Taking orostanal (a compound from a Japanese marine sponge, Stelletta hiwasaensis) as a lead compound, some novel B-norcholesteryl benzimidazole and benzothiazole derivatives were synthesized. The antiproliferative activity of the compounds against human cervical carcinoma (HeLa), human lung carcinoma (A549), human liver carcinoma cells (HEPG2) and normal kidney epithelial cells (HEK293T) was assayed. The results revealed that the benzimidazole group was a better substituent than benzothiazole group for increasing the antiproliferative activity of compounds. 2-(3B'-Acetoxy-5B'-hydroxy-6'-B-norcholesteryl)benzimidazole (9b) with the structure of 6-benzimidazole displays the best antiproliferative activity to the cancer cells in all compounds, but is almost inactive to normal kidney epithelial cells (HEK293T). The assay of compound 9b to cancer cell apoptosis by flow cytometry showed that the compound was able to effectively induce cancer cell apoptosis. The research provided a theoretical reference for the exploration of new anti-cancer agents and may be useful for the design of novel chemotherapeutic drugs.

Keywords: cholesterol; B-norcholesteryl benzimidazoles; B-norcholesteryl benzothiazoles; antiproliferative activity; apoptosis
1. Introduction

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries [1]. WHO reported that the incidence of cancer will increase by 50%, and the global annual increase of the number of cancer patients will reach 15,000,000 in 2020. So, finding novel chemotherapeutic agents with excellent antiproliferative activity and high therapeutic compounds remain an important target for scientists [2].

The discovery of new compounds from natural sources has been very important in pharmacologic science research. The past decade has witnessed an increase in the number of compounds from the screening of diverse marine invertebrates, such as soft corals, sponges and tunicates. In 2001, a novel sterol, named orostanal (1) (Figure 1), possessing a contracted cyclopentane B-ring was isolated from a Japanese marine sponge, Stelletta hiwasaensis. Orostanal induced apoptosis in HL-60 cells at 10 μg/mL, and inhibited 50% cell growth at 1.7 μM [3]. After that, another novel 5(6-7)abeo-sterol named parguesterol B (2) was obtained from the Caribbean Sea sponge Svenzea zeai, and it was a moderately strong anti-tuberculosis molecule with a MIC value of 7.8 μg/mL [4].

Since compound 1 displays an excellent antiproliferative activity by inducing the apoptosis of tumor cell, it aroused our great interest to design and synthesize some steroidal compounds with 6,5,6,5 fused rings and assay their antiproliferative activity. In previous work, taking orostanal as a lead compound, we had prepared a series of analogs of compound 1 or 2 with different substituted groups and various side chains, and evaluated their antiproliferative activities [5,6]. The results showed that the presence of a cholesterol-type side chain was very important in determining the cytotoxicity of these compounds, and the presence of a thiosemicarbazone group at the C-6 position of steroid nucleus could enhance the antiproliferative activity of the compounds. For example, compound 3 exhibited excellent antiproliferative activities with an IC_{50} value of 13.8 and 5.4 μM against SGC-7901 (human ventriculi carcinoma) and HeLa (human cervical carcinoma) cells [7].

Heterosteroids have been accredited with a great amount of attention over the years by medicinal chemists for drug discovery. The incorporation of a heterocyclic ring or a heteroatom in the steroidal skeleton affects the chemical properties of a steroid and often results in useful alterations in its biological activities [8,9]. Some heterosteroids display an excellent anticancer activity, e.g., anticancer
agents like 2-methoxyestradiol [10], exemestane [11], estramustine phosphate sodium [12] and abiraterone [13]. So far, the steroids containing heterocycles had been widely explored and reported [14]. Literatures suggested that such compounds displayed distinct cytotoxicity against cancer cell lines [15–19].

In order to obtain biologically potent anticancer compounds with diverse structures, as an extension of our previous work, a series of B-norsteroidal compounds possessing a 6,5,6,5 fused ring and the structure of 6-benimidazole or 6-benzothiazole had been prepared starting from cholesterol in the present study. Meanwhile the antiproliferative activity of the compounds in vitro was evaluated further.

2. Results and Discussion

2.1. Chemistry

Scheme 1 outlines the synthetic procedure of B-norcholesteryl benimidazole compounds (7–11). Compound 6 was prepared according to Gan, C.F. [5]. The configurations of C-5 and C-7 in compound 6 had been described in references [20–22]. The reaction of compound 6 with O-phenylenediamine afforded a corresponding benimidazole group in the 6-position of steroidal nucleus [23]. To investigate the effect of various substituent groups in benzene ring on the antiproliferative activity of compounds, compounds (7–11) were synthesized by the reaction of 6 with different O-phenylenediamine derivatives. Their structures were confirmed on analytical and spectral data. In the NMR spectrum, resonances signals of Ar-H at 6.93, 7.22, 7.44 ppm and Ar-C at 160.7–110.0 ppm proved the formation of benimidazole in compound 7a.

![Scheme 1. Synthesis of compounds 7–11.](image)

However, when compound 6 reacted with 4-trifluoromethyl-O-phenylenediamine, the anticipated compound, 2-(3β,5β-dihydroxy-6′-B-norcholesteryl)-5-trifluoromethylbenzimidazole was not produced. Interestingly, we obtained compound 12 possessing an isoxazolidine ring structure (Scheme 2). Because the nucleophilicity of 2′-NH₂ is largely decreased due to a negative inductive effect of trifluoromethyl group, it cannot form the structure of benzimidazole, and compound 12 is generated.
The structure of 12 was confirmed by analysis of $^1$H, $^{13}$C NMR, DEPT135/90 and HMQC (see Supplementary Information).

\[ \text{Scheme 2. Synthesis of compounds 12.} \]

A proposed mechanism for the formation of compounds 12 is shown in Scheme 3. First, 1-NH$_2$ of 4-trifluoromethyl-O-phenylenediamine attacks 6-aldehyde group of compound 6 to afford the intermediate A (Because of electron-withdrawing effect of trifluoromethyl, the nucleophilicity of 2-NH$_2$ was decreased largely.). Subsequently, the coupling of imine with 5-hydroxyl in intermediate A gives compound 12 via migration of a hydrogen on hydroxyl.

\[ \text{Scheme 3. The mechanism of formation of compound 12.} \]

In order to investigate the effect of S-containing benzimidazole on the antiproliferative activity, we prepared B-norcholesteryl benzothiazoles (13) by reacting compound 6a with 2-aminothiophenol (Scheme 4). The structure of 13 was confirmed by analysis of IR, NMR and HRMS.

\[ \text{Scheme 4. Synthesis of compound 13.} \]

Last, in order to determine the effect of benzene ring in benzimidazole on the cytotoxicity of compounds, we hoped to prepare compound 14 possessing a structure of pyridine ring but the
anticipated compound 14 was not formed (Scheme 5). However, we obtained compound 15 with a structure of imine. The structure of 15 was confirmed by analysis of IR, NMR and HRMS.

\[ \text{Scheme 5. Synthesis of compound 15.} \]

2.2. Biological Results and Discussion

Lung cancer and liver cancer are a main cause of death in cancer patients. To evaluate the antiproliferative activity of the new compounds, we determined their IC\textsubscript{50} values on A549 (human lung carcinoma), HEPG2 (human liver carcinoma) and HeLa (human cervical carcinoma) using a MTT assay, and non-cancer cells HEK293T (Normal Kidney Epithelial Cells) were chosen as a control. MTT 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 at 492 nm is in linear proportion to cell numbers. The results were summarized as IC\textsubscript{50} values (concentration of a compound allowing survival of 50% of the cells in a population) in \( \mu \text{mol/L} \) in Table 1.

Table 1. \textit{In vitro} antiproliferative activities (IC\textsubscript{50} in \( \mu \text{mol/L} \)) of compounds 7–15.

| Compounds | Cells | HeLa | A549 | HEPG2 | HeLa |
|-----------|-------|------|------|-------|------|
| 7a        |       | 4.2  | 66.7 | >40   | 19.1 |
| 7b        |       | 15.9 | 28.2 | 21.2  | 20.3 |
| 8a        |       | 3.6  | 47.2 | 21.8  | 37.2 |
| 8b        |       | 31.2 | 50.6 | 30.3  | >80  |
| 9a        |       | 4.9  | 61.2 | 29.6  | 53.3 |
| 9b        |       | 4.7  | 11.9 | 4.2   | >80  |
| 10a       |       | 7.5  | 13.1 | 4.5   | >80  |
| 10b       |       | 2.2  | 31.2 | >40   | >80  |
| 11a       |       | 7.5  | 14.0 | 8.4   | >80  |
| 11b       |       | 11.8 | >80  | >40   | >80  |
| 12a       |       | 16.6 | 27.0 | 12.2  | >100 |
| 12b       |       | 3.7  | >80  | >80   | >100 |
| 13        |       | 22.0 | >80  | >80   | 58.3 |
| 15        |       | 20.6 | >80  | >80   | >80  |
As showed in Table 1, B-norcholesteryl benzimidazole derivatives (7–11) exhibited an excellent antiproliferative activity against HeLa cells. Except for compounds 7b, 8b and 11b, the IC50 values of all compounds are under 10 µM. Thereinto, the most active compounds were 7a, 8a, 9a, 9b and 10a (IC50 values: 4.2, 3.6, 4.9, 4.7 and 2.2, respectively). Obviously, compound 9b with the structure of 6-benzimidazole displayed the best antiproliferative activity against A549 and HEPG2 cells while compound 10b showed the best activity against HeLa cells in all compounds. Among all compounds synthesized, compound 9b with benzimidazole, 10a and 11a with electron-donating groups in the benzimidazole displayed excellent cytotoxicity for HEPG2 cells (IC50: 4.2, 4.5 and 8.4, respectively).

After an electron-withdrawing group was introduced into the skeleton of benzimidazole, the cytotoxicity of the compounds 7b–8b possessing 5-fluoro and 5-nitro resulted in slightly decreased. However, compounds 10a, 10b and 11a with electron-donating groups in the benzene ring showed a similar cytotoxicity compare with compounds 9a and 9b. Interestingly, except compound 9a, compound 9b and compounds 10–11 with the electron-donating groups were almost inactive to normal kidney epithelial cells (HEK293T), but compounds 7 and 8a with the electron-withdrawing groups showed distinct cytotoxicity to same kind of cells.

After the 3-hydroxyl group on 8a was transformed into 3-acetoxyl, the antiproliferative activity of compound 8b against these cells decreased greatly, suggesting the importance of the hydroxyl group in the compound 8. However, after the 3-hydroxyl group in 9a was transformed into 3-acetoxyl, the antiproliferative activity of forming compound 9b obtained a prominent increase against the cancer cells and an obvious decrease on normal kidney epithelial cells. These results demonstrated that compound 9b based on the 3-acetoxyl and 6-benzimidazole was a potent antiproliferative agent.

Compound 12a bearing an isoxazolidine ring structure displayed also distinct cytotoxicity against these cancer cells. However, compound 12b showed an excellent selective cytotoxicity against HeLa cells with an IC50 value of 3.7 µM, and was almost inactive on other cancer cells. Both of 12a and 12b were inactive on normal kidney epithelial cells.

Apparently, after the N atom of benzimidazole was substituted by S atom, the cytotoxic activity of the forming compound 13 was markedly decreased (compare 9a and 13). The results demonstrate that benzimidazole group is a better substituent than benzothiazole group for increasing the antiproliferative activity of compounds and suggest that the analogs based on the 6-benzimidazole scaffold may constitute a novel class of antiproliferative agents, which deserve further study. Obviously, compound 15 with the structure of pyridine ring didn’t display distinct cytotoxicity against all these cells except HeLa cells.

The Selectivity Index (SI) was defined as the ratio of the cytotoxicity of a compound with respect to normal cells (IC50 HEK293T) versus cancer cells and used to determine the criterion of effectiveness of the compounds. The SI values of the compounds are listed in Table 2.

One important criterion for a therapeutic drug for cancer is to have minimal or no side effects to normal body cells of patients undergoing chemotherapy. Taking into account that a higher SI corresponds to greater overall anticancer activity, we can identify the leading compounds as 9b, 10a and 11a (SI values for HeLa cells: 17.0, 10.7, 10.7; A549 cells: 6.7, 6.1, 5.7; HEPG2: 19.0, 17.8, 9.5). Meanwhile, compound 10b displayed the best excellent selective inhibition against HeLa cells, and the SI was 36.4. So, comparison of the cytotoxicity of the compounds with the SI values suggested that the compounds 9b, 10a and 11a may be potent anticancer agents and compounds 10b and 12b are excellent inhibitors against human cervical carcinoma (HeLa), which deserve further study.
Table 2. SI values of the compounds 7–15.

| Compounds | SI_{HeLa} | SI_{A549} | SI_{HEPG2} |
|-----------|-----------|-----------|-----------|
| 7a        | 4.5       | -         | -         |
| 7b        | 1.3       | -         | -         |
| 8a        | 10.3      | -         | 1.7       |
| 8b        | 2.6       | 1.6       | 2.6       |
| 9a        | 10.9      | -         | 1.8       |
| 9b        | 17.0      | 6.7       | 19.0      |
| 10a       | 10.7      | 6.1       | 17.8      |
| 10b       | 36.4      | 2.6       | -         |
| 11a       | 10.7      | 5.7       | 9.5       |
| 11b       | 6.8       | -         | -         |
| 12a       | 6.0       | 3.7       | 8.2       |
| 12b       | 27.0      | -         | -         |
| 13        | 2.7       | -         | -         |
| 15        | 3.9       | -         | -         |

A comparison of the structures of the synthesized compounds with pronounced biological activity makes it possible to identify some structure/biological activity relationships for these B-norcholesteryl benzimidazole and benzothiazole derivatives:

(1) The 6-benzimidazole group is a better substituent than 6-benzothiazole group for increasing the antiproliferative activity of compounds (compare 9a and 13).

(2) The presence of electron-withdrawing groups in the benzimidazole will decrease the cytotoxicity of the compounds and electron-donating groups show not an obvious effect for cytotoxicity of compounds (compare 7b, 8b and 9b or 9a, 10a and 11a).

(3) Introduction of an isoxazolidine ring joined with ring B or a 6-imine moiety cannot increased the cytotoxicity of the compounds (compare 7a and 12a, 12b). The introduction of pyridine ring in benzimidazole deceases the cytotoxicity of the compound on HeLa and HEPG2 cells (compare 9a, 12a and 15).

To further disclose the molecular mechanism by which the compounds inhibit cancer cell proliferation, the HeLa cells were treated with compounds 9b, 10a and 11a, and Annexin V assay was performed. 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 Figure 2, treatment with 5 μM of 9b, 10a and 11a resulted in 54.2%, 54.0% and 65.1% PI/Annexin V double-labeled apoptotic cells after 24 h incubation (the lower right quadrant and the upper right quadrant which contains early and late apoptotic cells, respectively), and necrotic cells are only 0.43%, 0.36% and 0.67% (the upper left quadrant), suggesting these compounds are potent apoptotic inducers in cervical carcinoma cells. There into, compound 11a is more potent in induction of apoptosis in HeLa cells (Compare 11a with 9b and 10a).
Figure 2. HeLa cells were double-stained with annexin V/PI and analyzed by flow cytometry. Treatment with compounds 9b, 10a and 11a (5 μM) for 24 h induced apoptosis of HeLa cells.

Similar results were observed after HeLa cells were treated with compounds 9b and 10a in a dose dependent manner (Figures 3 and 4). Treatment with 5 μM of 9b for 24 h resulted in 54.2% PI/Annexin V double-labeled apoptotic cells while 10a could produce 78.4% in 10 μM condition.

Figure 3. Dose depended apoptosis induced by compound 9b for 24 h.
Figure 4. Dose depended apoptosis induced by compound 10a for 24 h.

3. Experimental Section

3.1. Chemistry

The sterols were purchased from Sinopharm Chemical Reagent Co., Ltd, Shanghai, China. All chemicals and solvents were analytical grade. Melting points were determined on an X4 apparatus (Beijing Tech Instrument Co. Ltd., Beijing, China) and were uncorrected. Infrared spectra were measured with a Thermo Scientific Nicolet IS-10 Spectrophotometer (Thermo Fisher Scientific, New York, NY, USA). The 1H and 13C NMR spectra were recorded in CDCl3 on a Bruker AV-600 spectrometer at working frequencies 600 and 150 MHz, and a Bruker AV-300 spectrometer at working frequencies 300 and 75 MHz, respectively. Chemical shifts are expressed in parts per million (δ) values and coupling constants (J) in Hertz. HREIMS was measured on an Agilent 6210 TOFMS instrument (Agilent Technologies, Palo Alto, CA, USA). The cell proliferation assay was undertaken by a MTT method using 96-well plates on a MLLTISKAN MK3 analysis spectrometer (Thermo Scientific, Shanghai, China). Annexin V assay was performed using FACS Calibur flow cytometry (Becton Dickinson, Biosciences, Franklin Lakes, NJ, USA).

Compound 6 was prepared according to the method in reference [5].

3.1.1. General Procedure for the Synthesis of Compounds 7–11

O-Phenylenediamine derivative (1.6 mmol) was added to a solution of compound 6 (1.0 mmol) in THF (50 mL). The solution was stirred for 8–24 h at room temperature until no starting material was observed (the progress of the reaction was monitored by TLC, petroleum ether/ethyl acetate = 2:1). Then the reaction was stopped and the majority of solvent was evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel (300–400 mesh) to afford the corresponding target products 7–11.

2-(3β',5β'-Dihydroxy-6'-B-norcholesteryl)-5-fluorobenzimidazole (7a) White solid, yield: 45%, m. p. 139–140 °C. IR (KBr) ν/cm⁻¹: 3252, 2945, 1626, 1599, 1524, 1444, 1382, 1142; 1H NMR (CDCl3, 300 MHz) δ: 0.64 (3H, s, 18'-CH3), 0.85 (6H, d, J = 6.6, 26'- and 27'-CH3), 0.91 (3H, d, J = 6.3, 21'-CH3), 1.06 (3H, s, 19'-CH3), 2.16–2.04 (2H, m, C8'-H and C4'-H), 2.70 (1H, d, J = 10.8, C7'-H), 3.89 (1H, br s, C3'-H), 6.93 (1H, td, J = 9.6, 2.4, 6-ArH), 7.22 (1H, d, J = 8.7, 7-ArH), 7.44 (1H, dd,
$J = 8.4, 4.5, 4$-ArH); $^{13}$C NMR (CDCl$_3$, 75 MHz): δ: 160.7 (2'-C), 157.6 (5'-C), 156.8 (8'-C and 9'-C), 110.3 (6'-C and 7'-C), 110.0 (4'-C), 82.3 (5'-'C), 66.9 (3'-'C), 56.1 (17'-C), 55.4 (14'-C), 55.0 (7'-C), 54.6 (9'-C), 45.3 (13'-C), 45.2 (12'-C), 44.5 (24'-C), 42.3 (10'-C), 39.6 (8'-C), 39.4 (4'-C), 36.2 (22'-C), 35.5 (20'-C), 31.6 (1'-'C), 28.4 (16'-C), 28.3 (25'-C), 28.0 (15'-C), 24.0 (2'-C), 23.8 (23'-C), 22.8 (26'-C), 22.5 (27'-C), 21.7 (11'-C), 18.8 (21'-C), 17.6 (19'-C), 12.4 (18'-C); HREIMS m/z: 525.3843 [M + H]$^+$ (calcd. for C$_{35}$H$_{50}$FN$_2$O$_2$, 525.3856).

2-(3β'-Acetoxy-5β'-hydroxy-6'-B-norcholesteryl)-5-fluorobenzimidazole (7b) Yellow oil, yield: 69%. IR (KBr) v/cm$^{-1}$: 3274, 2942, 1729, 1534, 1464, 1237, 1018; $^1$H NMR (CDCl$_3$, 600 MHz): δ: 0.56 (3H, s, 18'-CH$_3$), 0.83 (6H, d, $J = 4.2$, 26'- or 27'-CH$_3$), 0.89 (3H, s, 21'-CH$_3$), 1.07 (3H, s, 19'-CH$_3$), 1.74 (3H, s, COCH$_3$), 2.28 (1H, q, $J = 10.8$, C$_8$-'H), 2.95 (1H, d, $J = 11.4$, C$_7$-'H), 4.88 (1H, m, C$_3$-'H), 6.89 (1H, t, $J = 8.4$, 6-ArH), 7.23 (1H, d, $J = 7.8$, 7-ArH), 7.44 (1H, s, 4-ArH); $^{13}$C NMR (CDCl$_3$, 150 MHz): δ: 170.8 (3'-'C=O), 160.8 (2'-C), 157.7 (5'-C), 155.9 (8'-C and 9'-C), 110.3 (6'-C and 7'-C), 109.9 (4'-C), 81.4 (5'-C), 69.7 (3'-C), 55.7 (17'-C), 55.5 (14'-C), 54.3 (9'-C), 44.7 (12'-C), 44.4 (13'-C), 44.3 (10'-C), 39.8 (24'-C), 39.4 (8'-C), 38.6 (4'-C), 36.2 (22'-C), 35.4 (20'-C), 32.8 (1'-'C), 28.5 (16'-C), 28.0 (25'-C), 25.3 (15'-C), 24.2 (2'-C), 23.7 (23'-C), 22.8 (26'-C), 22.5 (27'-C), 21.9 (COCH$_3$), 21.0 (11'-C), 18.8 (21'-C), 18.4 (19'-C), 12.4 (18'-C); HREIMS m/z: 567.3951 [M + H]$^+$ (calcd. for C$_{35}$H$_{52}$FN$_2$O$_3$, 567.3962).

2-(3β',5β'-Dihydroxy-6'-B-norcholesteryl)-5-nitrobenzimidazole (8a) Yellow oil, yield: 78%. IR (KBr) v/cm$^{-1}$: 3369, 2935, 1587, 1517, 1462, 1317, 1165, 1070; $^1$H NMR (CDCl$_3$, 300 MHz): δ: 0.65 (3H, s, 18'-CH$_3$), 0.87 (6H, d, $J = 6.3$, 26'- and 27'-CH$_3$), 0.93 (3H, s, 21'-CH$_3$), 1.09 (3H, s, 19'-CH$_3$), 2.16-2.05 (1H, m, C$_8$-'H and C$_4$-'H), 2.72 (1H, d, $J = 10.5$, C$_7$-'H), 4.00-3.90 (1H, m, C$_3$-'H), 6.95 (1H, td, $J = 9.6$, 2.1, 7-ArH), 7.24 (1H, dd, $J = 7.8$, 6-ArH), 7.45 (1H, s, 4-ArH); $^{13}$C NMR (CDCl$_3$, 75 MHz): δ: 160.8 (2'-C), 157.7 (5'-C), 156.8 (8'-C), 131.0 (9'-C), 128.9 (6'-C), 110.4 (7'-C), 110.1 (4'-C), 82.3 (5'-C), 67.0 (3'-C), 56.1 (17'-C), 55.4 (14'-C), 54.9 (7'-C), 54.4 (9'-C), 45.6 (13'-C), 45.3 (12'-C), 44.6 (24'-C), 42.4 (10'-C), 39.6 (8'-C), 39.4 (4'-C), 36.2 (22'-C), 35.5 (20'-C), 31.3 (1'-'C), 29.7 (16'-C), 28.4 (25'-C), 28.0 (15'-C), 24.2 (2'-C), 23.8 (23'-C), 22.8 (26'-C), 22.5 (27'-C), 21.7 (11'-C), 18.8 (21'-C), 17.7 (18'-C), 12.5 (19'-C); HREIMS m/z: 552.3805 [M + H]$^+$ (calcd. for C$_{35}$H$_{50}$N$_2$O$_4$, 552.3801).

2-(3β'-Acetoxy-5β'-hydroxy-6'-B-norcholesteryl)-5-nitrobenzimidazole (8b) Yellow oil, yield: 41%. IR (KBr) v/cm$^{-1}$: 3428, 2945, 1706, 1615, 1517, 1467, 1337, 1253, 1068, 1018; $^1$H NMR (CDCl$_3$, 600 MHz): δ: 0.63 (3H, s, 18'-CH$_3$), 0.80 (3H, d, $J = 6.6$, 26'- or 27'-CH$_3$), 0.81 (3H, d, $J = 6.6$, 26'- or 27'-CH$_3$), 0.87 (3H, d, $J = 6.6$, 21'-CH$_3$), 1.06 (3H, s, 19'-CH$_3$), 1.93 (3H, s, 3'-COCH$_3$), 2.26 (1H, q, $J = 11.4$, C$_8$-'H), 2.88 (1H, d, $J = 11.4$, C$_7$-'H), 3.53 (1H, br s, -OH), 5.04-5.00 (1H, m, C$_3$-'H), 7.53 (1H, br s, 7-ArH), 8.10 (1H, dd, $J = 8.4$, 1.8, 6-ArH), 8.45 (1H, br s, 4-ArH); $^{13}$C NMR (CDCl$_3$, 75 MHz): δ: 170.6 (3'-'C=O), 168.2 (2'-C), 143.3 (5'-C), 143.2 (8'-C), 133.3 (9'-C), 118.1 (6'-C), 110.5 (7'-C), 109.7 (4'-C), 81.7 (5'-C), 69.9 (3'-C), 55.6 (17'-C), 55.4 (14'-C), 54.7 (7'-C), 54.6 (9'-C), 45.2 (13'-C), 45.1 (12'-C), 44.5 (24'-C), 39.6 (10'-C), 39.4 (8'-C), 39.3 (4'-C), 36.1 (22'-C), 35.5 (20'-C), 32.6 (1'-'C), 28.4 (16'-C), 28.3 (25'-C), 28.0 (15'-C), 25.3 (2'-C), 23.8 (23'-C), 22.8 (26'-C), 22.5 (27'-C), 21.9 (CH$_3$CO), 21.3 (11'-C), 18.6 (21'-C), 18.0 (18'-C), 12.5 (19'-C); HREIMS m/z: 594.3895 [M + H]$^+$ (calcd. for C$_{35}$H$_{52}$N$_2$O$_5$, 594.3907).
2-(3β',5β'-Dihydroxy-6'-B-norcholesterol)ybenzimidazole (9a) Colourless oil, yield: 40%; IR (KBr) v/cm−1: 3385, 2946, 2863, 1615, 1524, 1463, 1415, 1353, 1063, 1021; 1H NMR (CDCl3, 300 MHz) δ: 0.65 (3H, s, 18'-CH3), 0.86 (1H, d, J = 6.6, 26'- and 27'-CH3), 0.93 (1H, d, J = 6.3, 21'-CH3), 1.13 (1H, s, 19'-CH3), 2.77 (1H, d, J = 10.5, C7'-H), 4.07-3.99 (1H, m, C3'-H), 7.24 (2H, dd, J = 6.0, 3.0, 5-ArH and 6-ArH), 7.59 (2H, br s, 4-ArH and 7-ArH); 13C NMR (CDCl3, 75 MHz) δ: 169.2 (2-C), 155.4 (8-C and 9-C), 122.4 (5-C and 6-C), 122.2 (4-C and 7-C), 82.3 (5'-C), 67.1 (3'-C), 56.2 (17'-C), 55.5 (14'-C), 54.5 (7'-C), 53.9 (9'-C), 45.7 (13'-C), 45.3 (12'-C), 44.7 (24'-C), 42.8 (10'-C), 39.7 (8'-C), 39.4 (4'-C), 36.2 (22'-C), 35.6 (20'-C), 30.7 (1'-C), 29.7 (16'-C), 28.5 (25'-C), 28.0 (15'-C), 23.8 (2'-C), 22.8 (23'-C), 22.7 (26'-C), 22.6 (27'-C), 21.7 (11'-C), 18.8 (21'-C), 17.9 (19'-C), 12.5 (18'-C); HREIMS m/z: 507.3965 [M + H]+ (calcd. for C35H31N2O2, 507.3951).

2-(3β'-Acetoxy-5β'-hydroxy-6'-B-norcholesterol)ybenzimidazole (9b) White solid, yield: 63%, m.p.: 160–162 °C. IR (KBr) v/cm−1: 3451, 2945, 1714, 1614, 1526, 1454, 1362, 1265, 1023, 1023, 958; 1H NMR (CDCl3, 600 MHz) δ: 0.63 (3H, s, 18'-CH3), 0.83 (1H, d, J = 6.6, 26'- or 27'-CH3), 0.84 (1H, d, J = 6.6, 26'- or 27'-CH3), 0.89 (1H, d, J = 6.3, 21'-CH3), 1.08 (1H, s, 19'-CH3), 1.93 (1H, s, 3'-CH2CO), 2.06 (1H, d, J = 13.2, C4'-H), 2.21 (1H, q, J = 10.8, C8'-H), 2.82 (1H, d, J = 11.4, C7'-H), 5.01–4.95 (1H, m, C3'-H), 7.21–7.19 (2H, m, 5-ArH and 6-ArH), 7.55 (2H, br s, 4-ArH and 7-ArH); 13C NMR (CDCl3, 75 MHz) δ: 170.9 (3'-C=O), 170.6 (2-C), 154.7 (8-C and 9-C), 122.4 (5-C and 6-C), 122.1 (4-C and 7-C), 81.3 (5'-C), 70.0 (3'-C), 55.8 (17'-C), 55.4 (14'-C), 54.9 (7'-C), 54.9 (9'-C), 45.1 (13'-C), 44.9 (12'-C), 44.5 (24'-C), 39.7 (10'-C), 39.4 (8'-C), 39.3 (4'-C), 36.2 (22'-C), 35.5 (20'-C), 32.9 (11'-C), 28.5 (16'-C), 28.0 (25'-C), 25.4 (15'-C), 23.9 (2'-C), 23.8 (23'-C), 22.8 (26'-C), 22.5 (27'-C), 21.9 (CH3CO), 21.4 (11'-C), 18.8 (21'-C), 18.1 (19'-C), 12.5 (18'-C); HREIMS m/z: 549.4054 [M + H]+ (calcd. for C35H31N2O3, 549.4056).

2-(3β',5β'-Dihydroxy-6'-B-norcholesterol)-5, 6-dimethylbenzimidazole (10a) Brownish oil, yield: 77%. IR (KBr) v/cm−1: 3429, 2945, 1634, 1534, 1464, 1377, 1232, 1018; 1H NMR (CDCl3, 600 MHz) δ: 0.63 (3H, s, 18'-CH3), 0.85 (3H, d, J = 6.6, 26'- or 27'-CH3), 0.86 (3H, d, J = 6.6, 26'- or 27'-CH3), 0.91 (3H, d, J = 6.0, 21'-CH3), 1.08 (3H, s, 19'-CH3), 2.11–2.04 (2H, m, C4'-H and C8'-H), 2.34 (6H, s, 5-CH3 and 6-CH3), 2.71 (1H, d, J = 10.8, C7'-H), 3.86 (1H, br s, C2-H), 7.34 (2H, br s, 4-ArH and 7-ArH); 13C NMR (CDCl3, 150 MHz) δ: 154.8 (2-C), 132.3 (5-C), 130.9 (6-C), 128.0 (8-C and 9-C), 118.6 (4-C and 7-C), 82.2 (5'-C), 67.0 (3'-C), 56.3 (17'-C), 55.5 (14'-C), 54.7 (7'-C), 54.4 (9'-C), 45.6 (13'-C), 45.2 (12'-C), 44.6 (24'-C), 42.6 (10'-C), 39.7 (8'-C), 39.5 (4'-C), 36.2 (22'-C), 35.5 (20'-C), 31.5 (11'-C), 28.5 (2'-C), 28.0 (16'-C), 24.2 (25'-C), 23.8 (15'-C), 22.8 (26'-C), 22.5 (27'-C), 21.7 (23'-C), 20.3 (11'-C), 18.9 (5-CH3 and 6-CH3), 18.8 (21'-C), 17.7 (19'-C), 12.5 (18'-C); HREIMS m/z: 535.4270 [M + H]+ (calcd. for C35H35N2O2, 535.4264).

2-(3β'-Acetoxy-5β'-hydroxy-6'-B-norcholesterol)-5, 6-dimethylbenzimidazole (10b) White solid, yield: 63%, m.p.: 218–219 °C. IR (KBr) v/cm−1: 3274, 2942, 1729, 1534, 1465, 1359, 1237, 1018; 1H NMR (CD3OD, 300 MHz) δ: 0.74 (3H, s, 18'-CH3), 0.86 (6H, d, J = 6.6, 26'- and 27'-CH3), 0.94 (4H, d, J = 6.3, 21'-CH3), 1.07 (3H, s, 19'-CH3), 1.96 (1H, s, 3'-CH2CO), 2.12–2.03 (2H, m, C4'-H and C8'-H), 2.35 (6H, s, 5-CH3 and 6-CH3), 2.80 (1H, d, J = 11.7, C7'-H), 5.14–5.05 (1H, m, C3-H), 7.29 (2H, s, 4-ArH and 7-ArH); 13C NMR (CD3OD, 150 MHz) δ: 171.1 (3'-C=O), 153.6 (2-C), 130.4 (5-C, 6-C, 8-C and 9-C), 114.0 (4-C and 7-C), 80.8 (5'-C), 69.8 (3'-C), 55.8 (17'-C), 55.4 (14'-C), 54.4 (7'-C), 53.8 (9'-C), 44.5 (13'-C), 44.1 (12'-C), 43.8 (24'-C), 39.8 (10'-C), 39.2 (8'-C), 38.5 (4'-C), 36.0 (22'-C),...
35.5 (20′-C), 32.4 (1′-C), 28.2 (2′-C), 27.7 (16′-C), 25.0 (25′-C), 23.5 (15′-C), 23.3 (CH3CO), 21.8 (26′-C), 21.6 (23′-C), 21.5 (27′-C), 19.8 (11′-C), 19.0 (5′-CH and 6′-CH), 17.9 (21′-C), 17.3 (19′-C), 11.5 (18′-C); HREIMS m/z: 577.4390 [M + H]+ (calcd. for C37H37N2O5, 577.4369).

2-(3β,5β-Dihydroxy-6′-B-norcholesteryl)-5-methoxybenzimidazole (11a) Brownish oil, yield: 49%. IR (KBr) v/cm⁻¹: 3420, 2942, 1629, 1516, 1452, 1377, 1198, 1152, 1028; ¹H NMR (CDCl₃, 300 MHz) δ: 0.65 (3H, s, 18′-CH₃), 0.86 (3H, d, J = 6.6, 26′- or 27′-CH₃), 0.87 (3H, d, J = 6.6, 26′- or 27′-CH₃), 0.92 (3H, d, J = 6.6, 21′-CH₃), 1.10 (3H, s, 19′-CH₃), 2.16–2.05 (2H, m, C4′′-H and C8′′-H), 2.71 (1H, d, J = 10.8, C7′′-H), 3.83 (3H, s, 5-OCH₃), 3.98–3.91 (1H, m, C3′′-H), 6.85 (1H, dd, J = 8.7, 2.4, C6-H), 7.06 (1H, s, C4-H), 7.44 (1H, d, J = 8.7, C7-H); ¹³C NMR (CDCl₃, 75 MHz) δ: 156.1 (2-C), 155.3 (5-C), 155.2 (8-C), 130.1 (9-C), 120.4 (7-C), 111.53 (6-C), 111.52 (4-C), 82.2 (5′-C), 67.0 (3′-C), 56.2 (17′-C), 55.8 (3-OCH₃), 55.5 (14′-C), 54.7 (7′-C), 54.2 (9′-C), 45.6 (13′-C), 45.2 (12′-C), 44.6 (24′-C), 42.6 (10′-C), 39.4 (4′-C), 39.4 (8′-C), 36.2 (22′-C), 35.5 (20′-C), 31.2 (1′-C), 29.7 (2′-C), 28.5 (16′-C), 28.0 (25′-C), 24.2 (15′-C), 23.8 (23′-C), 22.8 (27′-C), 22.5 (26′-C), 21.7 (11′-C), 18.8 (21′-C), 17.8 (19′-C), 12.5 (18′-C); HREIMS m/z: 537.4057 [M + H]+ (calcd. for C34H33N2O3, 537.4056).

2-(3β′-Acetoxy-5β′-hydroxy-6′-B-norcholesteryl)-5-methoxybenzimidazole (11b) Yellow solid, yield: 29%, m.p.: 135–137 °C. IR (KBr) v/cm⁻¹: 3456, 2942, 2863, 1716, 1629, 1594, 1486, 1417, 1372, 798; ¹H NMR (CDCl₃, 300 MHz) δ: 0.65 (3H, s, 18′-CH₃), 0.85 (6H, d, J = 6.6, 26′- and 27′-CH₃), 0.91 (3H, d, J = 6.3, 21′-CH₃), 1.09 (3H, s, 19′-CH₃), 1.96 (3H, s, 3-COCH₃), 2.20 (1H, q, J = 6.3, C8′′-H), 2.76 (1H, d, J = 10.8, C7′′-H), 3.68 (1H, br s, -OH), 3.83 (3H, s, 5-OCH₃), 5.01–4.93 (1H, m, C3′′-H), 6.85 (1H, dd, J = 8.7, 2.1, C6-H), 7.19 (1H, br s, C4-H), 7.55 (1H, br s, C7-H); ¹³C NMR (CDCl₃, 75 MHz) δ: 170.7 (3′-C=O), 156.1 (2-C), 155.3 (5-C), 155.2 (8-C), 130.1 (9-C), 120.4 (7-C), 111.53 (6-C), 111.52 (4-C), 81.1 (5′-C), 70.0 (3′-C), 55.8 (17′-C), 55.7 (3-OCH₃), 55.4 (14′-C), 54.7 (7′-C), 54.4 (9′-C), 45.1 (13′-C), 44.8 (12′-C), 44.5 (24′-C), 39.7 (10′-C), 39.4 (4′-C), 39.3 (8′-C), 36.2 (22′-C), 35.5 (20′-C), 32.8 (1′-C), 29.7 (2′-C), 28.5 (16′-C), 28.0 (25′-C), 25.4 (15′-C), 23.8 (23′-C), 22.8 (27′-C), 22.5 (26′-C), 21.9 (CH3CO), 21.4 (11′-C), 18.8 (21′-C), 18.1 (19′-C), 12.5 (18′-C); HREIMS m/z: 579.4162 [M + H]+ (calcd. for C38H58N2O4, 579.4142).

3.1.2. Compounds 12a–12b Were Prepared Similarly According to the Procedure of 7–11, but Using 4-Trifluoromethyl-O-phenylenediamine as Reagent

N-(2′-amino-5′-trifluoromethyl)phenyl-3β-hydroxy-B-norcholestano[7,5-d] isoxazolidine (12a) Brownish oil, yield: 40%; IR (KBr) v/cm⁻¹: 3424, 2947, 1627, 1524, 1447, 1329, 1232, 1110, 931; ¹H NMR (CDCl₃, 300 MHz) δ: 0.66 (3H, s, 18-CH₃), 0.87 (6H, d, J = 6.6, 26- and 27-CH₃), 0.94 (3H, d, J = 6.3, 21-CH₃), 1.08 (3H, s, 19-CH₃), 2.08 (1H, d, J = 12.9, C6-H), 2.17 (1H, q, J = 10.8, C6-H), 2.79 (1H, d, J = 10.5, C7-H), 3.88 (1H, br s, C3-H), 4.30 (1H, br s, -OH), 7.44 (1H, d, J = 8.4, 6-ArH), 7.60 (1H, br s, 3-ArH), 7.88 (1H, br s, 5-ArH); ¹³C NMR (CDCl₃, 75 MHz) δ: 158.3 (1-PhC), 130.3 (CF₃), 126.7 (2-PhC), 124.7 (4-PhC), 124.2 (5-PhC), 123.1 (3-PhC), 119.1 (6-PhC), 82.5 (5-C), 70.0 (3-C), 56.1 (17-C), 55.3 (14-C), 55.1 (9-C), 54.7 (7-C), 45.8 (8-C), 45.3 (13-C), 44.6 (10-C), 42.3 (6-C), 39.5 (12-C), 39.4 (24-C), 36.2 (4-C), 35.5 (20-C), 31.6 (22-C), 29.7 (1′-C), 28.4 (2′-C), 28.3 (16′-C), 28.0 (25′-C), 24.2 (15′-C), 23.7 (23′-C), 22.8 (26′-C), 22.5 (27′-C), 21.7 (11′-C), 18.8 (21′-C), 17.5 (19′-C), 12.4 (18′-C); HREIMS m/z: 577.3902 [M + H]+ (calcd. for C38H58F3N2O2, 577.3981).
N-(2'-amino-5'-trifluoromethyl)phenyl-3β-acetoxy-B-norcholestanol[7,5-d] isoxazolidine (12b)

Yellow solid, yield: 87.8%, m.p.: 155–157 °C. IR (KBr) v/cm⁻¹: 3461, 2948, 2858, 1711, 1629, 1527, 1417, 1362, 1330, 1263, 1115, 1052; ¹H NMR (CDCl₃, 300 MHz) δ: 0.61 (3H, s, 18-CH₃), 0.85 (6H, d, J = 6.6, 26- and 27-CH₃), 0.91 (3H, d, J = 6.3, 21-CH₃), 1.10 (3H, s, 19-CH₃), 1.86 (3H, s, COCH₃), 2.28 (2H, q, J = 10.8, C6-H), 2.96 (1H, d, J = 11.7, C7-H), 3.38 (1H, br s, -NH), 5.02–4.89 (1H, m, C3-H), 7.45 (1H, d, J = 8.4, 6-ArH), 7.74 (1H, s, 3-ArH), 7.98 (1H, br s, 5-ArH); ¹³C NMR (CDCl₃, 75 MHz) δ: 170.6 (COCH₃), 157.3 (1-PhC), 130.3 (C₃F₃), 126.7 (2-PhC), 124.6 (4-PhC), 124.2 (5-PhC), 123.1 (3-PhC), 119.1 (6-PhC), 81.6 (5-C), 69.7 (3-C), 55.7 (17-C), 55.4 (14-C), 54.7 (9-C), 54.6 (7-C), 44.9 (8-C), 44.8 (10-C), 44.5 (13-C), 39.7 (6-C), 39.4 (12-C), 39.0 (24-C), 36.1 (22-C), 35.5 (20-C), 32.7 (4-C), 29.7 (1-C), 28.4 (16-C), 28.0 (25-C), 25.4 (2-C), 24.0 (15-C), 23.7 (23-C), 22.8 (26-C), 22.5 (27-C), 21.9 (11-C), 21.1 (CH₃CO), 18.8 (21-C), 18.2 (19-C), 12.4 (18-C); HREIMS m/z: [M + H]+ 619.3996 (calcd. for C₃₈H₅₄F₃N₂O₃S, 619.4087).

3.1.3. Compound 13 Was Prepared Similarly According to the Procedure of 7–11, but Using 2-Mercaptophenylamine as Reagent

2-(3β',5β'-Dihydroxy-6'-B-norcholesterolyl)benzothiazole (13) Light yellow solid, yield: 55%, m.p.: 180–182 °C. IR (KBr) v/cm⁻¹: 3453, 2920, 2726, 1674, 1589, 1462, 1377, 1165, 1073, 951; ¹H NMR (CDCl₃, 300 MHz) δ: 0.69 (3H, s, 18'-CH₃), 0.87 (3H, d, J = 6.6, 26'- or 27'-CH₃), 0.87 (3H, d, J = 6.6, 26'- or 27'-CH₃), 0.94 (3H, d, J = 6.3, 21'-CH₃), 1.13 (3H, s, 19'-CH₃), 2.14–2.06 (2H, m, C4'-H), 2.25 (1H, q, J = 10.8, C8'-H), 3.04 (1H, d, J = 9.6, C7'-H), 3.30 (1H, br s, -OH), 3.95 (1H, s, -OH), 4.10–4.04 (1H, m, C3'-H), 7.37 (1H, td, J = 8.1, 1.5, 5-ArH), 7.47 (1H, td, J = 7.8, 1.5, 6-ArH), 7.85 (1H, d, J = 7.5, 4-ArH), 7.99 (1H, d, J = 7.8, 7-ArH); ¹³C NMR (CDCl₃, 75MHz) δ: 173.1 (2-C), 153.4 (8-C), 134.5 (9-C), 126.0 (5-C), 124.7 (6-C), 122.7 (7-C), 121.4 (4-C), 83.2 (5'-C), 67.1 (3'-C), 58.3 (17'-C), 56.8 (14'-C), 55.5 (7'-C), 51.8 (9'-C), 47.2 (13'-C), 45.3 (8'-C), 44.8 (12'-C), 44.1 (24'-C), 39.7 (10'-CH₃), 39.5 (4'-C), 36.2 (22'-C), 35.6 (20'-C), 28.9 (1'-C), 28.5 (2'-C), 28.4 (16'-C), 28.0 (25'-C), 24.7 (15'-C), 23.8 (23'-C), 22.8 (27'-C), 22.5 (26'-C), 21.6 (11'-C), 18.8 (21'-C), 18.2 (19'-C), 12.5 (18'-C); HREIMS m/z: 524.3562 [M + H]+ (calcd. for C₃₃H₃₅NO₂S, 524.3562).

3.1.4. 3β,5β-Dihydroxy-6-(N-3'-(2-amino)pyridyl)imine-B-norcholestan (15)

Compound 15 was prepared similarly according to the procedure of 7–11, but using 2,3-diaminopyridine as reagent.

Brown oil, Yield: 49%. IR (KBr) v/cm⁻¹: 3454, 2920, 1716, 1671, 1589, 1462, 1377, 1165, 1073, 1013; ¹H NMR (CDCl₃, 300 MHz) δ: 0.67 (3H, s, 18-CH₃), 0.84 (6H, d, J = 6.9, 26- and 27-CH₃), 0.90 (3H, d, J = 6.3, 21-CH₃), 0.95 (3H, s, 19-CH₃), 2.35–2.30 (1H, m, C7-H), 3.38 (1H, s, -OH), 4.12–4.07 (1H, m, C3-H), 4.34 (2H, br s, -NH₂), 5.00 (1H, s, -OH), 6.52 (1H, dd, J = 7.5, 5.1, 5'-PhH), 6.94 (1H, dd, J = 7.5, 1.5, 4'-PhH), 7.77 (1H, dd, J = 5.1, 1.2, 6'-PhH), 7.85 (1H, d, J = 6.6, C6-H); ¹³C NMR (CDCl₃, 75 MHz) δ: 170.7 (6-C), 153.2 (2-PhC), 144.4 (6-PhC), 133.5 (3-PhC), 125.0 (4-PhC), 113.8 (5-PhC), 84.3 (5-C), 66.7 (3-C), 60.4 (17-C), 59.4 (14-C), 56.2 (7-C) 55.6 (9-C), 51.4 (13-C), 45.5 (10-C), 44.7 (8-C), 44.1 (12-C), 42.6 (24-C), 39.7 (4-C), 39.4 (22-C), 36.2 (20-C), 35.6 (1-C), 28.5
(2-C), 28.0 (16-C), 24.9 (25-C), 23.8 (15-C), 22.8 (26-C), 22.6 (27-C), 21.7 (23-C), 21.0 (11-C), 18.8 (21-C), 14.2 (19-C), 12.5 (18-C); HREIMS m/z: 510.4090 [M + H]^+ (calcd. for C_{32}H_{52}N_{3}O_{2}, 510.4060).

3.2. Biological Assays

3.2.1. Materials

Stock solutions of the compounds were prepared in sterile dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) at a concentration of 10 mg/mL and afterward diluted with complete nutrient medium (RPMI-1640) supplemented with 10% heat inactivated fetal bovine serum and 0.1 g/L penicillin G + 0.1 g/L streptomycin sulfate.

3.2.2. Cell Culture

HeLa, A549, HEPG2 cancer cells and HEK293T cells were grown in the medium (RPMI-1640) supplemented with 10% heat inactivated fetal bovine serum and 0.1 g/L penicillin G + 0.1 g/L streptomycin sulfate in a humidified atmosphere of 5% CO₂ at 37 °C.

3.2.3. Assay for Cell Viability

The anticancer activity in vitro was measured using the MTT assay. Briefly, cells (1~2 × 10⁴ cells per well) were seeded in 96-wells plates for 24 h. Different concentrations of the test compound were added to the cells. An equal amount of DMSO was added to the cells used as negative controls. Triplicate wells were prepared for each individual dose. After reincubated for 72 h, the cells were washed with sterile phosphate buffer saline (PBS). 190 µL of RPMI-1640 and 10 µL of the tetrazolium dye (MTT) (5 mg/mL) solution were added to each well, and the cells were incubated for additional 4 h. After the supernatant was discarded, 200 µL of DMSO was added to dissolve the purple formazan crystals formed. The absorbance values (A) at 492 nm were determined using a MLLTISKAN MK3 analysis spectrometer (Thermo Scientific, Shanghai, China). The IC_{50} values were calculated as the concentration of drug yielding 50% cell survival.

3.2.4. Annexin V Staining Assay

Apoptosis was detected with an annexin V-FITC kit purchased from BD Pharmingen (San Diego, CA, USA) according to the manufacturer’s instructions. HeLa cells were seeded in 35 mm culture dishes and allowed to attach overnight. The cells were treated with 9b, 10a and 11a for 24 h, respectively, collected, and washed twice with PBS. To detect early and late apoptosis, both adherent and floating cells were harvested together and resuspended in annexin V binding buffer at a concentration of 10⁶ cells/mL. Subsequently, 5 µL of FITC-conjugated annexin V and 5 µL of propidium iodide were added to 100 µL of the cell suspension (10⁵ cells). The cells were incubated for 15 min at room temperature in the dark. Finally, 400 µL of annexin V binding buffer was added to each tube, and cells were analyzed by a two-color cytometry using FACS Calibur flow cytometry (Becton Dickinson, Biosciences, Franklin Lakes, NJ, USA).
4. Conclusions

We synthesized some novel B-norcholesteryl benzimidazole and benzothiazole derivatives. The antiproliferative activity of the compounds against human cervical carcinoma (HeLa), human lung carcinoma (A549), human liver carcinoma cells (HEPG2) and normal kidney epithelial cells (HEK293T) was assayed. The results showed that some B-norcholesteryl benzimidazole compounds exhibited an excellent antiproliferative activity and almost inactive to normal kidney epithelial cells (HEK293T). In addition, the results revealed that the benzimidazole group was a better substituent than benzothiazole group for increasing the antiproliferative activity of compounds. The most potent compound 9b with the structure of 6-benzimidazole exhibited excellent antiproliferative activities with an IC$_{50}$ value of 4.7, 11.9 and 4.2 μM against HeLa, A549 and HEPG2 cells, respectively, and was able to effectively induce tumor cells apoptotic. The results suggest that B-norcholesteryl derivatives based on benzimidazole group may constitute a novel class of antiproliferative agents, which deserve further study.

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Author Contributions

Y.M. Huang was responsible for the natural science foundation of China (No:21462009). J.G. Cui conceived the design of synthetic route and was responsible for the guidance of all synthetic experiments. B.B. Qi, Q.F. Lin and D.D. Zhao synthetized B-norcholesteryl Benzimidazole and Benzothiazole Derivatives. Z.P. Liu and H. Huang screened cell lines in vitro. C.F. Gan performed the apoptotic experiment.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. World Health Organization. *The Global Burden of Disease: 2004 Update*; World Health Organization: Geneva, Switzerland, 2008.
2. Jemal, A.; Siegel, R.; Ward, E.C.A. Cancer Statistics, 2010. *Cancer J. Clin.* **2010**, *60*, 277–300.
3. Miyamoto, T.; Kodama, K.; Aramaki, H.Y.R.; van Soest, R.W.M. Orostanal, a novel abeo-sterol inducing apoptosis in leukemia cell from a marine sponge, *Stelletta hiwasaensis*. *Tetrahedron Lett.* **2001**, *42*, 6349–6351.
4. Wei, X.; Rodriguez, A.D.; Wang, Y.; Franzblau, S.G. Novel ring B abeo-sterols as growth inhibitors of *Mycobacterium tuberculosis* isolated from a Caribbean Sea sponge, *Svenzea zeai*. *Tetrahedron Lett.* **2007**, *48*, 8851–8854.
5. Gan, C.F.; Fan, L.H.; Cui, J.G.; Huang, Y.M.; Jiao, Y.X.; Wei, W.X. Synthesis and in vitro antiproliferative evaluation of some ring B abeo-sterols. Steroids 2012, 77, 1061–1068.

6. Gan, C.F.; Fan, L.H.; Huang, Y.M.; Liu, Z.P.; Cui, J.G. Synthesis of novel ring B abeo-sterol derivatives and their antiproliferative activities. Med. Chem. 2013, 9, 846–854.

7. Gan, C.F.; Lin, Q.F.; Cui, J.G.; Feng, J.D.; Guo, J.N.; Liao, H.Y.; Huang, Y.M. Synthesis and in vitro antiproliferative evaluation of some novel B-norcholesterols. Steroids 2014, 79, 37–43.

8. Singh, H.; Kapoor, V.K.; Paul, D. Heterostereoids and drug research. In Progress in Medicinal Chemistry; Ellis, G.P., West, G.B., Eds.; Springer: Amsterdam, The Netherlands, 1979; Volume 16, pp. 35–150.

9. Singh, H.; Jindal, D.P.; Yadav, M.R.; Kumar, M. Heterostereoids and drug research. In Progress in Medicinal Chemistry; Ellis, G.P., West, G.B., Eds.; Elsevier science publishers: Amsterdam, The Netherlands, 1991; Volume 28, pp. 233–300.

10. Lakhani, N.J.; Sarkar, M.A.; Venitz, J.; Figg, W.D. 2-Methoxyestradiol, a promising anticancer agent. Pharmacotherapy 2003, 23, 165–172.

11. Carlini, P.; Frassoldati, A.; Marco, S.D.; Casali, A.; Ruggeri, E.M.; Nardi, M.; Papaldo, P.; Fabi, A.; Paoloni, F.; Cognetti, F. Formestane, a steroidal aromatase inhibitor after failure of non-steroidal aromatase inhibitors (anastrozole and letrozole): Is a clinical benefit still achievable? Ann. Oncol. 2001, 12, 1539–1543.

12. Simpson, D.; Wagstaff, A.J. Estramustine Phosphate Sodium. Am. J. Can. 2003, 2, 373–390.

13. Attard, G.; Reid, A.H.M.; Yap, T.A.; Raynaud, F.; Dowsett, M.; Settatree, S.; Barrett, M.; Parker, C.; Martins, V.; Folkerd, E.; et al. Phase I Clinical trial of a selective inhibitor of CYP17, abiraterone acetate, confirms that castration-resistant prostate cancer commonly remains hormone driven. J. Clin. Oncol. 2008, 26, 4563–4571.

14. Cui, J.G.; Liu, L.; Gan, C.F.; Xiao, Q.; Huang, Y.M. Recent progress in synthesis and biological activity of steroids bearing aromatic rings and heterocycles. Progress Chem. 2014, 26, 320–333.

15. Ma, B.; Xiao, Z.Y.; Chen, Y.J.; Lei, M.; Meng, Y.H.; Guo, D.A.; Liu, X.; Hu, L.H. Synthesis and structure activity relationships study of cytotoxic bufalin 3-nitrogen-containing-ester derivatives. Steroids 2013, 78, 508–512.

16. Guo, H.; Zhang, G.L.; Zhang, T.; He, X.R.; Wu, Z.Y.; Xiao, Y.L.; Pan, Y.H.; Qiu, G.F.; Liu, P.; Hu, X.M. Synthesis, characterization and biological evaluation of some 16b-azoly-3bamino-5a-androstone derivatives as potential anticancer agents. Eur. J. Med. Chem. 2011, 46, 3662–3674.

17. Huang, L.H.; Zheng, Y.F.; Lu, Y.Z.; Song, C.J.; Wang, Y.G.; Yu, B.; Liu, H.M. Synthesis and biological evaluation of novel steroidal[17,16-d][1,2,4]triazolo [1,5-a]pyrimidines. Steroids 2012, 77, 710–715.

18. Kovács, D.; Mótyán, G.; Wölfling, J.; Kovács, I.; Zupkó, I.; Frank, É. A facile access to novel steroidal 17-2’-(1’,3’,4’)-oxadiazoles, and anevaluation of their cytotoxic activities in vitro. Bioorg. Med. Chem. Lett. 2014, 24, 1265–1268.

19. Zhang, B.L.; Zhang, E.; Pang, L.P.; Song, L.X.; Li, Y.F.; Yu, B.; Liu, H.M. Design and synthesis of novel D-ring fused steroidal heterocycles. Steroids 2013, 78, 1200–1208.

20. Liu, B.; Zhou, W.S. The first stereoselective synthesis of orostanal isolated from a marine sponge Stelletta hiwasaensis. Tetrahedron 2003, 59, 3379–3384.
21. Wentworth, P., Jr.; Nieva, J.; Takeuchi, C.; Galve, R.; Wentworth, A.D.; Dilley, R.B.; DeLaria, G.A.; Saven, A.; Babior, B.M.; Janda, K.D.; et al. Evidence for ozone formation in human atherosclerotic arteries. *Science* **2003**, *302*, 1053.

22. Natalie, K.C.; Johanna, C.S.; Terry, D.B.; Wentworth, P., Jr. Adduction of cholesterol 5,6-secosterol aldehyde to membrane-bound myelin basic protein exposes an immunodominant epitope. *Biochemistry* **2011**, *50*, 2092–2100.

23. Lin, S.N.; Yang, L.H. A simple and efficient procedure for the synthesis of benzimidazoles using air as the oxidant. *Tetrahedron Lett.* **2005**, *46*, 4315–4319.

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