Research Article

Ginsengenin derivatives synthesized from 20(R)-panaxotriol: Synthesis, characterization, and antitumor activity targeting HIF-1 pathway

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ABSTRACT

Background: Ginseng possesses antitumor effects, and ginsenosides are considered to be one of its main active chemical components. Ginsenosides can further be hydrolyzed to generate secondary saponins, and 20(R)-panaxotriol is an important sapogenin of ginsenosides. We aimed to synthesize a new ginsengenin derivative from 20(R)-panaxotriol and investigate its antitumor activity in vivo and in vitro.

Methods: Here, 20(R)-panaxotriol was selected as a precursor and was modified into its derivatives. The new products were characterized by 1H-NMR, 13C-NMR and HR-MS and evaluated by molecular docking, MTT, luciferase reporter assay, western blotting, immunofluorescent staining, colony formation assay, Edu labeling and immunofluorescence, apoptosis assay, cells migration assay, transwell assay and in vivo antitumor activity assay.

Results: The derivative with the best antitumor activity was identified as 6,12-dihydroxy-4,4,8,10,14-pentamethyl-17-(2,6,6-trimethyltetrahydro-2H-pyran-2-yl)hexadecahydro-1H-cyclopenta[a]phenanthren-3-yl(tert-butoxycarbonyl)glycinate (A11). The focus of this research was on the antitumor activity of the derivatives. The efficacy of the derivative A11 (IC50 < 0.3 μM) was more than 100 times higher than that of 20(R)-panaxotriol (IC50 > 30 μM). In addition, A11 inhibited the protein expression and nuclear accumulation of the hypoxia-inducible factor HIF-1α in HeLa cells under hypoxic conditions in a dose-dependent manner. Moreover, A11 dose-dependently inhibited the proliferation, migration, and invasion of HeLa cells, while promoting their apoptosis. Notably, the inhibition by A11 was more significant than that by 20(R)-panaxotriol (p < 0.01) in vivo.

Conclusion: To our knowledge, this is the first study to report the production of derivative A11 from 20(R)-panaxotriol and its superior antitumor activity compared to its precursor. Moreover, derivative A11 can be used to further study and develop novel antitumor drugs.

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1. Introduction

Cancer can be described as the uncontrolled growth and spread of abnormal cells, which can also affect other body parts and may spread to other organs. Etiology and pathogenesis of tumor are not fully understood. Tumor may act as a leading trigger for death worldwide [1], with its incidence increasing every year [2]. The discovery of cancer drugs has presented a significant challenge for the scientific community due to the challenges associated with evaluating and verifying various possible targets [3]. Globally, cervical cancer is the fourth most common cancer amongst women,
heterodimeric transcription factor HIF-1 [10]. The level of HIF-1 agent). In addition, there are many studies on the biological activity and exhibit more antitumor properties. Furthermore, these functionality structural fragments, containing cinnamic acid [41,42], carbamates [43,44], and amino acids [45], are extensively present in numerous molecule which have biological activity and exhibit more antitumor properties. Based on the principle of drug combination and the above findings, we designed and synthesized four new 20(R)-panaxatriol derivatives by attaching these pharmacophores to the C-3 or C-6 position of panaxatriol. The anti-proliferative activity of the target compound against human hepatoma cancer (Hep3B) was evaluated. In addition, we selected the derivative A11 (Fig. 1) with the strongest anti-proliferative activity, studied its possible mechanism of action of HIF-1, and particularly focused on the activity of this panaxatriol derivative on cell apoptosis and cell migration. Finally, we conducted in vivo experiments to verify its antitumor properties.

2. Materials and methods

2.1. Materials

20(R)-panaxatriol was obtained from Nanjing Dilger Medical Technology Co.Ltd. All organic reagents used for the experiment were analytically pure, and other chemicals were purchased from Aladdin reagent. Hep3B cells and Hela cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT, USA), penicillin (100 U/mL), and streptomycin (100 U/mL) (Invitrogen, Carlsbad, CA, USA). The hypoxic culture was kept in a gas-controlled chamber (Thermo Electron Corp., Marietta, OH, USA) and maintained at 37 °C under 5% CO2, 94% N2, and 1% O2. Antibodies against the hypoxia-inducible factor (HIF)-1α were purchased from BD Biosciences (San Diego, CA, USA). Antibodies against the vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)-9, HIF-1α, B cell lymphoma-2 (Bcl-2), Bcl-2-associated X (Bax), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and topoisomerase (Topo)-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against cleaved Caspase-3 were purchased from Cell Signaling Technology. All other reagents were obtained from local reagent companies.

2.2. Synthesis of 20(R)-panaxatriol derivatives

2.2.1. Procedure for the preparation of compounds A1-A3, B1-B3, and C1-C3

First, 20(R)-panaxatriol (0.4 mmol, 190 mg) was mixed with toluene (5 mL) in an ice bath and slowly different substituted isocyanates (0.8 mmol) and triethanolamine (110 μL, 0.24 mmol) were added. Then stirred for 24h at 100 °C under nitrogen protection. The crude compounds were obtained by evaporating the solvent after thin-layer chromatography (TLC), confirming the completion of the reaction. The desired compounds were purified by silica gel column chromatography (dichloromethane/methanol, 100:1-50:1 v/v).

2.2.2. Procedure for the preparation of compounds A4-A12, B4-B12, and C4-C12

First, 20(R)-panaxatriol (0.4 mmol, 190 mg) was mixed with different carboxyl compounds (1.2 mmol), N-ethyl-N’-(3-(dimethylamino) propyl) carbodiimide (1.2 mmol) and 4-(dimethylamino) pyridine (0.4 mmol) in CH2Cl2 (5 mL) at 37 °C, and stirred for 24 h. After TLC confirmed the end of the reaction, it was poured into water (10 mL) and extracted three times with dichloromethane (3 × 10 mL); the organic phase was washed with water (1 × 10 mL) and saturated NaCl (1 × 10 mL) and dried over Na2SO4 to obtain crude compounds. The desired compounds were purified by silica gel column chromatography (dichloromethane/methanol, 200:1-50:1 v/v).

2.2.3. Procedure for the preparation of compounds A13-A15

The mixture of compounds A10-A12 (50 mg) and 200–300 mesh silicone (150 mg) was stirred in 2 mL of toluene at 120 °C for 6 h. The crude compounds were obtained by evaporating the solvent after TLC, confirming the completion of the reaction. The desired compounds were then purified by silica gel column chromatography (dichloromethane/methanol, 100:1-30:1 v/v).

2.3. Analysis of 20(R)-panaxatriol derivatives

Silica gel plate GF254 was used for TLC analysis to detect the reaction. The instrument used for the 1H-NMR and 13C-NMR spectroscopic analyses of all compounds was: AV-300 (Bruker BioSpin, Switzerland), with CDCl3 as the solvent and trimethylsilylane as the internal label. The coupling constant is expressed in Hz, and the chemical shift is indicated in ppm. The high-resolution
Fig. 1. Reagents and conditions: (A) (a) (i) 10% HCl, NaNO₂, H₂O, 0–5 °C, 30 min; (ii) NaN₃, H₂O, 0–5 °C, 2–4 h. (b) propionic acid, L-ascorbic acid sodium salt, CuSO₄·5H₂O, n-BuOH/H₂O, r.t., 24 h. (c) pyridine in DMF, 90 °C, 6 h. (B) (a) different substituted isocyanates, TEA, methylbenzene, 100 °C, 24 h. (b) 2a–2c, EDC, DMAP, CH₂Cl₂, 37 °C, 24 h. (c) 3a–3c, EDC, DMAP, CH₂Cl₂, 37 °C, 24 h. (d) different amino acids with t-butoxycarbonyl, EDC, DMAP, CH₂Cl₂, 37 °C, 24 h. (e) silica gel column chromatography, methylbenzene, 120 °C, 6 h.
mass spectra (HR-MS) were analyzed using electrospray ionization (ESI) on a Thermo Scientific LTQ Orbitrap XL spectrometer. The Compound, 6,12-dihydroxy-4,8,10,14-pentamethyl-17-(2,6,6-trimethyltetralyhydro-2H-pyran-2-yl)hexacyclodehydro-1H-cyclopenta[a]phenanthren-3-yl-(ter-butoxy)carbonyl)glycinate (A11) was further characterized using physical and spectral data. (Spectra of other 20(R)-panaxotriol derivatives are included in the Supporting Materials). White powder; Mp: 233-234 °C; yield 52%. 1H-NMR (CDCl3, 300 MHz, ppm): δ 6.28 (s, 1H, OH-12), 5.09-5.01 (m, 1H, CH-3), 4.55-4.50 (m, 1H, CH-3), 4.15-4.06 (m, 1H, CH-6), 3.91 (d, J = 4.9 Hz, 2H, -CH2-C=O), 3.53 (s, 9H, -(CH3)3), 1.98-1.51 (m, 15H, panaxotriol-H), 1.45 (s, 9H, -(CH3)3), 1.06 (s, 6H, panaxotriol-CH3), 1.00 (s, 1H, panaxotriol-H), 0.96 (s, 3H, panaxotriol-CH3), 0.90 (s, 3H, panaxotriol-CH3), 0.87-0.81 (m, 1H, panaxotriol-H). 13C-NMR (CDCl3, 75 MHz, ppm): δ 170.18, 155.61, 82.06, 79.86, 77.24, 73.15, 69.72, 68.34, 61.10, 54.64, 50.99, 49.29, 48.76, 47.11, 42.66, 40.96, 38.99, 38.29, 36.41, 35.70, 33.01, 31.08, 30.72, 30.40, 29.68, 28.33 (3C), 27.15, 25.11, 23.37, 19.39, 17.23, 17.16, 17.01, 16.54, 16.23. ESI-HRMS (m/z): calcld for C37H64NO5: [M+H]+: 634.4677, found: 634.4665.

2.4. Cytotoxic (MTT) assay

HeP3B cells were cultured in a 96-well plate at a density of 5 × 10^3/well, 200 mL medium, and cultured for 12 h to allow them to adhere. Then, 20(R)-panaxotriol and its derivatives were processed according to a concentration gradient (final concentrations of 0.3, 1, 3, 10, and 30 μM) for 4 h. The incubation was continued for 4 h after the addition of the thiazole blue reagent in the dark. After removing the thiazole blue reagent, 150 μL of dimethyl sulfoxide was added for coloring and dissolved by shaking for 10 min. The absorbance (optical density, OD) was measured at a wavelength of 492 nm on a microplate reader, and the data were analyzed.

2.5. Luciferase reporter assay

When the Hep3B cells reached 60%–80% confluence, they were used for plasmid transfection. The medium was changed. Lipofectamine 2000 was used to transfect the cells with pGL-HRE-Luc plasmid, and the cells were cultured for 24 h. After digesting the cells with trypsin, they were seeded into a 96-well plate (5 × 10^3/well, 200 mL medium, and cultured for 12 h to allow them to adhere. Then, 20(R)-panaxotriol and its derivatives were processed according to a concentration gradient (final concentrations of 0.3, 1, 3, 10, and 30 μM), each concentration was repeated three times, and the cells were cultured for 24 h. The incubation was continued for 4 h after the addition of the thiazole blue reagent in the dark. After removing the thiazole blue reagent, 150 μL of dimethyl sulfoxide was added for coloring and dissolved by shaking for 10 min. The absorbance (optical density, OD) was measured at a wavelength of 492 nm on a microplate reader, and the data were analyzed.

2.6. Molecular docking

A molecular docking study was performed to investigate the binding mode of the compound A11 to HIF-1α protein using the Discovery Studio 2017/DOCKER protocol (Accelrys, San Diego, USA), according to a previously described method [46]. HIF-1α structure was obtained from the Protein Data Bank (https://www.rcsb.org). PDB ID: 4ZPR. Compound A11 was treated with the ligand preparation and minimization models in Discovery Studio 2017 to investigate the binding patterns of compound A11 and HIF-1α LBD.

2.7. Western blotting

Cell extracts were analyzed using western blotting [47,48]. HeLa cells were incubated with 3, 10, and 30 μM of compound A11 for 12 h. Cells were lysed in an ice-cold lysis buffer to obtain the nuclear extract protein. A bicinechinic acid protein quantitative detection kit was used to quantify the protein at 570 nm. Sodium dodecyl sulfate-polyacrylamide gel was used to separate 30 μg of nuclear extract protein per lane, and then the protein bands were transferred to polyvinylidene fluoride membranes (Hybond-P). Membranes were blocked with 5% nonfat milk and then incubated with the corresponding antibodies at 4 °C overnight, followed by incubation with secondary antibodies for 2 h, and detection with a high-efficiency chemiluminescence kit.

2.8. Immunofluorescence staining

HeLa cells were cultured for 24 h on a 24-well plate (1 × 10^4/well) to facilitate adherence, and treated with the derivative A11 (final concentration 30 μM) for 12 h. Cells were rinsed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde at room temperature for half an hour, and then permeabilized in 0.2% Triton X-100. They were blocked in 5% bovine serum albumin for half an hour, incubated with the HIF-1α antibody at 4 °C overnight, followed by incubation with a secondary antibody at room temperature for half an hour. They were finally stained with 4',6-diamidino-2-phenylindole for half an hour, and observed under a fluorescence microscope. Color blue represents the nucleus and green represents the HIF-1α protein. The green and blue-colored images were merged using NIS-Elements software.

2.9. Colony formation assay

HeLa cells were incubated for 12 h in a 6-well plate (1 × 10^3/well). After changing to a new medium, the cells were treated with the derivative A11 (final concentrations of 3, 10, and 30 μM) and incubated for 10 days until the colonies were clearly visible. Next, the cells were rinsed in 1 mL of PBS, fixed in 700 μL of 10% formaldehyde, stained in 700 μL of 1% crystal violet, washed with 1 mL of PBS three times, and naturally air-dried.

2.10. 5-ethynyl-2'-deoxyuridine (EdU) labeling and immunofluorescence

HeLa cells were incubated for 24 h in a 96-well plate (5 × 10^4/well). After treatment with the derivative A11 (final concentrations of 3, 10, and 30 μM), the cells were incubated for 12 h, followed by incubation with EdU (RiboBio, Guangzhou, China) for 1 h. The cells were then fixed with 4% paraformaldehyde for 30 min, treated with 0.5% Triton X-100 for 10 min, and washed in PBS. They were then incubated in a 1 × Apollo® reaction cocktail for half an hour and stained with Hoechst 33,342. Finally, the stained cells were visualized using an Olympus IX83 inverted fluorescence microscope.

2.11. Apoptosis assay

Apoptosis was analyzed using flow cytometry, as previously described [49]. Apoptosis was detected using the Annexin V–FITC Apoptosis Detection Kit (BD Biosciences). Briefly, the HeLa cells were cultured for 24 h in a 6-well plate (5 × 10^3/well) and incubated for 24 h after treatment with derivative A11 (final concentrations of 3, 10, and 30 μM). Then, the cells were collected, washed
twice with PBS, and mixed with the binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). The cells were then stained with Annexin V-FITC and 2 μg/ml propidium iodide in a dark environment at 37 °C for 15 min. Analysis was performed by flow cytometry after the addition of 400 μl binding buffer to the samples. Cell Quest software (Becton-Dickinson, Franklin Lakes, NJ, USA) was used to analyze the data.

2.12. Cell migration assay

Scratch experiments were performed to detect the migration of HeLa cells. The cells were inoculated in a 24-well plate (1 × 10⁵/ well) and incubated for 24 h to facilitate adherence. A sterile pipette tip was used to make vertical scratches in each hole, and the plates were washed thrice with PBS to remove the scratched cells. The medium was then replaced with a serum-free medium. After treatment with the derivative A11 (final concentrations of 3, 10, and 30 μM), the cells were incubated for 12 h. Images were taken with a fluorescence microscope to observe the degree of migration of the cells to the scratched area.

2.13. Transwell assay

The invasion assay was performed using a Transwell chamber (Costar, MA, USA). The Transwell membrane was coated with Matrigel (BD Biocoat, Bedford, MA, USA) for the invasion assay and the cells pretreated with or without A11 (3, 10, and 30 μM) in serum-free DMEM were added to the upper compartment of the chamber. The lower compartment contained DMEM supplemented with 10% FBS. After 24 h of invasion at 37 °C, the cells migrated to the bottom chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Then, the cells were photographed under a microscope.

2.14. In vivo antitumor activity

Male athymic Nu/Nu nude mice (4–5 weeks of age, 24 ± 2 g; Vital River Laboratory Animal, Beijing, China) were reared under specific pathogen-free conditions as per the Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Normal saline (200 μL) was injected subcutaneously into the mice with HeLa cells. Two weeks later, the mice were randomly divided into five groups (n = 4), 50 mg/kg A11, 30 mg/kg A11, 30 mg/kg 20(R)-panaxotriol, 30 mg/kg 5-fluorouracil, and a negative control group. An intraperitoneal injection was administered every two days, and the body weights and tumor volumes of mice were simultaneously measured for 20 days. On the 21st day, the mice were sacrificed, and the tumors were excised and weighed. The following standard formula was used to calculate the tumor volume (mm³): \( \text{tumor volume} = \text{length} \times \text{width}^2 / 2 \). Ratio of inhibition of tumor (%) = \( \frac{1 - \text{average tumor weight of treated group/average tumor weight of control group}}{100 \%} \).

2.15. Statistical analysis

All data are presented as the mean ± standard deviation (SD), and all experiments were independently performed in triplicate. A comparison of the results was performed using one-way analysis of variance and Tukey’s multiple comparison tests (GraphPad Software, Inc. San Diego, CA, USA). Statistically significant differences between the groups were defined as p < 0.05.

3. Results and discussion

3.1. Chemistry

Fig. 1A illustrates the synthesis of intermediate. Compounds 1a-1c were obtained through diazotation and azidation of different anilines [50]. After that, the click process between propionic acid and intermediates 1a-1c yielded intermediates 2a-2c [51,52]. Cinnamic acid intermediates 3a-3c were synthesized by the Knoevenagel condensation reaction of various benzaldehydes and malonic acid [53].

Fig. 1B shows the general process for the synthesis of target 20(R)-panaxotriol analogs A1-A15, B1-B12, and C1-C12. Derivatives A1-A3, B1-B3, and C1-C3 are formed by the condensation of 20(R)-panaxotriol with isocyanates under alkaline conditions, and the yield ranged from 8% to 64%. In addition, 20(R)-panaxotriol undergoes a condensation reaction with various carboxylic acids to generate derivatives A4-A12, B4-B12, and C4-C12, with DMAP and EDC used as condensing agents, and yields ranging from 5% to 58%. Derivatives A13-A15 were derived from derivatives A10-A12 after removing t-butyloxycarbonyl, with yields ranging from 75% to 97%. The structures of the desired compounds were characterized by 1H-NMR and 13C-NMR spectroscopy and high-resolution mass spectrometry (Supporting Materials).

3.2. Biological evaluation

3.2.1. In vitro HIF-1α inhibitory activity and SAR study

MTT assay determined the cytotoxicity of 20(R)-panaxotriol and its derivatives in Hep3B cells. The results in Figure 2A show that, except for A13 (IC₅₀ = 6.99 μM), all other compounds exhibited no cytotoxicity (IC₅₀ > 30 μM). To detect the effect of 20(R)-panaxotriol and its derivatives on the transcriptional activity of HIF-1, a luciferase reporter gene experiment was performed on human liver cancer cells Hep3B (Fig. 2A), with 20(R)-panaxotriol as a positive control. The great majority of the derivatives had inhibitory effects on HIF-1α and were stronger than the positive control 20(R)-panaxotriol. Among them, the compound A11 with t-butyloxycarbonyl-glycine was the most effective derivative, and its IC₅₀ for inhibiting HIF-1α transcription activity in the Hep3B cell line was <0.3 μM. Its efficacy was over 100 times higher than that of 20(R)-panaxotriol. The derivatives C1-C12 inhibited transcription of HIF-1α weaker than those of derivatives A1-A12 and B1-B12, especially amino-triazoles and cinnamic acids (derivatives C4-C9: IC₅₀ > 30 μM), indicating that the double-modified derivatives at the C-3 and C-6 positions of 20(R)-panaxotriol have weaker activity than the C-3 or C-6 single-modified derivatives. Among 20(R)-panaxotriol isocyanate derivatives, the C-3 monosubstituted derivatives had the best activity with a benzene ring A2 (IC₅₀ = 1.58 μM), and the C-6 monosubstituted derivatives with cycloalkyl B3 had the best activity (IC₅₀ = 1.08 μM), the activity of the cycloalkyl group in the C-3 and C-6 dissubstituted derivatives C3 was the highest (IC₅₀ = 14.85 μM). This shows that the aromatic ring and cycloalkyl are equally important in 20(R)-panaxotriol isocyanate derivatives. For the 20(R)-panaxotriol derivatives with phenyl-1,2,3 triazole mono-substituted at the C-3 position, the electron donating group showed better effects than the electron withdrawing group (p-OCH₃ > p-H > p-Cl) (IC₅₀ are 0.33 μM, 0.43 μM, and >30 μM, respectively). Unfortunately, no clear structure-activity relationship has been found in cinnamic acid-substituted 20(R)-panaxotriol derivatives. It can be seen from the derivatives A10-A15 that the influence of the protection of the tert-butoxycarbonyl group in amino acids on the inhibition of HIF-1α transcription activity is not obvious (Fig. 2B). These findings indicate that 20(R)-panaxotriol...
In vitro inhibition of HIF-1α transcriptional activity in cell-based HRE reporter assay under hypoxia conditions and cytotoxic activity. The inhibitory effects of all the derivatives on HIF-1α transcriptional activity were tested by HRE luciferase reporter assay after 24 h treatment of Hep3B cells under hypoxic conditions. Values were shown as mean ± SD, n = 3. The cytotoxic activity was evaluated by MTT assay after 24 h treatment of compounds under normoxic conditions. Values were shown as mean ± SD, n = 3. The structures of 39 derivatives of 20(R)-panaxotriol for their structure-activity-relationships. Effect of compound A11 on HRE-mediated reporter gene expression. Hep3B cells were transiently co-transfected with a pGL3-HRE-Luciferase and pRL-CMV vectors. Following 24 h incubation, the cells were incubated under hypoxia in the absence or presence of the indicated concentrations of compound A11. Luciferase activities were determined as described in "Materials and Methods". Data are represented as the mean ± standard deviation of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, significant with respect to the hypoxia control. Hep3B cells were treated with the indicated concentrations of compound A11. After 24 h incubation, cell viability was determined by MTT assays.
derivatives effectively reduced the level of HIF-1α without significant cytotoxicity in Hep3B cells.

Further activity analysis was conducted on derivative A11 with the best HIF-1α inhibitory activity. The results in Fig. 2C and Fig. 2D show that, compared with 20% oxygen situation, under 1% oxygen situation, the transcription activity of HIF-1α was significantly increased (p < 0.0001). After adding 0.3 μM of derivative A11, the transcription activity of HIF-1α was reduced. As the concentration of the derivative A11 drug increased, the transcriptional activation of HIF-1α decreased. The results indicated that derivative A11 dose-dependent inhibited the transcriptional activity of HIF-1α (Fig. 2C). Derivative A11 at concentrations as high as 30 μM had no adverse effects on cell viability (Fig. 2D).

3.2.2. Compound A11 inhibits HIF-1α protein expression in HeLa cells

To explore the interaction between compound A11 and HIF-1α, we investigated its binding mode through a molecular docking study. As shown in Fig. 3A, the central structure of compound A11 was considered to form conventional hydrogen bond interactions with surrounding residues (Cys351, Gln359, and His286), and the t-butylcarbonyl part of compound A11 was considered to form a mixed pi/alkyl hydrophobic interaction with the surrounding residues (Arg156, Phe54 and Met354). Moreover, the nitrogen atom of the amino acids of compound A11 formed a hydrogen bond with Ser355. We also found that the compound A11 showed good binding interactions with the related protein (AZPR) in the binding pocket. Western blotting determined the effect of compound A11 on the expression of HIF-1α protein in HeLa cells. As shown in Fig. 3B, under normoxic conditions, HIF-1α was not expressed. Under 1% O2 conditions, HIF-1α was shown to accumulate in large amounts. The addition of different concentrations of A11 dose-dependently reduced the HIF-1α protein content in HeLa cells. However, A11 had almost no influence on the expression level of HIF-1β. Next, we performed an immunofluorescence experiment to determine whether compound A11 inhibited the increase of HIF-1α in the nucleus of HeLa cells. The results in Fig. 3C show that, in the nucleus, the HIF-1α protein was not expressed under normoxic conditions. Under 1% O2 conditions, in the nucleus, the HIF-1α protein was largely amassed; however, after adding 30 μM of compound A11, HIF-1α expression in the nucleus was not detected. These results indicate that compound A11 inhibited nuclear accumulation of HIF-1α.

HIF-1α is known to regulate the expression of VEGF, a crucial growth factor involved in tumor cell proliferation, angiogenesis, invasion, and metastasis [9,54]. Therefore, in this study, we tested the effect of A11 on VEGF protein expression by western blotting. We found that hypoxic conditions significantly increased the expression levels of VEGF, while treatment with A11 resulted in a dose-dependent decrease in its levels (Fig. 3D), and the effective concentrations were comparable to those inhibiting HIF-1α protein expression.

3.2.3. Compound A11 inhibits HeLa cell proliferation and promotes apoptosis

Malignant tumor cells can grow without restriction. Colony forming test can determine the size of cell colony formation [35]; thus, the colony formation test can evaluate the inhibitory ability of A11 on cell multiplication more directly. The results in Fig. 4A show that, compound A11 apparently inhibited the colony formation of cells, and the inhibitory effect became more obvious when the concentration was increased. The Edu experiment yielded similar results. As shown in Fig. 4B, compared with the control group, compound A11 concentration-dependently suppressed the number of Edu-positive cells. In addition, we wanted to confirm whether compound A11 could induce cell apoptosis in addition to inhibiting cell multiplication. Therefore, Annexin V-FITC/PI double staining was performed. As shown in Fig. 4C, compared with the control group, incubation with compound A11 (3, 10, and 30 μM) resulted in a concentration-dependent increase in the percentage of total apoptotic (early and late) (5.2%, 14.0%, and 24.2%, respectively). These results indicated that compound A11 could effectively induce cell apoptosis. To further understand the anti-apoptotic effects, western blotting analysis was used to determine the expression levels of cleaved-caspase 3, Bcl-2, and Bax proteins in vivo. As shown in Fig. 4D, the expression levels of Bax were increased in the compound A11-treated HeLa cells compared to those in the control group, whereas the levels of Bcl-2 were decreased after treatment with compound A11. Therefore, the ratio of Bax/Bcl-2 increased in a dose-dependent manner. In addition, it was observed that with the treatment of compound A11 (0, 3, 10, and 30 μM), the expression levels of cleaved caspase-3 in HeLa cells significantly increased in a concentration-dependent manner. Taken together, these data further confirmed that compound A11 could induce apoptosis in HeLa cells.

3.2.4. Compound A11 inhibits HeLa cell migration and invasion

Because HIF-1α is a key regulator of tumor invasion [56,57], we performed cell scratch assay and transwell assay to study the effect of compound A11 on HeLa cell motility. The results showed that the migration of HeLa cells in the cells treated with compound A11 decreased compared with the control group, and the degree of reduction in the migration of HeLa cells increased as the concentration of compound A11 increased. Treatment with compound A11 significantly reduced the number of tumor cells that crossed the transwell chamber compared to the control. Moreover, the invasion and migration of HeLa cells decreased to a greater extent at an increased dose of A11, suggesting that A11 inhibited the invasion and migration of HeLa cells in a concentration-dependent manner (Fig. 5A and Fig. 5B). Next, the effect of A11 on the expression of the invasion and migration-associated protein, MMP-9, was investigated. Western blotting experiments showed that treatment with A11 (10 μM) significantly reduced the expression levels of MMP-9 (Fig. 5C), further confirming that A11 plays an inhibitory role in the migration and invasion of HeLa cells.

3.2.5. In vivo antitumor activity of compound A11

Antitumor activity of compound A11 was remarkable in vitro, prompting us to verify its antitumor effect in vivo. The measured nude mouse body weight and nude mouse tumor volume results are shown in Fig. 6A and 6B. In the control group, the average tumor volume was obviously larger than that in the 20(R)-panaxotriol, A11, and 5-fluorouracil treatment groups. A11 significantly inhibit tumor volume (p < 0.001). But, the mice in the control group and other groups had very little difference in body weight, and this difference was not statistically significant. This indicates that the treatment could not produce toxicity in vivo. After administration of the treatment to nude mice, the nude mice were died by spinal dislocation, and the solid tumor was removed and photographed. The experimental results are shown in Fig. 6C. It can be seen more clearly from the figure that compared with the control group, the A11 group was associated with inhibition of cancer cell growth. The tumor growth inhibition rates in mice treated with 30 mg/kg 20(R)-panaxotriol, A11, and 5-fluorouracil were 40.15%, 70.33%, 76.21%, and 61.89%, respectively (Fig. 6D). According to the above pharmacological data, in vivo, the antitumor ability of derivative A11 was remarkably stronger than that of 20(R)-panaxotriol (p < 0.01). In addition, the antitumor activity of A11 increased as its concentration increased.

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Fig. 3. (A) Compound A11 was docked into the binding site of HIF-1α (2D binding mode and 3D binding mode) (PDB ID: 4ZPR). The ligand is shown in red. (B) HeLa cells were incubated under normoxia or hypoxia for 12 h in the absence or presence of indicated concentrations of A11. Whole-cell lysates for HIF-1β and nuclear extract for HIF-1α were analyzed using western blotting. Data are represented as the mean ± standard deviation of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, significant with respect to the hypoxia control. (C) HeLa cells were incubated with or without A11 (30 μM) under normoxia or hypoxia for 12 h and then analyzed for the intracellular distribution of HIF-1α by immunofluorescence assay (magnification, 400 x). (D) HeLa cells were incubated under normoxia or hypoxia for 12 h in the absence or presence of indicated concentrations of A11. VEGF were analyzed using western blotting. Data are represented as the mean ± standard deviation of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, significant with respect to the hypoxia control.
4. Conclusion

For the study, 39 new C-3 and C-6 derivatives of 20(R)-panaxotriol were designed and synthesized, and their HIF-1α transcription inhibitory activities were studied. Most of the derivatives showed strong HIF-1α transcription inhibitory activity. Among them, the derivative A11 with t-butyloxycarbonyl-glycine showed the strongest HIF-1α transcription inhibitory activity, and its IC₅₀ for inhibiting HIF-1α transcription activity in the Hep3B cell line was <0.3 μM. Its efficacy was shown to be over 100 times higher.
than that of 20(R)-panaxotriol. The results of molecular docking highlight that glycine with t-butyloxy carbonyl on C-3 of 20(R)-panaxotriol may be important for good HIF-1α inhibition. In addition, derivative A11 dose-dependently inhibited the transcriptional ability of HIF-1α, dose-dependently inhibited HIF-1α protein content in HeLa tumor cells, and inhibited the nuclear accumulation of HIF-1α. A11 was also observed to dose-dependently inhibit HeLa cell proliferation and promote apoptosis, as well as inhibit migration of HeLa cells. Furthermore, in the HeLa cervical cancer xenograft model, compound A11 significantly inhibited tumor volume by 76.21% (50 mg/kg) and 70.33% (30 mg/kg) (ip). It was better than 20(R)-panaxotriol (40.15%) and 5-fluorouracil (61.89%). In short, derivative A11 shows excellent antitumor activity in vivo and in vitro, and may be used as an effective antitumor agent in the future after more in-depth research. So far, only a few types of 20(R)-panaxotriol derivatives with amino acids have been synthesized, and there may be certain compounds that have better activity than A11. Therefore, more follow-up studies are needed to explore and analyze such compounds in the future.

Fig. 5. (A) HeLa cells were incubated under hypoxic for 12 h in the absence or presence of A11 (3, 10 and 30 μM). HeLa cell migrations were photographed at identical locations and cell migration was analyzed by comparing final gap width to initial gap width. Original magnification 200×. (B) HeLa cells were seeded into the matrigel coated transwell chamber and exposed to A11 (3, 10 and 30 μM) for 12 h to evaluated the invasion activity. The cells at the lower side of the membrane were then stained using Crystal Violet. Original magnification 200×. (C) HeLa cell was treated with or without A11 (3, 10 and 30 μM) for 12 h. Whole cell extracts were analyzed by Western blot analysis using MMP-9. Data represented as mean ± standard deviation of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, significantly different when compared with control group.
Declaration of competing interest

The authors have no conflicts of interest to report.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2022.03.001.

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