Ginkgolide B derivative synthesis and their effects on the viability of SKOV3 cells

Zi-Li Feng
Shaanxi University of Technology
Zhi-Bin Zhu
Shaanxi University of Technology
Wang Chen (chenwang@snut.edu.cn)
Shaanxi University of Technology https://orcid.org/0000-0003-0565-9157
Jia-Yuan Xu
Shaanxi University of Technology
Yu Bai
Shaanxi University of Technology
Dai-Hua Hu
Shaanxi University of Technology
Zheng-Dong Zhao
Shaanxi Cuicheng Biomedical Tehnology CO., Ltd.

Research Article

Keywords: ginkgolide, derivatives, ovarian cancer, SKOV3 cell viability, apoptosis

DOI: https://doi.org/10.21203/rs.3.rs-266392/v1

Abstract

The natural product GB was used as a raw material and modified by esterification on C10-OH or C1-OH to obtain 11 derivatives (**1** to **11**), which were structurally characterized with nuclear magnetic resonance spectroscopy. An MTT assay-based *in vitro* tumor proliferation inhibitory activity test showed that compounds **2**, **3**, **6**, **7**, **10**, and **11** exhibited strong inhibitory activity against the human ovarian cancer cells SKOV3, with IC_{50} values of 16.05 μmol/L, 15.65 μmol/L, 32.00 μmol/L, 63.30 μmol/L, 23.20 μmol/L, and 31.10 μmol/L, respectively. Annexin V/PI double staining assay showed that compound **2** induced apoptosis in SKOV3 cells to a slightly greater extent than GB and compounds **5**, and **9**, with an apoptosis rate of 31.68%.

Introduction

Ginkgolide B (GB) is a diterpenoid isolated from Ginkgo biloba, which is associated with various pharmacological activities, such as anti-platelet aggregation, anti-inflammatory, antioxidant, and free radical scavenging [1–5]. In recent years, GB has also been reported to have anti-tumor effects [6]; it inhibits the growth and proliferation of various tumor cells, such as ovarian, breast, lung, colorectal, bladder, and prostate cancers, with a rich set of anti-tumor targets and pathways [7–15].

The PAF/PAFR signaling axis has emerged as an important determinant of the aggressive phenotype in several malignancies, including ovarian cancer [16–17]. GB, as a potent PAFR antagonist, may be a key regulator in treating tumor cells. Jiang Wei et al. [7–8] conducted *in vivo* and *in vitro* experiments and found that GB could inhibit the proliferation of ovarian cancer cells and growth of nude mice tumors with a tumor suppression rate of 48%, and up to 78.2% in combination with cisplatin (CDDP), which could be used as an adjuvant drug for the treatment of ovarian cancer. The mechanism may be that GB inhibits the expression of platelet-activating factor (PAF), platelet-activating factor receptor (PAFR), and the tyrosine kinase Src and the p38 mitogen-activated protein kinase (p38MAPK), preventing p38MAPK from activating downstream transcription factors [18], including the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), which is closely related to cell invasion. This results in the inability of CREB to bind to the promoter in the upstream of the matrix metalloproteinase 2/9 (MMP2/MMP9) gene and the inability of MMP2 mRNA/MMP9 mMRA to be expressed, which inhibits the migration of ovarian cancer cells. In addition, ginkgolide B upregulated 25 proteins with antitumor effects (e.g., p53) and downregulated 22 proteins associated with tumor migration (e.g., β-catenin).

Yu Y et al. [19] demonstrated that the PAFR upregulation in CDDP-treated ovarian cancer cells provided additional support for the function of the PAF/PAFR signaling axis as a resistance mechanism, with the possible mechanism being that cisplatin (CDDP) acts on the ovarian cancer cells and causes nuclear factor kappa-B (NF-KB) and hypoxia-inducible factor (HIF-1α) to accumulate in the nucleus, leading to the upregulation of the platelet-activating factor receptor PAFR. In addition, GB inhibited the PAFR activity, which may block the downstream signaling pathways of phosphoinositide 3-kinase (PI3K) and the

extracellular regulated protein kinase (ERK), thereby enhancing the sensitivity of ovarian cancer cells to CDDP, intensifying drug efficacy and significantly reducing tumor growth.

Ginkgolide B has a unique rigid caged dodeca-carbon skeleton structure, and it was found that the synthesis of GB ester derivatives by converting the hydroxyl groups at the C-1 and (or) C-10 positions of GB into aromatic-containing groups such as ester groups significantly enhanced the anti-PAF activity [20–21]. To explore the effect of GB parent structure and the introduction of small molecule side chains of benzoic acid series on the hydroxyl groups at C-1 and (or) C-10 positions on pharmacological activity, this study constructed GB ester derivatives by the esterification reaction of GB as the parent nucleus with compounds with aromatic groups such as p-chlorobenzoic acid, p-fluorobenzoic acid, p-nitrobenzoic acid, p-methoxybenzoic acid, 3-methoxybenzoic acid, and 3'5'-dinitrobenzoic acid by reference to the design of nicotinic acid esters, cinnamic acid esters, and benzoic esters [22–30]. The GB derivatives were structurally characterized by nuclear magnetic resonance spectroscopy (NMR), tested for *in vitro* tumor proliferation inhibitory activity by thiazolyl blue (MTT), and detected for ovarian cancer SKOV3 cell apoptosis by the Annexin V/PI double staining assay.

Materials And Methods

Chemistry

All reagents and solvents used in this study were purchased from Tianjin Fuyu Fine Chemical Co., Ltd. (P.R. China), Shanghai Macklin Biochemical Technology Co., Ltd. (P.R. China), and Energy Chemical (P.R. China), and used as received without further purification unless otherwise noted. In addition, some biological reagents were purchased from Sigma or GIBCO, USA.

TLC was carried out on Silica Gel GF254 plates (Qingdao Haiyang Chemical Co., Ltd) and spots were visualized by iodine vapors or by irradiation with UV light (254 nm). All water employed was ultrapure (> 18.2 MΩcm-1 at 25℃, Milli-Q, Millipore, Billerica, MA). The NMR spectra were measured by Bruker Avance 600 NMR analyzer (Bruker, Switzerland) in the indicated solvents. Chemical shifts are expressed in ppm (δ units) relative to TMS signal as internal reference, and the coupling constant values (J) are shown in Hertz. Signal multiplicities are reported by the following abbreviations: s (singlet), d (doublet), t (triplet), dd (double doublet), q (quartet), m (multiplet), and brs (broad signal). HB DAC-50 was used for preparative liquid chromatograph (Jiangsu Hanbon Science & Technology Co., Ltd.). Agilent 1260 high performance liquid chromatograph (Agilent Technologies Co. Ltd, USA), SCIENTZ-10N vacuum freeze dryer (Ningbo Xinzhi Biotechnology Co. Ltd.), YG-875B ultra clean bench (Suzhou Medical Equipment Factory), inverted phase contrast microscope (Zeiss, Germany), enzyme marker (Biotek, USA), CO_2 cell incubator (Thermo Fisher Scientific), and GB (98.24% purity, lab-made) were used.

Tumor cell growth inhibitory assay

The SKOV3 cells used in the following cell experiments were obtained from the Shanghai EK-Bioscience Co., Ltd. (P.R. China). SKOV3 cells were maintained in DMEM containing 10% fetal bovine serum (FBS).

All cells were grown at 37°C in a humidified atmosphere of 5% CO_2. All the reagents used in this study, unless otherwise indicated, were purchased from Sigma (USA). The compounds with inhibitory activity against human ovarian cancer cells SKOV3 were screened by the MTT assay [31]. Briefly, SKOV3 cells were cultured at a density of 80 % to 90 % and inoculated in 96-well plates at 2×10^4/well. The attached cells were placed overnight and treated with increasing concentrations of drug for 24 h. Blank controls (no drug treatment), and experiment groups (containing 5 μmol/L, 10 μmol/L, 20 μmol/L, 40 μmol/L, 60 μmol/L, 80 μmol/L, and 100 μmol/L) of ginkgolide B derivatives compounds **1** to **11** were used to treat ovarian cancer cells, respectively. After 24 h of cell culture, 20 μL of MTT was added to the cell medium of each well, and the plates were mixed by gently tapping. After continuing incubation for 4 h, the culture supernatant in the wells was aspirated and discarded, 150 μL of DMSO solution was added to each well and shaken for 10 min on a shaker. Cell growth was examined by detecting the absorbance value (A) of each well at a wavelength of 550 nm by an enzyme marker to preliminarily investigate the optimal concentration of compounds **1**~**11** on the viability of SKOV3 cells. Three replicate wells were set up for each concentration. The inhibition rate was calculated according to the following formula, and IC_{50} was calculated using the software Prism 5 (GraphPad Software, Inc.).

Inhibition rate = [1 − (*OD* of treatment − *OD* of blank)/(*OD* of control − *OD* of blank)] × 100%

lg $IC_{50} = Xm - I[P - (3 - Pm - Pn)/4]$

Xm − lg Maximum dose

I − lg (maximum dose/adjacent dose)

P − (sum of positive rate)

Pm − (Maximum positive rate)

Pn − (minimum positive rate)

Annexin V/PI double staining assay to observe SKOV3 cell apoptosis

Annexin V-FITC Apoptosis Detection Kit was purchased from Sigma (USA) and used according to the manufacturer's instructions. Human ovarian cancer cells SKOV3 at the logarithmic growth stage were inoculated in 6-well plates at 37°C, 5% CO_2, and cultured overnight. Drug-treated groups were treated with compound **1**, **3**, **6**, and **10** at the optimal mass concentration for 48 h. The negative control group was treated with McCoy's 5A complete medium only. After digestion of cells with EDTA-free trypsin, cells were collected by centrifugation at 4 °C, 7000 rpm/min for 5 min. Cells were resuspended in precooled sterilized PBS at 4°C and 7000 rpm/min for 5 min, and the supernatant was discarded. The 1 × binding buffer (195 μL) was added. After suspending the cells, 5 μL of Annexin V-FITC was added and mixed gently. Ten μL of propidium iodide staining solution was added, mixed gently, and incubated at room temperature for 15 min protected from light. Stained samples were analyzed immediately by a FACScan

flow cytometer (Beckman coulter EPICS xL). The fractions of cell population in different quadrants were analyzed using quadrant statistics. Cells in the lower right quadrant represented apoptosis while cells in the upper right quadrant represented necrosis or post apoptotic necrosis.

General procedure for the synthesis of GB derivatives 1-11

The synthetic route was shown in Fig. 1. A 100.0 mL round bottom flask was added with 212.2 mg of GB (0.5 mmol), 122.2 mg of 4-dimethylaminopyridine (DMAP) (1.0 mmol), 191.7 mg of 1-ethyl-carbodiimide hydrochloride (EDC-HCl) (1.0 mmol) and 6 benzoic acids (*p*-chlorobenzoic acid, *p*-fluorobenzoic acid, *p*-nitrobenzoic acid, *p*-methoxybenzoic acid, 3-methoxybenzoic acid, 3,5-dinitrobenzoic acid) with 1.0 mmol, respectively. Anhydrous dichloromethane 50.0 mL was added and stirred under room temperature. The reaction was detected by TLC after 6 h at 1 h intervals and fumigated with sulfuric acid-ethanol solution or acetic acid-iodine. The reaction was considered complete when the spots of GB disappeared. After recovering the dichloromethane solution under reduced pressure, the residue was dissolved with 30.0 mL of ethyl acetate and underwent reverse extraction three times with water, with 15.0 mL/time. The ethyl acetate layer was dried by anhydrous sodium sulfate and concentrated under reduced pressure to dryness. The product of each synthesis was dissolved in acetonitrile to make a sample solution of 30.0 mg/mL. After passing through a 0.45 μm filter membrane, the product was purified by high pressure preparative chromatography eluting with acetonitrile/water to produce the corresponding derivatives.

1-(4-chlorobenzoyl) GB (1)

White powder, yield 12.12%, λ_{max} = 254 nm. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.04 (m, 2H), 7.65 (dd, J = 11.2, 4.4 Hz, 2H), 6.71 (d, J = 4.9 Hz, 1H), 6.67 (s, 1H), 6.16 (d, J = 93.6 Hz, 1H), 5.69 (dd, J = 61.2, 3.8 Hz, 1H), 5.64 (d, J = 3.6 Hz, 1H), 4.96 (m, 1H), 4.93 (d, J = 5.6 Hz, 1H), 2.97 (m, 1H), 2.22 (dd, J = 13.6, 4.5 Hz, 1H), 2.00 (ddd, J = 36.1, 28.3, 24.2 Hz, 1H), 1.79 (ddd, J = 31.8, 14.3, 4.4 Hz, 1H), 1.13 (dd, J = 28.9, 5.1 Hz, 3H), 1.01 (d, J = 6.7 Hz, 9H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 176.45, 173.78, 170.41, 163.44, 139.07, 131.89, 129.51, 128.51, 109.59, 99.31, 91.71, 83.85, 79.84, 75.40, 71.04, 68.72, 68.30, 48.88, 41.70, 40.54, 37.07, 32.45, 29.35, 8.62. ESI-MS: *m/z* 563.3 $[M+H]^+$.

10-(4-chlorobenzoyl) GB (2)

White powder, 15.48% yield, λ_{max} = 254 nm. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.06 (m, 2H), 7.54 (m, 2H), 6.82 (s, 1H), 6.48 (s, 1H), 6.25 (s, 1H), 6.22 (s, 1H), 5.46 (s, 1H), 4.62 (t, *J* = 21.1 Hz, 1H), 4.09 (d, *J* = 5.3 Hz, 1H), 2.86 (q, *J* = 7.0 Hz, 1H), 2.14 (m, 1H), 1.75 (d, *J* = 12.6 Hz, 2H), 1.07 (d, *J* = 7.2 Hz, 3H), 0.90 (s, 9H, t-Bu); ^{13}C NMR (151 MHz, DMSO-d_6) δ 176.72, 170.41, 168.96, 164.18, 139.94, 132.47, 129.55, 127.05, 110.46, 100.16, 94.36, 83.33, 78.66, 74.38, 72.38, 70.72, 67.45, 48.89, 41.88, 40.53, 37.22, 32.21, 30.57, 28.89, 8.51. ESI-MS: *m/z* 563.3 $[M+H]^+$.

1-(4-fluorobenzoyl) GB (3)

White powder, yield 15.13%, λ_{max} = 210 nm,^{1}H NMR (600 MHz, DMSO-d_6) δ 8.00 (m, 2H), 7.32 (m, 2H), 6.64 (t, J = 4.3 Hz, 1H), 6.61 (s, 1H), 6.01 (s, 1H), 5.68 (d, J = 4.1 Hz, 1H), 5.46 (dd, J = 14.8, 5.1 Hz, 1H), 4.91 (d, J = 6.4 Hz, 1H), 4.84 (m, 1H), 2.91 (m, 1H), 2.14 (m, 1H), 1.98 (m, 1H), 1.71 (m, 1H), 1.07 (m, 3H), 0.92 (m, 9H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 176.46, 173.78 , 170.44 , 166.64 , 165.18 , 163.31 , 133.00 , 126.30, 116.54 , 109.58, 99.36 , 91.78, 83.85, 79.86, 75.31, 70.10 , 69.14 , 68.31 , 48.88, 41.69 , 40.48 , 37.07, 32.45 , 29.35 , 8.64. ESI-MS: *m/z* 547.2 $[M+H]^+$.

10-(4-fluorobenzoyl) GB (4)

White powder, 36.47% yield, λ_{max} = 210 nm, ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.21 (m, 2H), 7.32 (t, J = 8.8 Hz, 2H), 6.82 (d, J = 4.9 Hz, 1H), 6.47 (s, 1H), 6.25 (s, 1H), 6.22 (s, 1H), 5.46 (d, J = 2.9 Hz, 1H), 4.64 (d, J = 6.4 Hz, 1H), 4.09 (dd, J = 6.3, 5.1 Hz) 6.3, 5.1 Hz, 1H), 2.87 (q, J = 7.1 Hz, 1H), 2.14 (m, 1H), 1.77 (m, 2H), 1.07 (m, 3H), 0.90 (s, 9H,t-Bu); ^{13}C NMR (151 MHz, DMSO-d_6) δ 176.72, 170.42, 169.03, 165.41, 164.00, 133.74, 133.68, 124.83, 116.62, 110.84, 100.16, 94.39, 83.35, 78.66, 74.40, 72.39, 70.59, 67.47, 48.89, 41.88, 40.48, 37.23, 32.21, 28.89, 8.51. ESI-MS: *m/z* 547.2 $[M+H]^+$.

1-(4-methoxybenzoyl) GB (5)

White powder, yield 8.40%, molecular formula: $C_{28}H_{30}O_{12}$, λ_{max} = 254 nm, ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.93 (dd, J = 12.1, 8.8 Hz, 2H), 7.08 (dd, J = 8.8, 3.8 Hz, 2H), 6.66 (m, 1H), 6.07 (s, 1H), 5.93 (m, 1H), 5.74 (m, 1H), 5.52 (dd, J = 14.8, 4.9 Hz, 1H), 4.90 (m, 1H), 3.85 (d, J = 5.6 Hz, 3H), 2.92 (m, 1H), 2.21 (dd, J = 12.2, 8.1 Hz, 1H), 2.05 (m, 1H), 1.92 (td, J = 13.6, 3.7 Hz, 1H), 1.79 (m, 1H), 1.27 (m, 2H), 1.16 (d, J = 7.2 Hz, 1H), 0.98 (s, 9H,t-Bu); ^{13}C NMR (151 MHz, DMSO-d_6) δ 176.82, 169.76, 164.33, 163.83, 131.98, 121.01, 114.54, 110.03, 108.64, 104.27, 98.48, 85.44, 83.90, 80.24, 73.23, 70.20, 68.88, 68.44, 56.03, 49.92, 48.38, 42.03, 36.72, 31.48, 29.45, 8.72. ESI-MS: *m/z* 559.3 $[M+H]^+$.

10-(4-methoxybenzoyl) GB (6)

White powder, yield 55.57%, λ_{max} = 254 nm, ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.15 (dd, J = 16.9, 8.8 Hz, 2H), 7.08 (m, 2H), 6.80 (t, J = 47.4 Hz, 1H), 6.53 (s, 1H), 6.23 (m, 1H), 5.52 (m, 1H), 4.65 (dd, J = 50.4, 15.3 Hz, 1H), 4.16 (dd, J = 41.0, 35.2 Hz, 1H), 3.79 (m, 3H), 2.93 (m, 1H), 2.17 (m, 1H), 1.80 (m, 1H), 1.13 (m, 4H), 1.03 (dd, J = 16.2, 9.1 Hz, 1H), 0.96 (s, 9H,t -Bu); ^{13}C NMR (151 MHz, DMSO-d_6) δ 176.74, 169.88, 169.25, 164.54, 132.92, 131.08, 120.43, 114.64, 110.35, 100.27, 94.51, 83.44, 78.66, 74.46, 72.48, 70.16, 67.52, 56.04,48.91, 41.85, 40.48, 37.17, 32.19, 30.61, 28.90, 8.60. ESI-MS: *m/z* 559.3 $[M+H]^+$.

1-(4-Nitrobenzoyl) GB (7)

White powder, 38.62% yield, λ_{max} = 254 nm, ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.38 (m, 2H), 8.24 (m, 2H), 6.72 (m, 1H), 6.09 (d, J = 11.5 Hz, 1H), 5.78 (t, J = 11.0 Hz, 1H), 5.53 (m, 1H), 5.05 (t, J = 11.1 Hz, 1H), 4.93 (m, 1H), 2.98 (m, 1H), 2.24 (m, 1H), 2.03 (m, 1H), 1.78 (m, 1H), 1.28 (m, 1H), 1.14 (m, 3H), 1.01 (s, 9H,t-Bu);

^{13}C NMR (151 MHz, DMSO-d_6) δ 176.44, 173.81, 170.33, 162.89, 150.94, 134.95, 131.44, 124.39, 109.66, 99.24, 91.52, 83.85, 79.83, 75.87, 70.99, 69.17, 68.30, 48.87, 41.73, 40.55, 37.12, 32.49, 29.34, 14.07, 8.56. ESI-MS: *m/z* 574.4 $[M+H]^+$.

10-(4-Nitrobenzoyl) GB (8)

White powder, 26.1% yield, λ_{max} = 254 nm, ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.38 (m, 2H), 8.23 (dd, J = 18.7, 8.7 Hz, 2H), 6.77 (d, J = 4.7 Hz, 1H), 6.07 (m, 1H), 5.74 (t, J = 30.6 Hz, 1H), 5.55 (m, 1H), 5.05 (t, J = 11.1 Hz, 1H), 4.91 (dd, J = 34.0, 4.7 Hz 34.0, 4.7 Hz, 1H), 2.92 (m, 1H), 2.24 (m, 1H), 2.05 (m, 1H), 1.79 (m, 1H), 1.28 (ddd, J = 60.3, 40.8, 20.6 Hz, 1H), 1.18 (s, 3H), 1.01 (s, 9H,t-Bu); ^{13}C NMR (151 MHz, DMSO-d_6) δ 176.44, 173.81, 170.33, 162.89, 150.94, 134.96, 131.53, 124.43, 110.88, 99.25, 91.52, 83.85, 80.54, 75.87, 70.99, 68.74, 68.30, 48.88, 41.72, 40.50, 37.12, 32.49, 30.79, 29.34, 8.56. ESI-MS: *m/z* 574.4 $[M+H]^+$.

1-(5-methoxybenzoyl) GB (9)

White powder, yield 8.72%, λ_{max} = 254 nm, ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.78 (ddd, J = 13.3, 4.6, 3.6 Hz, 2H), 7.47 (m, 2H), 6.82 (t, J = 41.7 Hz, 1H), 6.52 (d, J = 45.7 Hz, 1H), 6.31 (s, 1H), 6.29 (s, 1H), 5.51 (m, 1H), 4.64 (m, 1H), 4.42 (m, 1H), 4.17 (dt, J = 22.0, 11.0 Hz, 1H), 3.80 (m, 1H), 2.88 (m, 1H), 2.21 (m, 1H), 1.85 (m, 1H), 1.14 (d, J = 7.3 Hz, 1H), 1.03 (m, 4H), 0.96 (s, 9H,t-Bu); ^{13}C NMR (151 MHz, DMSO-d_6) δ 177.64, 169.76, 169.06, 165.10, 159.72, 130.23, 129.47, 122.12, 114.87, 110.40, 100.19, 94.27, 83.60, 79.06, 74.39, 72.59, 70.54, 67.89, 55.85, 48.89, 42.27, 33.73, 31.75, 30.61, 29.12, 8.43. ESI-MS: *m/z* 559.3 $[M+H]^+$.

10-(5-methoxybenzoyl) GB (10)

White powder, yield 38.90%, λ_{max} = 254 nm, ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.56 (m, 1H), 7.49 (m, 2H), 7.27 (m, 1H), 6.69 (dt, J = 35.6, 19.0 Hz, 1H), 6.06 (m, 1H), 5.76 (m, 1H), 5.56 (ddd, J = 31.9, 19.4, 5.0 Hz, 1H), 5.02 (dd, J = 36.5, 17.5 Hz, 1H), 4.89 (dd, J = 33.1, 5.1 Hz, 1H), 4.08 (s, 1H), 3.87 (m, 3H), 2.96 (m, 1H), 2.25 (m, 1H), 2.01 (m, 1H), 1.81 (m, 1H), 1.23 (m, 1H), 1.18 (m, 2H), 0.99 (s, 9H,t-Bu); ^{13}C NMR (151 MHz, DMSO-d_6) δ 176.48, 173.38, 170.44, 164.25, 159.74, 131.14, 122.09, 119.67, 114.76, 109.59, 99.26, 91.79, 83.46, 79.49, 76.46, 73.32, 71.06, 68.50, 55.83, 49.40, 40.89, 36.97, 32.49, 29.34, 13.16, 8.60. ESI-MS: *m/z* 559.3 $[M+H]^+$.

1-(3-5-Dinitrobenzoyl) GB (11)

White powder, 61.25% yield, λ_{max} = 254 nm, ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.06 (m, 1H), 8.98 (dd, J = 15.0, 2.1 Hz, 2H), 6.82 (m, 2H), 6.12 (s, 1H), 5.86 (d, J = 4.1 Hz, 1H), 5.53 (dd, J = 30.6, 5.3 Hz, 1H), 5.21 (d, J = 6.9 Hz, 1H), 4.93 (m, 1H), 2.98 (m, 1H), 2.24 (dt, J = 41.8, 21.0 Hz, 1H), 1.97 (m, 1H), 1.73 (m, 1H), 1.13 (m, 3H), 0.99 (m, 9H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 176.43, 173.80, 170.22, 161.54, 148.97, 132.60,

130.40, 129.27, 123.39, 109.78, 99.04, 91.18, 83.58, 79.67, 76.52, 70.86, 69.22, 68.25, 48.86, 41.76, 40.53, 36.94, 30.88, 29.30, 8.37. ESI-MS: *m/z* 619.4 $[M+H]^+$.

Results And Discussion

According to the reaction described in Scheme 1, eleven novel GB derivatives **1**–**11** were synthesized by esterification using GB as a raw material. Two products can be simultaneously obtained in one pot reaction. Due to the higher reaction activity of C-10 hydroxyl in GB, the yields of esterification at the C-10 position were higher than at the C-1 position. Conformational analysis showed that compounds **1**, **3**, **5**, **7**, **9** and **11** were hydroxyesterification products at the C-1 position of GB, and compounds **2**, **4**, **6**, **8**, and **10** were hydroxyesterification products at the C-10 position of GB. A method for the rapid separation and purification of GB derivatives by preparative chromatography was established. The structure of all the final compounds **1**–**11** was confirmed by ^{1}H NMR and ^{13}C NMR. For the NMR spectra of compounds **1**–**11**, a singlet at δ 1.0-0.9 ppm that corresponded to tert butyl functionality, signals at δ 9.1-6.0 ppm revealed the presence of the benzene ring.

On the basis of previous studies, the final products **1**–**11** and GB were evaluated for their *in vitro* antiproliferative activity against ovarian cancer SKOV3 cell lines by MTT assay. The proliferation inhibitory activity of these compounds was measured at different concentrations. The IC_{50} values for each sample were obtained by plotting the inhibition rate against the drug concentration. The results are presented in Table 1. As we defined that compounds showing less than 50% inhibitory rate at 100 μM were inactive. Compounds **3**, **4**, **7**, **8** and **11** hardly showed any activity, the IC_{50} values against the SKOV3 cell lines are all above 100 μM. The rest of the compounds showed moderate to good activity on SKOV3 cell lines with IC_{50} values 15.65–63.30 μM. Obviously, as compared with compound **1** and **2**, the esterification position of C-1 or C-10 has little effect on the activity of the compounds. However, the inhibitory activities were increased by introduce of the electron-donating group into the benzyl group.

Table 1
In vitro antiproliferative activity of GB derivatives. IC_{50} values are shown in μM against SKOV3 cell line, respectively.

Compounds	IC_{50}	Compounds	IC_{50}
GB	45.4 ± 1.24	6	63.30 ± 2.04
1	16.05 ± 086	7	> 100
2	15.65 ± 0.78	8	> 100
3	> 100	9	23.20 ± 0.97
4	> 100	10	31.10 ± 1.05
5	32.00 ± 1.12	11	> 100

The apoptosis of ovarian cancer SKOV3 cells was induced by 100 μmol/L of GB, compounds **2**, **5**, and **9**. The apoptosis rates were found to be 7.98%, 31.68%, 8.82%, and 9.46%, respectively. Preliminary *in vitro* anti-tumor cell proliferation activity test and the Annexin V/PI double staining assay showed that compound **2** had the highest inhibition rate against ovarian cancer SKOV3 cells and could inhibit the proliferation of tumor cells and induce apoptosis. Ginkgolide B derivatives hydroxyesterified at the C-10 position showed significantly higher apoptosis rates than the hydroxyesterified product at the C-1 position in ovarian cancer cells SKOV3.

Conclusion

In this study, 11 novel GB derivatives **1**–**11** were synthesized and evaluated for their *in vitro* antiproliferative activities against human ovarian cancer SKOV3 cell lines. The majority of the compounds showed moderate to good activity on SKOV3 cell lines. Among these derivatives, compound **2** was found to exhibit better cytotoxic activity on against SKOV3 cells with IC_{50} values 15.65 μM. Annexin V/PI double staining assay showed that compound **2** induced apoptosis in SKOV3 cells to a slightly greater extent than GB and compounds **5**, and **9**, with an apoptosis rate of 31.68%. The above findings will be of great significance for the development of GB derivatives as potential antitumor agents.

Declarations

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31702206), Key projects of science and Technology Department of Shaanxi Province (2018ZDXM-SF-083), and Natural Science Foundation of Shaanxi Province of China (2019JQ-530).

Conflict of 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.

References

1. Ahlemeyer B, Krieglstein J (2003) Pharmacological studies supporting the therapeutic use of Ginkgo biloba extract for Alzheimer's disease. Pharmacopsychiatry 36(1):8–14
2. Bate C, Salmona M, Williams A (2004) Ginkgolide B inhibits the neurotoxicity of prions or amyloid-beta1-42. J Neuroinflamm 1(1):4
3. Tian QY, Gong LL (2016) Research progress of ginkgolide. Zhongnan Pharmacol 14(8):838–841
4. Nabavi SM, Habtemariam S, Daglia M, Braidy N, Loizzo MR, Tundis R, Nabavi SF (2015) Neuroprotective Effects of Ginkgolide B Against Ischemic Stroke: A Review of Current Literature. Curr Top Med Chem 15(21):2222–2232

5. Pan M, Yuan Y, Hui A-L et al (2012) Anti-platelet aggregation activity of ginkgolide B precursor drugs. Chin J Pharmacol 28(10):1435–1438
6. Huaian W, Jinsheng G (2017) Research progress of ginkgolide B for the treatment of malignant tumors. Guangming TCM 32(17):2589–2590
7. Gao T, Zhao R, Yao L, et al (2020) Platelet-activating factor induces the stemness of ovarian cancer cells via the PAF/PAFR signaling pathway[J]. American Journal of Translational Research 12(11):7249–7261
8. Jiang W, Cong Q, Wang Y, Ye B, Xu C (2014) Ginkgo may sensitize ovarian cancer cells to cisplatin: antiproliferative and apoptosis-inducing effects of ginkgolide B on ovarian cancer cells. Integr Cancer Ther 13(3):NP10–N7
9. Chan W-H (2007) The signaling cascades of ginkgolide B-induced apoptosis in MCF-7 breast cancer cells. Int J Mol Sci 8(11):1177–1195
10. Yang B, Shuan S, Shengqiong C et al (2015) Study of ginkgolide B induced apoptosis in human colorectal cancer HCT116 cells. J Chengdu Med Coll 10(3):281–286
11. Zhi Y, Pan J, Shen W, He P, Zheng J, Zhou X et al (2016) Ginkgolide B inhibits human bladder cancer cell migration and invasion through MicroRNA-223-3p. Cell Physiol Biochem 39(5):1787–1794
12. Chan WH, Hsuuw YD (2007) Dosage effects of ginkgolide B on ethanol-induced cell death in human hepatoma G2 cells. Ann NY Acad Sci 1095:388–398
13. Lou C, Lu H, Ma Z, Liu C, Zhang Y (2019) Ginkgolide B enhances gemcitabine sensitivity in pancreatic cancer cell lines via inhibiting PAFR/NF-κB pathway. Biomed Pharmacother 109:563–572
14. Yao B, Liu B, Shi L, Li X, Ren C, Cai M et al (2017) PAFR selectively mediates radioresistance and irradiation-induced autophagy suppression in prostate cancer cells. Oncotarget 8(8):13846–13854
15. Coyne CP, Narayanan L (2019) Carnosic Acid, Tangeretin, and ginkgolide-B anti-neoplastic cytotoxicity in dual combination with dexamethasone-[anti-EGFR] in pulmonary adenocarcinoma (A549). Anti Cancer Agents Med Chem 19(6):802–819
16. Sahu RP, Turner MJ, DaSilva SC, Rashid BM, Ocana JA, Perkins SM et al (2012) The environmental stressor ultraviolet B radiation inhibits murine antitumor immunity through its ability to generate platelet-activating factor agonists. Carcinogenesis 33(7):1360–1367
17. Zhang L, Wang D, Jiang W et al (2010) Activated networking of platelet activating factor receptor and FAK/STAT1 induces malignant potential in BRCA1-mutant at-risk ovarian epithelium. BioMed Central 8(1):74
18. Aponte M, Jiang W, Lakkis M, Li MJ, Edwards D, Albitar L et al (2008) Activation of platelet-activating factor receptor and pleiotropic effects on tyrosine phospho-EGFR/Src/FAK/paxillin in ovarian cancers. Cancer Res 68(14):5839–5848
19. Yu Y, Zhang X, Hong S, Zhang M, Cai Q, Zhang M et al (2014) The expression of platelet-activating factor receptor modulates the cisplatin sensitivity of ovarian cancer cells: a novel target for combination therapy. Br J Cancer 111(3):515–524

20. Chen Q, Chen L, Sun JB. Research progress on the sources, structural modifications and new dosage forms of ginkgolide compounds. J Nanjing. 2019 University of Traditional Chinese Medicine;35(3):344–50
21. Hui A-L, Wu Z-Y, Yuan Y, Zhou A, Pan M (2013) Synthesis of ginkgolide derivatives and analogs and progress in the study of PAFR and GlyR antagonistic activities. J Org Chem 33(6):1263–1272
22. Liu KZ, Xue LP, Xu YF et al (2018) Review and comparative analysis of benoxate synthesis processes. Henan Sci 36(09):1362–1366
23. Highest L, Jia L, Zhang Yin Y et al (2016) Optimization of experimental reaction conditions for the synthesis of benoxate. J Xiangnan Coll 37(05):18–21
24. Wang Y (2014) Synthesis and characterization of benzoate. Exp Sci Technol 12(04):32–34
25. Dai GX, Wang L, Shoutao C (2014) Study on the catalytic synthesis process of benzoate. Coal Chem Ind 37(08):79–81
26. Xiaozhong F, Zero L, Guangling R et al (2012) Improvement of the synthesis process of benoxate. J Guiyang Med Coll 37(04):440–1 + 443
27. Teng SB, Qian J (2009) Progress in the synthesis of benoxylates. Shandong Chem Ind 38(08):23–25
28. Feng ZIL, Jiayuan X, Zhu ZB et al (2020) Synthesis of ginkgolide B structural modifiers and their effects on 6-OHDA-induced SH-SY5Y cell viability. Chin J Med Chem 30(04):199–203
29. Yong-Ming L, Jian P, Zhang, Wen-Na et al (2015) Synthesis, in silico and in vivo blood brain barrier permeability of ginkgolide B cinnamate. Fitoterapia 106:110–114
30. Wu ZY, Pan J, Yuan Y, Hui A, Yang Y, Zhou A et al (2012) Brain-targeting research of 10-O-nicotinate ginkgolide B: a new prodrug of ginkgolide B. Med Chem Res 21(12):4028–4036
31. Sladowski D, Steer SJ, Clothier RH, Balls M (1993) An improved MTT assay. J Immunol Methods 157(1–2):203–207