2 induce apoptosis in cancer cells

To determine the molecular mechanism by which the compound 1 and 2 inhibit cancer cell proliferation, we further analyzed the cytotoxicity of the compound 1 and 2 in PC 3 cells. As shown in Fig. 2, both compound 1 and 2 induced PC 3 cell death could be clearly observed. To determine whether the decreased viability of PC 3 cells was due to the compound 1 or 2 induced apoptosis, the cells were treated with the compound 1 or 2, and subjected to Annexin V analysis. The translocation of membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane is an early event of cell apoptosis.

Annexin V is a 35 36 kD Ca^{2+} dependent, phospholipid binding protein that has a high affinity for PS. Therefore, FITC conjugated Annexin V is commonly used to determine apoptotic cells at an early stage. As shown in Fig. 3, treatment with compound 1 and 2 resulted in 35.2% and 61.5% PI/Annexin V double labeled apoptotic cells after 24 h incubation, suggesting both compounds are a potent apoptotic inducer in prostate cancer cells. The similar result was observed after PC3 cells were treated with compound 1 and 2 in a dose dependent manner (Fig. 4). Treatment with 10 $\mu\text{g/mL}$ of compound 1 for 24 h resulted in 42.2% PI/Annexin V double labeled apoptotic cells while compound 2 could produce 66.4% on the same condition, suggesting the compound 2 is more potent in induction of apoptosis in PC 3 cells.

To further evaluate compound 1 induced apoptosis in prostate cancer cells, we determined the activity of caspase 3 in the cells by a flow cytometry assay. Majority of the cells (81.28%) after 24 h treatment with the compound 1 exerted caspase 3 activity (Fig. 5).

Cytochrome c is an electron transporting protein within the inter membrane space of the mitochondria. It has been demonstrated that cytochrome c plays an important role in apoptotic

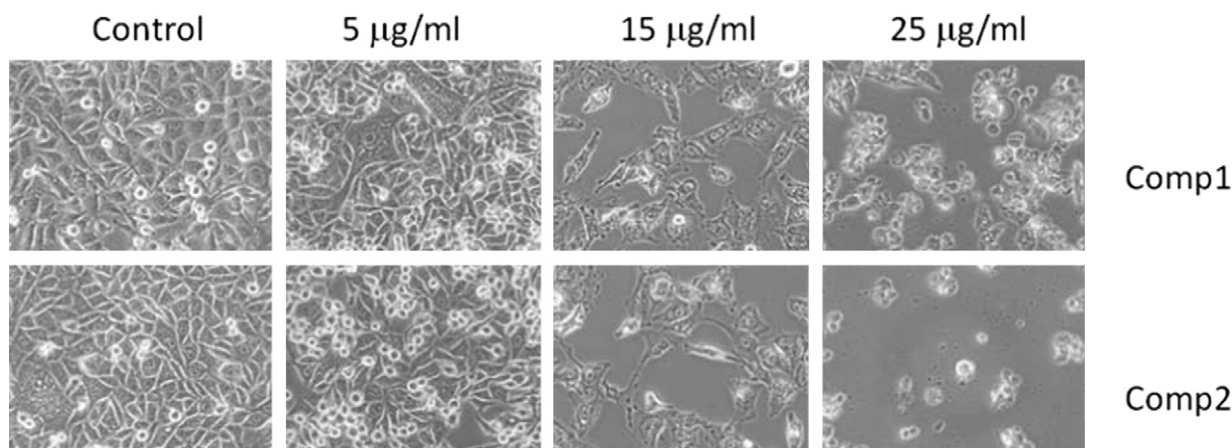


Fig. 2. Photographs of the unstained cells were taken under Olympus model CKX31 at 100 \times magnification after treatment of PC-3 cells with various doses of compound 1 or 2 for 48 h.

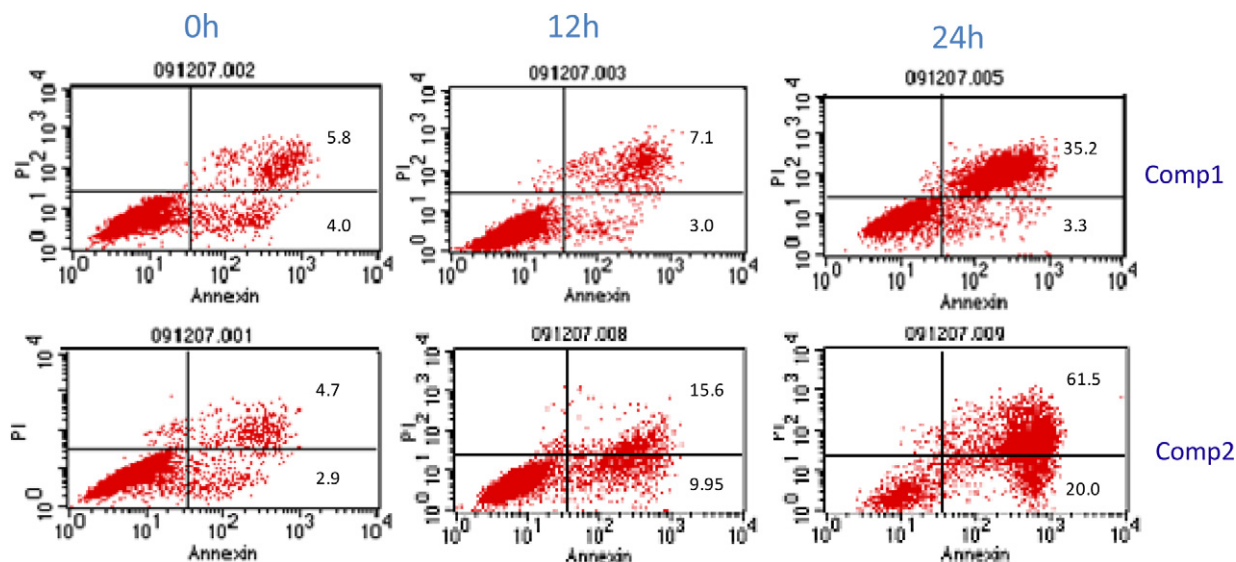
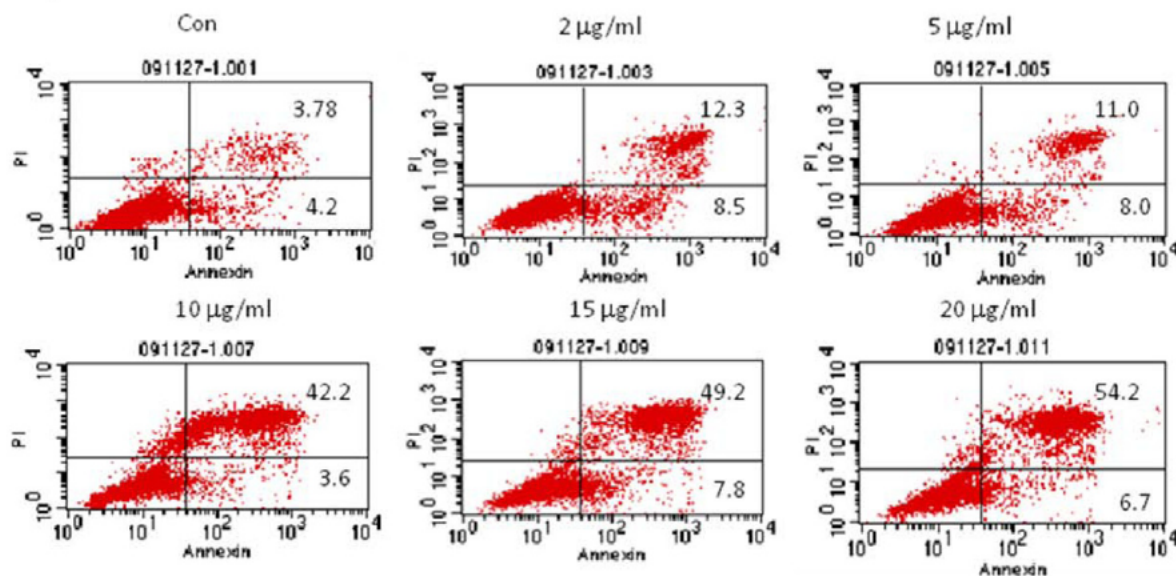


Fig. 3. Compound 1 and 2 inducing apoptosis in PC-3 cells is time-dependent PC-3 cells were treated with 10 $\mu\text{g/mL}$ of either compound 1 or 2 for 0, 12 and 24 h and subjected to Annexin V and PI double staining.

processes. In a healthy cell, cytochrome c is restricted within the mitochondrion. Upon apoptotic stimulation, cytochrome c is

rapidly released into the cytoplasm to activate caspases. To determine the release of cytochrome c, PC 3 cells were treated with the

Comp 1



Comp 2

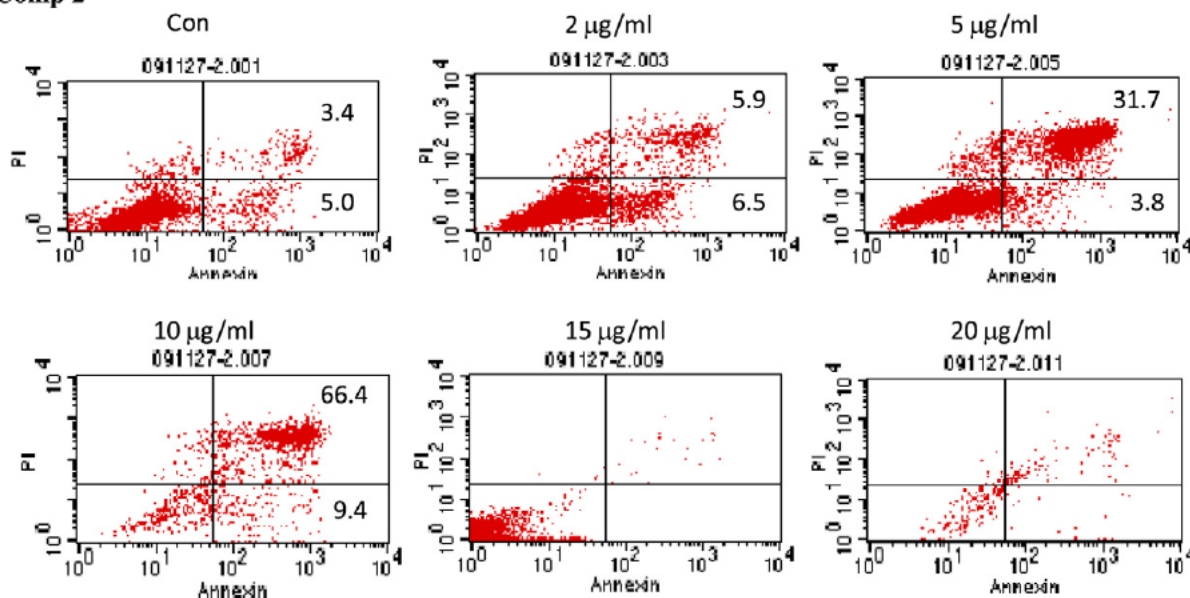


Fig. 4. Compound 1 and 2 inducing apoptosis in PC-3 cells is dose-dependent PC-3 cells were treated with different concentrations of compound 1 or 2 for 12 h and subjected to Annexin V and PI double staining.

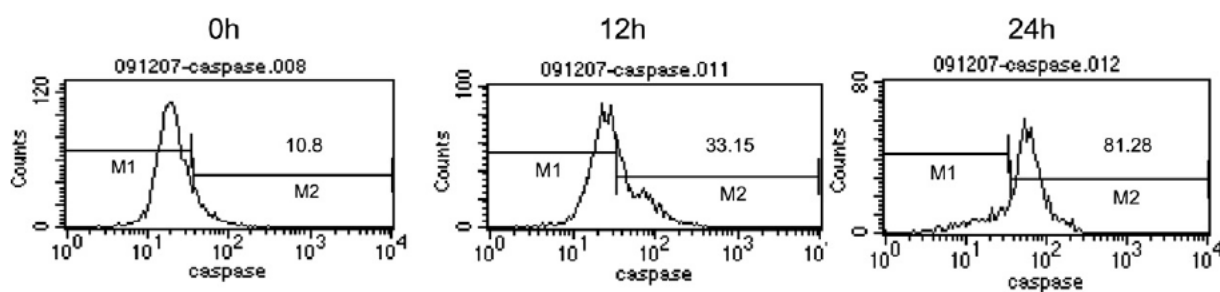


Fig. 5. Compound 1 activates caspase in PC-3 cells PC-3 cells were treated with 20 $\mu\text{g/mL}$ compound 1 for 12 and 24 h, and then incubated in PBS containing FITC-VAD-FMK at room temperature in dark for 15–25 min. After washing, the caspase activity in the cells was analyzed by flow cytometry using a FACScan™ (Becton Dickinson, Franklin Lake, NJ).

compound **1** for various times and presence of cytochrome c in cytoplasm was examined by Western blot analysis. Obviously cytosolic cytochrome c was not detectable in PC 3 cells without treatment. In contrast, cytosolic cytochrome c accumulated significantly after exposure to the compound **1** for 6 h (Fig. 6A). The entry of cytochrome c from mitochondria into the cytoplasm was confirmed by immunostaining with a monoclonal antibody against

human cytochrome c (Fig. 6B). It is obvious that the longer the cells were incubated with compound **1**, the more the cells were showing the release of cytochrome C as arrows pointed out.

In mammalian cells, there are two major apoptosis pathways termed "extrinsic" and "intrinsic." The extrinsic pathway is activated by the binding of a "death" ligand to its receptor. Subsequently the adapter proteins FADD and caspase 8 are recruited to

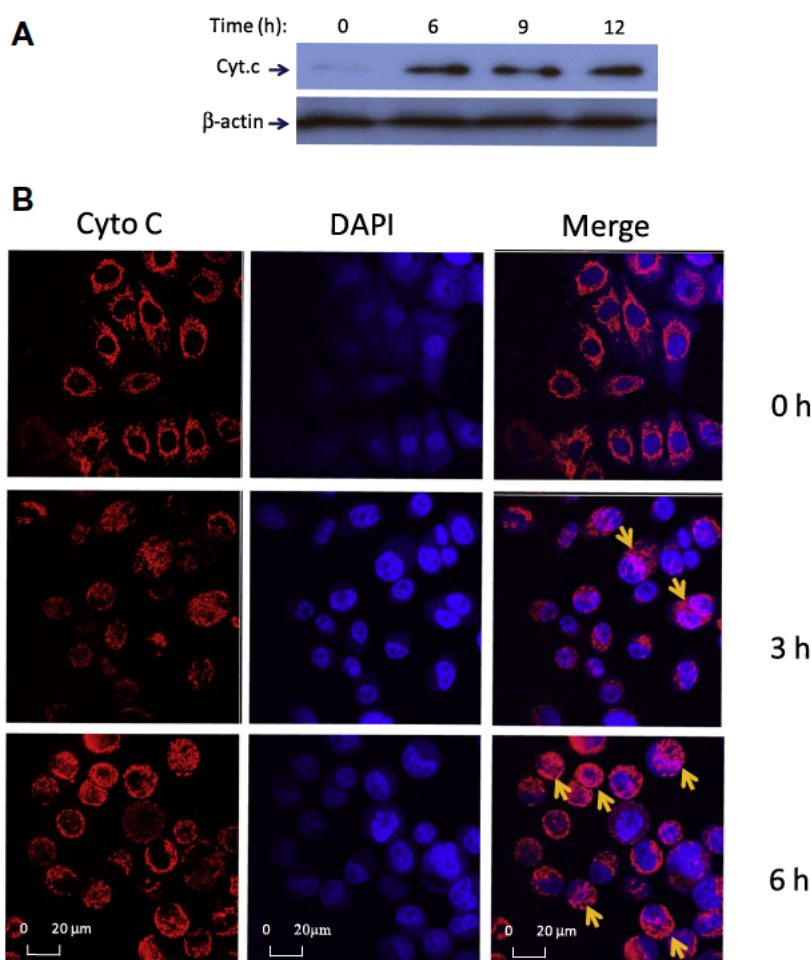


Fig. 6. Compound **1** induces release of cytochrome c in cytoplasm (A) PC-3 cells were treated with 20 µg/mL of compound **1** for 0, 6, 9 and 12 h, and cell homogenates were subjected to western blot analysis with a monoclonal antibody to human cytochrome C. (B) Immunostaining of cytochrome c in PC-3 cells after treatment with 20 µg/mL of compound **1** for 0, 3 and 6 h. Arrows indicate the cells with a release of cytochrome c (magnification 20×).

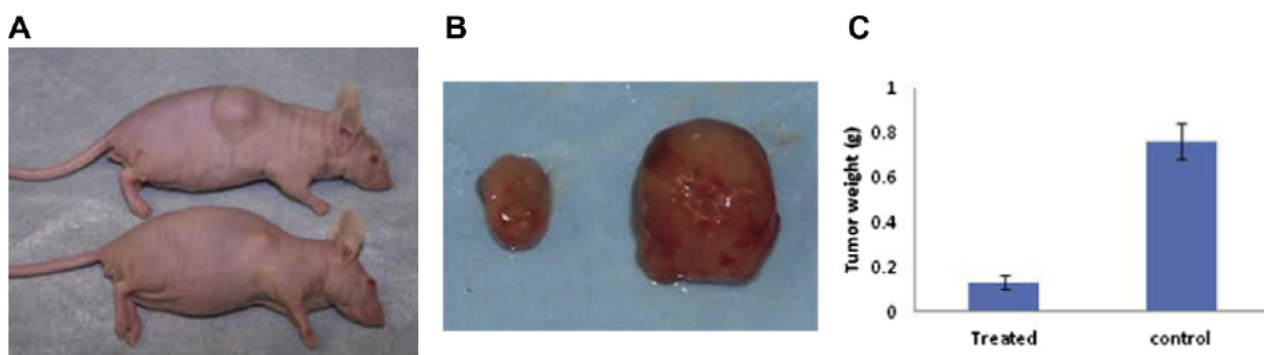


Fig. 7. Compound **1** effectively inhibits prostate tumor growth *in vivo* PC-3 cells (1×10^6) were injected subcutaneously into the anterior flank of each mouse in six nude mice. After tumors grew to a volume of 50 mm³, a half of the tumor-bearing mice were intraperitoneally injected 10 mg/kg body weight of compound **1** at the lower abdomen for five consecutive days. (A) Typical photo of tumor-bearing mice on day 21 of post-treatment with Compound **1** (Com **1**). (B) Comparison of the size of representative tumors from mice treated with or without Com **1**. (C) Tumors were weighed and represented as the mean \pm SD, $P < 0.05$.

the intracellular portion of the receptor, resulting in the activation of caspase 8 and 3 – the effector enzymes in cell apoptosis. The intrinsic apoptotic pathway is characterized by permeabilization of the mitochondria in the injured cells, resulting in release of cytochrome c into the cytoplasm. Cytochrome c then forms a multi protein complex known as the 'apoptosome' and initiates activation of the caspase cascade through caspase 9. Activated caspase 9 cleaves and activates caspase 3, leading to apoptosis. Our results implicate that the compound **1** may induce apoptosis in PC 3 cells through activation of the intrinsic pathway although the exact molecular mechanism remains to be further elucidated.

Inhibitory effect on the growth of xenografted tumors

Clearly, compound **1** is able to induce apoptosis in prostate cancer cells. The potential to be a drug candidate is dependent of the effectiveness on tumor growth *in vivo*. To determine the therapeutic role of the compound **1** in tumor growth, PC 3 cells were implanted on the back near anterior limbs as described in the experimental section. The compound **1** dissolved in DMSO and diluted in PBS containing 1% Tween 80 was intraperitoneally injected at the lower abdomen with 10 mg/kg body weight per day for five consecutive days. Obviously the compound was effective. The tumors on the mice treated with compound **1** were significantly growing slower. After termination of the experiment, the tumors were excised and weighed. As shown in Fig. 7A C, the average weight of tumors from mice treated with compound **1** was about 5.8 fold smaller than that in the control mice. The result suggests compound **1** may be a potent drug candidate for treating prostate cancer.

Conclusion

The antiproliferative activity of the new steroidal lactam 6 hydroximino 4 aza A homo cholest 3 one(**1**) and 6 hydroxyl 4 aza A homo cholest 3 one(**2**) against several cancer cell types

was investigated. Our results revealed that the steroidal lactam compounds **1** and **2** displayed a distinct cytotoxicity against these cancer cells through effectively inducing cancer cell apoptosis by activation of the intrinsic pathway. Furthermore, the compound **1** inhibited tumor growth in an athymic mouse model, suggesting a potential for the compound to be a therapeutic drug candidate for prostate cancer treatment.

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