Steroids 169 (2021) 108822

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Apoptosis inducing properties of 3-biotinylate-6-benzimidazole B-nor-cholesterol analogues

Zhiling Zhu, Zhiping Liu, Jianguo Cui, Yanmin Huang, Hualong Chen, Yulan Wu, Xiaotong Huang, Chunfang Gan *

Guangxi Key Laboratory of Natural Polymer Chemistry and Physics, Key Laboratory of Beibu Gulf Environment Change and Resources Utilization, School of Chemistry and Material, Nanning Normal University, Nanning 530001, PR China

ARTICLE INFO

Keywords:
B-nor-cholesterol
Benzimidazole
Synthesis
Cell-cycle
Apoptosis

ABSTRACT

In this work, a series of Biotin-substituted B-nor-cholesteryl benzimidazole compounds were synthesized. The antiproliferative activities of these compounds were evaluated *in vitro* using a series of human cancer cell lines, including HeLa (cervical cancer), SKOV3 (ovarian cancer), T-47D (thymus gland cancer), MCF-7 (human breast cancer) and HEK293T (normal renal epithelial) cells. These compounds displayed distinct antiproliferative activities against the currently tested cancer cells. The apoptotic properties induced by compound **6d** were further investigated. Our results showed that compound **6d** could induce the apoptosis of SKOV3 cells, blocking the cell growth in S-phase. Western blotting analyses revealed that compound **6d** can induce cell apoptosis via the mitochondria-dependent pathway.

1. Introduction

Despite the various preventive strategies, early diagnosis and drug development efforts, cancer is still considered as the second leading cause of death worldwide [1]. Cholesterol and its derivatives display a variety of biological functions. As one of the predominant components in the plasma membrane, cholesterol is found to participate in modulating the multidrug efflux pumps, contributing to the development of the multidrug resistance (MDR) phenotype in cancer cells [2]. On the other hand, cholesterol analogues, such as estramustine sodium phosphate, exemestane, abiraterone acetate or 2-methoxyl estradiol, have been developed for treating different cancers. Steroidal compounds are a class of drug molecules with polycyclic skeleton structure, which play an extremely important role in maintaining the normal physiological functions. Modifications on steroidal structure or changes on the steroid backbone may endow novel biological activities or improve its original biological function [3–5]. Cholesterol derivatives display anti-cancerous activities mainly through inducing cancer cell apoptosis and other cytotoxicity [6–8].

B-Nor steroidal compounds can be considered as a class of steroid derivatives, which are rarely found in the nature. Higuchi and colleagues have isolated a B-Nor compound, named *Orostanal*, from the Japanese sponge *Stelletta hiwasaensis* [9]. This compound was able to induce HL-60 cell apoptosis at the concentration of 10 μg/mL. Wei and colleagues have also obtained some B-Nor compounds from *Svenzea zeai* that possess good inhibitory activity against *Mycobacterium tuberculosis* [10]. In our previous work, we had prepared some different series of B-Nor steroidal compounds and evaluated their antiproliferative activity [11–13]. Among these compounds, some B-nor-steroids with cholesteryl side chain and 6-benzimidazole groups showed significant cytotoxic activity [14]. These compounds can inhibit cell proliferation by interfering with SKOV3 cell membrane structure and cell metabolic function [15]. In order to identify the biomolecule that bind to B-Nor cholesterols, a biotinylated derivative of B-nor-cholesteryl benzimidazole with considerable bioactivity should be designed and further synthesized. Using the B-nor benzimidazole as a lead compound, introduced a biotin group into the 3-position of cholesteryl, leading to the generation of four compounds with different benzimidazole rings [16–18]. A non-biotinylated linker could be incorporated at the suitable position, so the biological activity of the small molecule could be conserved.

The antiproliferative activity of these compounds in distinct types of cancer cells was evaluated, and the mechanism of action of the most potent anti-cancer compound was further evaluated.

* Corresponding author.
E-mail address: ganchunfang2008@126.com (C. Gan).

https://doi.org/10.1016/j.steroids.2021.108822
Received 10 November 2020; Received in revised form 1 March 2021; Accepted 4 March 2021
Available online 12 March 2021

2. Result and discussion

2.1. Chemistry

Generally, biotin can directly connect to the hydroxyl group of steroids by esterification. However, the 3-hydroxy group of cholesterol is β-configuration, which causes a large steric hindrance due to the 1,4-interaction between the 3-hydroxy group and the methyl group on the 10-position [17]. At the same time, biotin has a large molecular structure, so that its carboxyl group is not prone to be directly esterified with the 3-hydroxyl group of cholesterol. Therefore, a glycol linker on the 3-position of cholesterol was introduced and then the biotin group was connected, thereby the purpose of introducing biotin into B-nor-cholesterol was achieved.

Fig. 1 illustrates the synthesis of biotin-substituted B-nor-cholesteryl benzimidazole derivatives **6(a–d)**. In the presence of triethylamine, compound **1** was treated with methyl sulfonyl chloride to afford compound **2**, followed by etherification with glycol to generate compound **3** [19]. Thereafter, compound **3** was ozonized at −80℃ and the B-ring of the cholesterol was broken. Using aluminum oxide as a base, compound **4** was further obtained via aldol condensation. The reaction of compound **4** with different O-phenylenediamine derivatives allowed the formation of corresponding benzimidazole derivatives **5(a–d)**. Lastly, compounds **6(a–d)** were yielded by coupling compounds **5(a–d)** with D-Biotin. Their respective structures were confirmed by spectral data.

Reagents and conditions: (a) MsCl/Et_3N/DCM, −15 °C for 5 mins; (b) Glycol,1,4-dioxane at 90 °C for 18 h; (C) O_3 at −80 °C, for 55 mins, Al_2O_3, r.t for 12 h;(D) R_1, R_2-diaminobenzene, EtOH, 65 °C for 8 h;(e) DCC/DMAP/D-Biotin, DCM, 45 °C for 12 h.

2.2. Biological evaluation

2.2.1. Antiproliferative activity of compounds 5(a–d) and 6(a–d)

The antiproliferative activity of compound **5(a–d)** and **6(a–d)** was evaluated in four distinct types of human cancer cell lines (HeLa, SKOV3, T47D and MCF-7). Normal human kidney cells (HEK293T) were also tested for comparison. The results are summarized in Table 1. It was observed that, upon linkage of a short chain at 3-hydroxyl, the presence of an alkyl group on the benzimidazole structure could considerably affect the biological activity of B-nor-cholesteryl derivatives. Replacement of hydrogen by a methyl group almost abolished this inhibitory activity. It seems that the presence of an electron-attracting group in the benzimidazole is more efficient than that with an electron-donating group. Based on this data, we can find that by the addition of a short chain in the 3-positon of B-nor-cholesterol benzimidazole derivatives, the antiproliferative activity of compound **5a, 5c** and **5d** showed distinct activity to the test cancer cells, the antiproliferative activity of compound **5b** disappeared. In addition, compound **5c** (i.e. with electron-attracting group in benzimidazole structure) showed better biological activity.

A series of novel biotinylated compounds derived from B-nor-

Table 1
IC_{50} of compounds against human cancer cell lines. (IC_{50}[a], μM).

Compd.	HeLa	SKOV3	T47D	MCF-7	HEK293T
5a	18.19 ± 17.6	16.45 ± 32.8	9.85 ± 8.0	9.21 ± 0.96	23.80 ± 1.38
5b	>100	>100	>100	>100	17.81 ± 1.25
5c	12.42 ± 10.9	13.17 ± 6.5	11.25 ± 6.9	10.39 ± 1.06	28.07 ± 1.42
5d	47.46 ± 11.5	42.43 ± 13.0	50.02 ± 21.3	9.68 ± 0.99	24.22 ± 1.38
6a	24.06 ± 13.0	16.87 ± 3.8	6.88 ± 3.9	19.57 ± 6.0	25.4 ± 34.8
6b	>100	70.69 ± 1.8	>100	94.94 ± 1.9	>100
6c	18.92 ± 9.8	19.70 ± 10.4	22.43 ± 16.6	22.15 ± 6.4	14.7 ± 22.2
6d	72.52 ± 14.6	34.78 ± 13.8	>100	>100	>100

[a] :The data are processed as the mean ± SD of three independent experiments data.

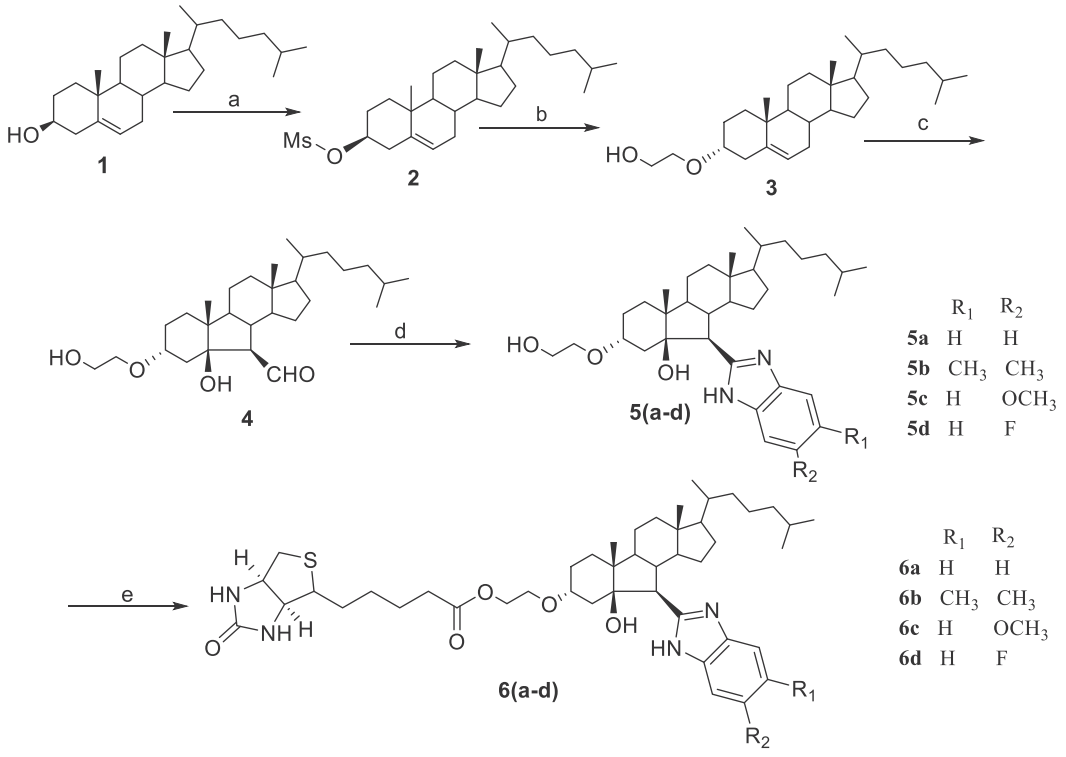

Fig. 1. The synthesis route of compounds **6(a–d)**.

cholesteryl benzimidazole **6(a–d)** efficiently yielded by esterification of intermediate compound **5** with biotin. The antiproliferation effects of compound **6** are summarized in Table 1. This biotinylated derivative had a cell growth inhibition potency that was comparable to the lead compound B-nor-cholesteryl benzimidazole. Of note, compound **6d** showed a better selectivity inhibition activity in SKOV3 cancer cells. As compared to HEK293T, the compound **6d** did not show any inactivation. In conclusion, a biotin moiety was successfully introduced into the steroidal structure without loss of the biological activity of the parental compound. Thus, **6d** was selected for further studies to identify a potential chemotherapeutic agent.

2.2.2. Cell apoptosis analysis

Apoptosis is the biological process underneath the programmed cell death. Basically, there are two main forms of cell death: (i) necrosis, which is also known as "pathological death", and (ii) apoptosis, while usually happens under certain physiological conditions [20]. Thus, chemically induced apoptosis in tumor cells is pivotal in cancer therapy [21]. FACS can be used to analyze the morphology and/or proliferative status of a cell population. In this study, FACS was used to evaluate cell fate (i.e. programmed cell death) upon treatment with different drug concentrations. The four quadrants shown in Fig. 2 represent distinct cell states: (i) dead cells (upper left); (ii) living cells (lower left); (iii) late apoptotic cells (upper right), and (iv) early apoptotic cells (lower right) [22]. To further certify that compound **6d** induces apoptosis in SKOV3 cells, annexin V-FITC/PI staining was performed. For this, SKOV3 cells were treated with compound **6d** at different concentrations (0, 35, 40 and 45 μM) for 48 h, and the percentage of apoptotic cells was measured by flow cytometry. Upon increasing the compound concentration, we observed that most of the cells were apoptotic (19.94% versus 88.32%), but the apoptotic state was mostly at the early stage (3.99% versus 59.37%). Thus, compound **6d** exhibited an antiproliferative effect possibly by inducing apoptosis in SKOV3 cells.

2.2.3. Transmembrane mitochondrial analysis

$DiOC_6$ (3) is a lipophilic fluorescent dye which used for labeling cell membrane and hydrophobic tissue. It is an environment-sensitive type of fluorescent dye whose fluorescence intensity is significantly enhanced when it binds to membranes or to lipophilic biomolecules [7]. The results (Fig. 3) shown that the percentage of cells with depolarized mitochondria decreased from 99.9% to 52.9%, indicated that the ΔΨm increased as compared to the control.

2.2.4. Cell cycle analysis

In most of the eukaryotic cells, including tumor cells, the cell cycle is divided in four stages: G1, S, G2, and M. According to each step of the cell cycle, the following biological changes occur: (i) RNA and ribosomes are mainly synthesized (G1 phase); (ii) enzymes needed for DNA replication are synthesized (S phase); (iii) large amounts of RNA and protein are produced (G2 phase); and (iv) cell division effectively arise (M phase) [23]. As a tool for cell cycle analysis, Propidium Iodide (PI) has been extensively used due to its capacity to stain DNA. Since PI freely

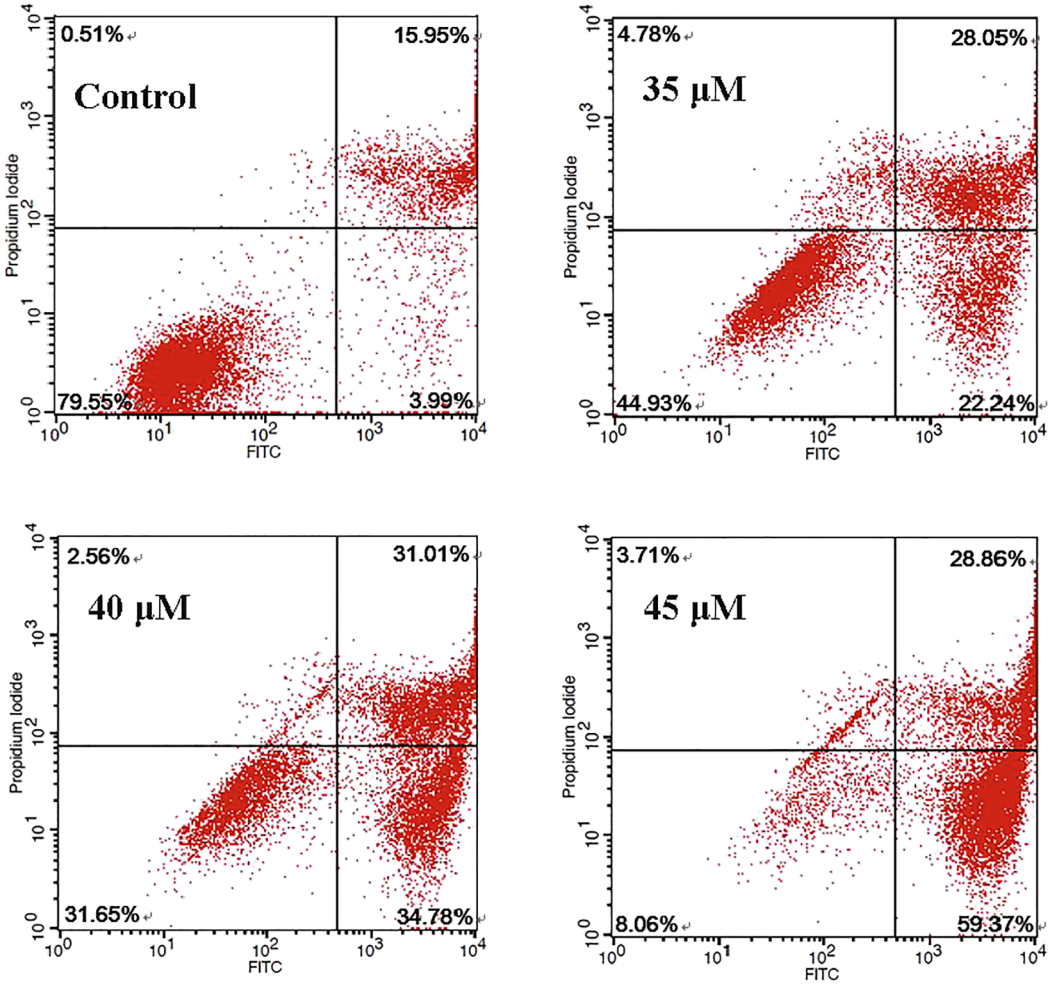

Fig. 2. Compound **6d** induce apoptosis in SKOV3 cells. Graphs indicate FACS analyses of cell populations treated with respective compound concentrations (0 (control), 35, 40 and 45 μM) for 48 h.

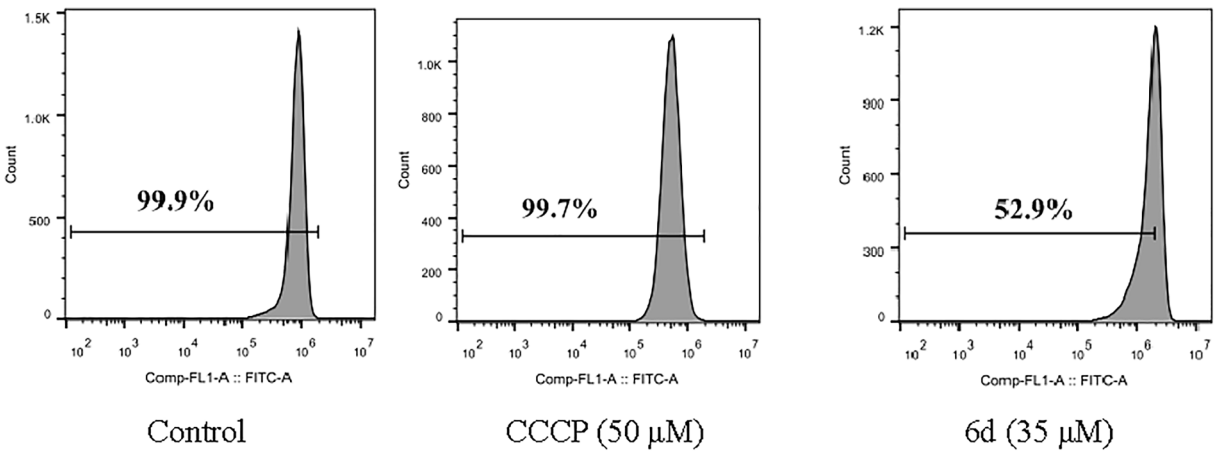

Fig. 3. The transmembrane potential of compound **6d** (0 (control), 35 μM) induce on SKOV3 cells for 48 h.

penetrate broken cell walls, apoptotic nuclei can be stained. In contrast, PI cann't penetrate the walls of living cells. Therefore, PI-stained cells were analyzed by flow cytometry, where both cell cycle and apoptotic levels were evaluated [24]. Different concentrations of compound **6d** (0, 35, 40 and 45 μM) were used to treat SKOV3 cells for 48 h, and then stained with propidium iodide (PI), followed by flow cytometry analysis. Non-treated cells were used as control. As shown in Fig. 4, control cells at the G1, S and G2/M phases accounted for 96.11%, 3.30% and 0.58%, respectively. Upon treatment with increasing amounts of compound **6d**, tumor cells at the S phase shifted from 3.30% to 31.90%. This result indicates that **6d** may blocks cell proliferation during the S phase.

2.2.5. *Effect on apoptosis-related proteins*

Disruption of the cell cycle regulation is one of the main reasons for the abnormal proliferation of cancer cells. So, the modulation of the cell cycle by certain therapeutic agents may lead to tumor growth arrest and, ultimately, to apoptosis, therefore contributing to cancer therapy [25]. Cell cycle progression is cooperatively regulated by cyclin/cyclin-dependent kinase (Cdk) complexes. Particularly, cyclin E/Cdk2 and cyclin A/cdk2 complexes play key roles in the initiation and progression

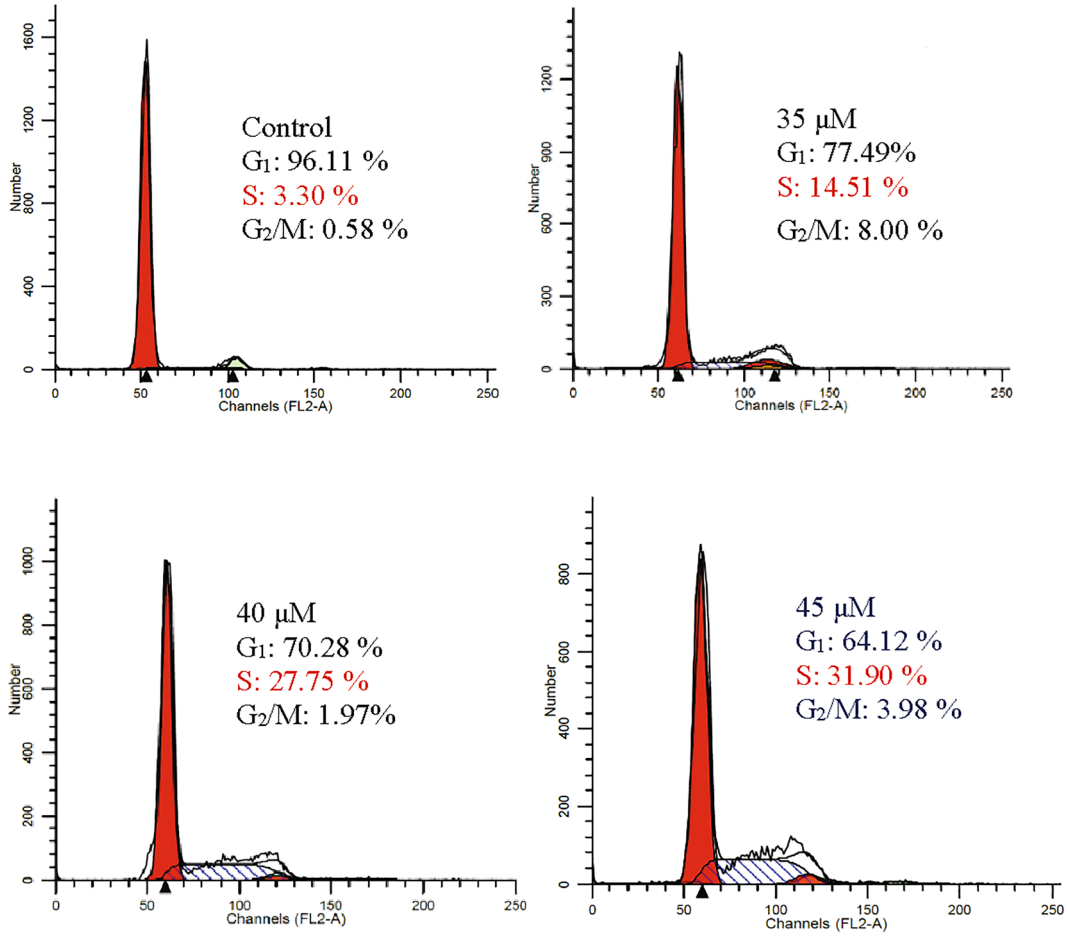

Fig. 4. Cell cycle analysis of SKOV3 cells treated with increasing concentration of compound **6d** (control), 35, 40 and 45 μM) for 48 h.

of the S phase [26]. In fact, S phase arrest of the cell cycle has been associated with the activation of Cdk2-cyclin E/cyclin A and caspases in ovarian cancer cells [27]. A Cdk inhibitor, p21, is known to be mediated by cellular events such as cell cycle arrest, senescence and apoptosis [28]. The functional activity of p21 is regulated by phosphorylation and dephosphorylation of cdk proteins [29]. The p21 expression is modulated by p53-dependent and independent mechanisms, and it is essential for DNA damage-induced cell cycle arrest [30–32]. p21 can also suppress the expression of antiapoptotic genes, such as Bcl-2 and Bcl-xl [33,34]. At the same time, Bax(a Bcl-2 family member) controls cell death through activation of caspase-9 and caspase-3 [35]. Therefore, using β-actin as a housekeeping/loading control, we further analyzed the protein levels of upstream apoptosis-initiating protein (i.e. caspase-9), downstream apoptosis-executing proteins (i.e. caspase-3), anti-and pro-apoptotic proteins (Bcl-2 and Bax, respectively) and cyclin dependent kinase inhibitors (i.e. p21), to determine whether SKOV3 cells would be undergoing an apoptotic process.

Thus, western blot analyses were performed, and the results are summarized in Fig. 5. After 48 h of cell treatment, compound **6d** was able to significantly upregulate the expression of p21. Moreover, other proteins including Bax, caspase-3, caspase-9, were also upregulated, while the levels of anti-apoptotic Bcl-2 protein were suppressed. Taken together, this data may indicate that **6d** can induce cell apoptosis via the mitochondria-dependent pathway.

3. Conclusions

In the present study, a series compounds with biotin group in the B-nor-cholesterol benzimidazole structure were designed and synthesized. The anti-proliferative activity of all targeted compounds was evaluated by MTT. Among these compounds, the most effective was **6d**, which presented a selective cytotoxicity effect towards SKOV3 cell line, with no effect on the control 293T cells. Therefore, the apoptotic properties of compound **6d** was further investigated by using the SKOV3 cell line. Western blot analysis confirmed that **6d** induces apoptosis by mitochondrial-related pathways, whereas **6d** increased the expression of Bax, caspase-9, caspase-3 and p21, but decreased the relative levels of Bcl-2. By comparing the mechanism of action of the lead compounds, it can be found that both of them block the normal metabolism of tumor cells by inhibiting the energy cycle in tumor cells. The proliferation signal and transduction of tumor cells were inhibited, the protein synthesis in tumor cells were slowed down, and the apoptosis of tumor cells were induced. Overtly, compound 6d displays better activity in inhibiting SKOV3 cells through inducing apoptosis. Most interestingly, it has low cytotoxicity to normal cells, which confers the potential for this compound to be a therapeutic drug candidate for treatment of ovarian cancer.

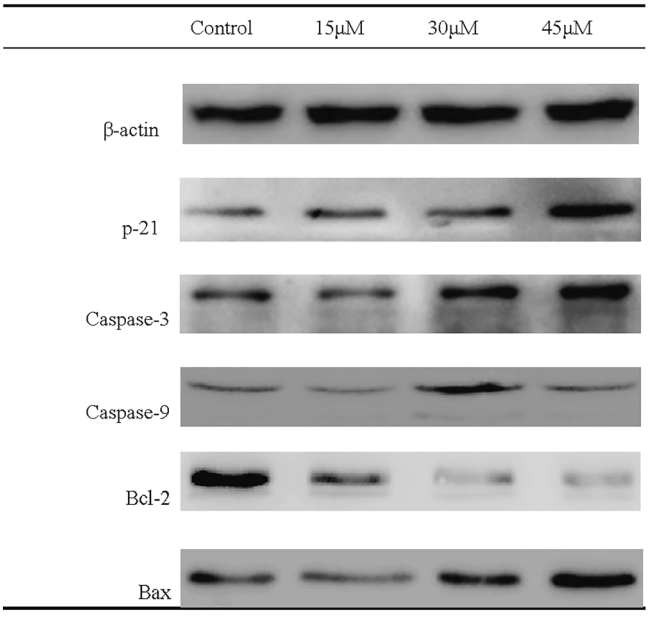

Fig. 5. Western blot analysis of β-actin, p-21, Caspase 3, Caspase 9, Bcl-2 and Baxproteins. SKOV3 cells were incubated with or without compound 6d, at different concentrations, for 48 h. The levels of respective proteins were detected using specific antibodies. Data shown are representative images from three separate experiments.

4. Experiment

4.1. Chemistry

Chemical reagents were purchased from J&K Scientific Co., Ltd. (Shanghai, China) with the purity > 98%. Anhydrous reagents were further purified by standard methods (when applicable). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV-300 NMR spectrometer using indicated solvents (TMS as internal standard). In this case, the values of the chemical shifts were expressed in δ values (ppm) and the coupling constants (J) in Hz. Mass spectra and High-resolution mass spectra (HR-MS) were carried out on Agilent LC QDECA-1000. The melting points were determined on an X-4 binocular microscope melting point apparatus (Beijing Tech Instrument Co., Ltd., Beijing, China) and not modified. Infrared spectra were measured with a Thermo Scientific Nicolet IS-10 Spectrophotometer using KBr pellets (Thermo Scientific, America).

4.1.1. General procedures to synthesize 2 ~ 4

Compound **1** (3.86 g, 10 mmol) was dissolved in 20 mL anhydrous DCM with Et_3N (3 mL, 15 mmol). Mixture was then dropped to −15 °C and stirred for 15 mins. MsCl (2 mL) was slowly added dropwise, and reaction was continued for 5 mins. Compound mixture was concentrated and purified by column chromatography (PE-EA 4:1elution.) to generate compound **2**.

Compound **2** (2.50 g, 5.38 mmol) was dissolved in 15 mL 1,4-dioxane with glycol (3.41 g, 5.50 mmol). The reaction was kept at 90 °C for 18 h, then cooled to room temperature. The mixture was extracted with EA. The combined layer was washed with water and saturated NaCl solution sequentially, dried over anhydrous Na_2SO_4 and filtered. The crude product was concentrated and purified by column chromatography (PE-EA 2:1 elution) to generate compound **3**.

Compound **3** (650 mg, 1.5 mmol) was dissolved in 20 mL DCM and 1 mL methanol. The reaction was maintained at −80 °C for 15 mins, then ventilated with ozone (2.5 mL/min) for 55 mins. Subsequently, dimethyl sulfide (2.5 mL) was added and the mixture was keep stirring for 12 h at room temperature. Compound mixture was concentrated and dissolved in 25 mL methylbenzene, and 6 g Al_2O_3 was added and maintained in reaction for 12 h at room temperature. Al_2O_3 was further filtered and removed. The crude product was concentrated and purified by column chromatography (PE-EA 1:1 elution) to generate compound **4**.

4.1.2. General procedures to synthesize 5a-d and 6a-d

Compound **4** (463 mg, 1.0 mmol) and different kinds of o-phenylenediamine (1.2 mmol) were respectively dissolved in 30 mL anhydrous ethyl alcohol, and reaction proceeded for 8 h at 65 °C. The crude products were concentrated and purified by column chromatography (DCM-MeOH 15:1 elution) to generate compound **5(a–d)**.

Compound **5** (0.65 mmol), D-Biotin (711 mg, 2.9 mmol) and DCC (801 mg, 3.8 mmol) were dissolved in 25 mL DCM with DMAP (119 mg, 0.97 mmol), and stirred for 12 h at 45 °C, respectively. The crude products were concentrated and purified by column chromatography (DCM-MeOH 10:1 elution) to generate compound **6(a–d)**.

4.2. Cell culture and reagents

HeLa (human cervical cancer), SKOV3 (human ovarian cancer), T47D (breast cancer), MCF-7 (human breast cancer) and HEK293T (normal renal epithelial) cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 0.1 g/L streptomycin sulfate. Respective cell cultures were incubated at 37 °C in 5% CO_2 incubator. All cell lines were purchased from Shanghai Gaining biotechnology Co. Ltd (China) 0.3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and chemical reagents (pure grade) were obtained from Sigma-Aldrich (St. Louis, MO). FITC-Annexin V, PI and cell cycle kit was from BD (USA). Antibodies such as Bax, Bcl-2, P-21, caspase-9, caspase-3, β-actin were from Cell Signaling Technology, Inc (USA).

4.3. MTT assay

The anti-proliferative activity of the synthesized compounds was evaluated by MTT assay, in a 96-well format. Briefly, 100 μL of respective cells, with a density of 10^4 cells/mL, were seeded into a 96-well plate and incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. Thereafter, cells were treated with compounds, at different concentrations. After 48 h, 20 μL MTT (5 mg/mL) was added to each well and cells were incubated for more 4 h. Subsequently, 200 μL DMSO was added to each well. The absorbance was measured on a Microplate Reader (BIO-RAD) at the wavelength of 570 nm. The IC_{50} values were calculated according to the inhibition ratios, $(OD_{experiment}\text{-}OD_{blank})/(OD_{control}\text{-}OD_{blank}) \times 100\%$. The data was processed by using GraphPad Prism 8.0.2.

4.4. Cell apoptosis assay

SKOV3 cells were seeded in 6-well plates overnight, then different concentrations of **6d** were added for 48 h incubation (in triplicates). After that, cells were washed with phosphate buffered saline (PBS) twice, then resuspended in Annexin V binding buffer. Annexin V-FITC was further added to the mixture, which was kept in dark for 15 mins. PI was applied right before acquisition. After Annexin V and PI double staining, apoptotic cells were analyzed, by flow cytometry (BD Calibur, USA). For this, the percentage of cells positive for PI and/or Annexin V-FITC was reported inside respective quadrants [36,37]. The percentage of apoptotic cells was determined by flow cytometry, according to the manufacturer's instructions (BD Calibur, USA).

4.5. Cell cycle assay

SKOV3 cells were seeded at 2×10^5 cells/ml in 6-well plates, and then treated with different concentration of compound **6d** (30 μM, 35 μM and 40 μM) for 48 h. After that, cells were washed with PBS and stained with propidium iodide (PI) solution in the dark, for 30 mins. The cell cycle distribution was analyzed by flow cytometry (FACS Calibur, BD biosciences, USA) and Cell-Quest software. The percentage of DNA content present at different phases of the cell cycle was analyzed with Modfit-software. Experimental procedures were repeated three times.

4.6. Transmembrane mitochondrial potential assay with DiOC6(3)

Well-grown SKOV3 cells (3×10^6/ mL) were inoculated on the six-well plate and cultured for 24 h with compound concentrations of 0, 35, 40 and 45 μM. After being treated for 48 h, the cells were processed by 3,3-dihexyloxacarbocyanine iodide ($DIOC_6$) kit (Abcam) [8]. First, $DiOC_6$, Carbonyl cyanide m-cholorophenyl hydrzone (CCCP; Multi Science) were balanced to room temperature. The cells were collected and centrifuged, then discarded the supernatant, each tube was added with 1 mL warm PBS (37 ℃) and mixed , 1 μL CCCP (50 mM) was added to the tube (50 mM) as positive, incubation at 37 ℃ for 10 min, after incubation, each tub e with 1 μL DiOC6 (1 μM), blending and incubate 15 min in 37 ℃ incubator, the cells was centrifuged and discarded supernatant, then, each tube was cleaned with 1 mL warm PBS 2 times, 100 μL PBS was added into each tube, and the mitochondrial depolarization was evaluated by BD Accuri^{TM}C6 PLUS (USA).

4.7. Western blotting

SKOV3 cells were incubated with indicated doses of 6d (15, 30 and 45 μM) in triplicates for 48 h. Cells were then harvested and resuspended in lysis buffer. Cell homogenates were centrifuged for 10 mins at 4 °C. After determination of protein concentration, respective cell lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gel, SDS-PAGE) and transferred onto nitrocellulose membranes. Membranes were blocked with 5% fat-free milk, and probed with respective primary antibodies, against Bcl-2, Bax, β-actin, caspase-9, caspase-3 and p21, at 4 °C overnight. The bound antibodies were detected using appropriate secondary antibodies and then visualized with an enhanced chemiluminescent reagent [38].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (21762008, 21562007), BAGUI Scholar Program of Guangxi Province of China, Guangxi Natural Science Foundation of China (2019JJA120066).

References

[1] W.H. Organization, The Global Burden of Disease: 2004 Update, World Health Organization, 2008.
[2] J. Kopecka, P. Trouillas, A.Č. Gašparović, E. Gazzano, Y.G. Assaraf, C. Riganti, Phospholipids and cholesterol: inducers of cancer multidrug resistance and therapeutic targets, Drug. Resist. Updat. 49 (2020) 100670, https://doi.org/10.1016/j.drup.2019.100670.
[3] M. El-Far, G.A. Elmegeed, E.F. Eskander, H.M. Rady, M.A. Tantawy, Novel modified steroid derivatives of androstanolone as chemotherapeutic anti-cancer agents, Eur. J. Med. Chem. 44 (10) (2009) 3936–3946, https://doi.org/10.1016/j.ejmech.2009.04.020.
[4] Y.K. Shi, B. Wang, X.L. Shi, Y.D. Zhao, B. Yu, H.M. Liu, Synthesis and biological evaluation of new steroidal pyridines as potential anti-prostate cancer agents, Eur. J. Med. Chem. 145 (2018) 11–22, https://doi.org/10.1016/j.ejmech.2017.12.094.
[5] M.R. Yadav, M.A. Barmade, R.S. Tamboli, P.R. Murumkar, Developing steroidal aromatase inhibitors-an effective armament to win the battle against breast cancer, Eur. J. Med. Chem. 105 (2015) 1–38, https://doi.org/10.1016/j.ejmech.2015.09.038.
[6] E. Sievänen, Exploitation of bile acid transport systems in prodrug design, Molecules 12 (2007) 1859–1889, https://doi.org/10.3390/12081859.
[7] M. Poirot, S. Silvente-Poirot, When cholesterol meets histamine, it gives rise to dendrogenin A: a tumour suppressor metabolite1, Biochem. Soc. Trans. 44 (2016) 631–637, https://doi.org/10.1042/BST20150232.
[8] K. Sassi, T. Nury, A. Zarrouk, R. Sghaier, A. Khalafi-Nezhad, A. Vejux, M. Samadi, F.B. Aissa-Fennira, G. Lizard, Induction of a non-apoptotic mode of cell death associated with autophagic characteristics with steroidal maleic anhydrides and 7β-hydroxycholesterol on glioma cells, J. Steroid Biochem. Mol. Bio. 191 (2019) 105371, https://doi.org/10.1016/j.jsbmb.2019.04.020.
[9] T. Miyamoto, K. Kodama, Y. Aramaki, R. Higuchi, R.W.M. Van Soest, Orostanal, a novel abeo-sterol inducing apoptosis in leukemia cell from a marine sponge, Stelletta hiwasaensis, Tetrahedron. Lett. 42 (36) (2001) 6349–6351, https://doi.org/10.1016/S0040-4039(01)01278-3.
[10] X. Wei, A.D. Rodríguez, Y. Wang, S.G. Franzblau, Novel ring B abeo-sterols as growth inhibitors of Mycobacterium tuberculosis isolated from a Caribbean Sea sponge, Svenzea zeai, Tetrahedron Lett. 48 (50) (2007) 8851–8854, https://doi.org/10.1016/j.tetlet.2007.10.070.
[11] C. Gan, L. Fan, Y. Huang, Z. Liu, J. Cui, Synthesis of novel ring B Abeo-sterol derivatives and their antiproliferative activities, Med. Chem. 9 (2013) 846–854, https://doi.org/10.2174/1573406411309060008.

[12] C. Gan, L. Fan, J. Cui, Y. Huang, Y. Jiao, W. Wei, Synthesis and in vitro antiproliferative evaluation of some ring B abeo-sterols, Steroids 77 (11) (2012) 1061–1068, https://doi.org/10.1016/j.steroids.2012.05.005.
[13] C. Gan, Q. Lin, J. Cui, J. Feng, J. Guo, H. Liao, Y. Huang, Synthesis and in vitro antiproliferative evaluation of some novel B-norcholesterols, Steroids 79 (2014) 37–43, https://doi.org/10.1016/j.steroids.2013.10.009.
[14] J. Cui, B. Qi, C. Gan, Z. Liu, H. Huang, Q. Lin, D. Zhao, Y. Huang, Synthesis and in vitro antiproliferative evaluation of some B-norcholesteryl benzimidazole and benzothiazole derivatives, Mar. Drugs 13 (2015) 2488–2504, https://doi.org/10.3390/md13042488.
[15] C. Gan, X. Huang, Y. Wu, J. Zhan, X. Zhang, Q. Liu, Y. Huang, Untargeted metabolomics study and pro-apoptotic properties of B-norcholesteryl benzimidazole compounds in ovarian cancer SKOV3 cells, J. Steroid Biochem. Mol. Bio. 202 (2020) 105709, https://doi.org/10.1016/j.jsbmb.2020.105709.
[16] T. Arshad, K.M. Khan, N. Rasool, U. Salar, S. Hussain, T. Tahir, M. Ashraf, A. Wadood, M. Riaz, S. Perveen, M. Taha, N.H. Ismail, Syntheses, in vitro evaluation and molecular docking studies of 5-bromo-2-aryl benzimidazoles asα-glucosidase inhibitors, Med. Chem. Res. 25 (9) (2016) 2058–2069, https://doi.org/10.1007/s00044-016-1614-y.
[17] A.T. Mavrova, D. Wesselinova, N. Vassilev, J.A. Tsenov, Design, synthesis and antiproliferative properties of some new 5-substituted-2-iminobenzimidazole derivatives, Eur. J. Med. Chem. 63 (2013) 696–701, https://doi.org/10.1016/j.ejmech.2013.03.010.
[18] M. Hranjec, G. Pavlović, G. Karminski-Zamola, Synthesis, crystal structure determination and antiproliferative activity of novel 2-amino-4-aryl-4,10-dihydro [1,3,5]triazino[1,2-a]benzimidazoles, J. Mol. Struct. 1007 (2012) 242–251, https://doi.org/10.1016/j.molstruc.2011.10.054.
[19] S. Bhattacharya, Y. Krishnan-Ghosh, Vesicle formation from oligo(oxyethylene)-bearing cholesteryl amphiphiles: site-selective effects of oxyethylene units on the membrane order and thickness, Langmuir 17 (2001) 2067–2075, https://doi.org/10.1021/la000498i.
[20] J.F.R. Kerr, G.C. Gobé, C.M. Winterford, B.V. Harmon, Methods in Cell Biology, Elsevier, 1995. https://doi.org/10.1016/S0091-679X(08)61921-4.
[21] S. Kasibhatla, B. Tseng, Why target apoptosis in cancer treatment? Mol. Cancer Ther. 2 (2003) 573–580. https://mct.aacrjournals.org/content/2/6/573.
[22] H.M. Shapiro, Overture - Practical Flow Cytometry, John Wiley & Sons, 2003. https://doi.org/10.1002/0471722731.ch1.
[23] L. Hartwell, M. Kastan, Cell cycle control and cancer, Science 266 (5192) (1994) 1821–1828, https://doi.org/10.1126/science:7997877.
[24] A. Krishan, Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining, J. Cell. Biol. 66 (1975) 188–193, https://doi.org/10.1083/jcb.66.1.188.
[25] X. Hu, R. Jiao, H. Li, X. Wang, H. Lyu, X. Gao, F. Xu, Z. Li, H. Hua, D. Li, Antiproliferative hydrogen sulfide releasing evodiamine derivatives and their apoptosis inducing properties, Eur. J. Med. Chem. 151 (2018) 376–388, https://doi.org/10.1016/j.ejmech.2018.04.009.
[26] A. Eastman, Cell cycle checkpoints and their impact on anticancer therapeutic strategies, J. Cell. Biochem. 91 (2) (2004) 223–231, https://doi.org/10.1002/(ISSN)1097-464410.1002/jcb.v91:210.1002/jcb.10699.
[27] L. Lei, D.B. Chen, L. Cao, C. Kang, W. Yang, X.Y. Zhao, C.L. Nie, Z. Yuan, Y. Wei, hPNAS-4 inhibits proliferation through S phase arrest and apoptosis: underlying action mechanism in ovarian cancer cells, Apoptosis 18 (2013) 467–479, https://doi.org/10.1007/s10495-012-0797-z.
[28] A. Besson, S.F. Dowdy, J.M. Roberts, C.D.K. Inhibitors, Cell cycle regulators and beyond, Dev. Cell. 14 (2008) 159–169, https://doi.org/10.1016/j.devcel.2008.01.013.
[29] J.W. Harper, G.R. Adami, N. Wei, K. Keyomarsi, S.J. Elledge, The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases, Cell 75 (1993) 805–816, https://doi.org/10.1016/0092-8674(93)90499-G.
[30] A.L. Gartel, A.L. Tyner, The role of the cyclin-dependent kinase inhibitor p21 in apoptosis 1 supported in part by NIH grant R01 DK56283 (to A. L. T.) for the p21 research and Campus Research Board and Illinois Department of Public Health Penny severns breast and cervical cancer grants (to A. L. G.).1, Mol. Cancer. Ther. 1 (8) (2002) 639–649. https://mct.aacrjournals.org/content/1/8/639.long.
[31] S. Parker, G. Eichele, P. Zhang, A. Rawls, A. Sands, A. Bradley, E. Olson, J. Harper, S. Elledge, p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells, Science 267 (5200) (1995) 1024–1027, https://doi.org/10.1126/science:7863329.
[32] Tarek Abbas, Anindya Dutta, p21 in cancer: intricate networks and multiple activities, Nat. Rev. Cancer 9 (6) (2009) 400–414, https://doi.org/10.1038/nrc2657.
[33] Jana Ćmielová, M. Řezáčová, Protein and its function based on a subcellular localization, J. Cell. Biochem. 112 (12) (2011) 3502–3506, https://doi.org/10.1002/jcb.v112.1210.1002/jcb.23296.
[34] Igor B Roninson, Oncogenic functions of tumour suppressor p21(Waf1/Cip1/Sdi1): association with cell senescence and tumour-promoting activities of stromal fibroblasts, Cancer Lett. 179 (1) (2002) 1–14, https://doi.org/10.1016/S0304-3835(01)00847-3.
[35] X. Wang, The expanding role of mitochondria in apoptosis, Genes Dev. 15 (2001) 2922–2933. https://pubmed.ncbi.nlm.nih.gov/11711427/.
[36] Shengtao Xu, Hong Yao, Shanshan Luo, Yun-Kai Zhang, Dong-Hua Yang, Dahong Li, Guangyu Wang, Mei Hu, Yangyi Qiu, Xiaoming Wu, Hequan Yao, Weijia Xie, Zhe-Sheng Chen, Jinyi Xu, A novel potent anticancer compound optimized from a natural oridonin scaffold induces apoptosis and cell cycle arrest through the mitochondrial pathway, J. Med. Chem. 60 (4) (2017) 1449–1468, https://doi.org/10.1021/acs.jmedchem.6b0165210.1021/acs.jmedchem.6b01652.s00110.1021/acs.jmedchem.6b01652.s002.
[37] J. Chen, T. Wang, S. Xu, P. Zhang, A. Lin, L. Wu, H. Yao, W. Xie, Z. Zhu, J. Xu, Discovery of novel antitumor nitric oxide-donating β -elemene hybrids through inhibiting the PI3K/Akt pathway, Eur. J. Med. Chem. 135 (2017) 414–423, https://doi.org/10.1016/j.ejmech.2017.04.045.
[38] T. Han, K. Tian, H. Pan, Y. Liu, F. Xu, Z. Li, T. Uchita, M. Gao, H. Hua, D. Li, Novel hybrids of brefeldin A and nitrogen mustards with improved antiproliferative selectivity: design, synthesis and antitumor biological evaluation, Eur. J. Med. Chem. 150 (2018) 53–63, https://doi.org/10.1016/j.ejmech.2018.02.088.