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Stereoisomeric guaiacylglycerol-β-coniferyl aldehyde ether induces distinctive apoptosis by downregulation of MEK/ERK pathway in hepatocellular carcinoma cells

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ABSTRACT

Two 8-*O*-4′-type neolignan epimers *erythro*-guaiacylglycerol-β-coniferyl aldehyde ether (**1**) and *threo*-guaiacylglycerol-β-coniferyl aldehyde ether (**2**) were isolated from the stems of *Picrasma quassioides*. Further chiral separation gave two pairs of enantiomers **1a**/**1b** and **2a**/**2b**. The cytotoxicity assay against hepatocellular carcinoma Hep3B and HepG2 cells was evaluated by MTT assay. The results showed that **1b** (IC_{50} = 45.56 μM) and **2b** (IC_{50} = 39.02 μM) had more cytotoxic effect than its enantiomers **1a** (IC_{50} = 82.66 μM) and **2a** (IC_{50} = 67.97 μM) in Hep3B cells, respectively. Moreover, **1b** and **2b** could induce more apoptotic cells as well as higher reactive oxygen species (ROS) generation than **1a** and **2a** at 50 μM. In addition, a further study on the phosphoinositide 3-kinase (PI3K)/AKT and mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathways was investigated. The results revealed that all compounds had no significant effect on PI3K/AKT pathway, however, **1b** and **2b** attenuated the relative levels of p-MEK and p-ERK when compared with **1a** and **2a**. Taken together, the absolute configurations of guaiacylglycerol-β-coniferyl aldehyde ether had an impact on the inhibitory effect on Hep3B cells. The inactivation of MEK/ERK signaling pathway might contribute to apoptosis induction and ROS generation in **1b**- and **2b**-treated cells.

1. Introduction

Lignans and neolignans are a large class of natural products that are widely distributed in plants and are derived from the oxidative coupling of two phenylpropane units [1]. Among them, 8-*O*-4′-type neolignans represent a classical group of *bis*-phenylpropanoids with an ether oxygen atom linkage between C-8 and C-4′ [2]. Generally, the stereochemistry at the C-7 and C-8 positions afforded *threo*- and *erythro*-configuration in naturally occurring 8-*O*-4′-type neolignans [3]. Recently, our group has found that 8-*O*-4′-type neolignans also exist as enantiomeric mixtures [4], it means that a planar structure of 8-*O*-4′-type neolignans (when C-7 and C-8 positions were chiral carbons) might provide four stereoisomers. Considering that sometimes enantiomers may have drastically different bioactivities [5], it was essential to resolve their absolute configurations and provide pure stereoisomers. Thus, these 8-*O*-4′-type neolignans have attracted our great attention in their enantioselectivity on bioactivities.

Picrasma quassioides Benn. (Simaroubaceae), one of the most important traditional Chinese medicines (TCM), is widely distributed in China, Korea, and Japan. To date, studies have demonstrated that the stems of this plant have potential anti-cancer activities [6,7]. Our team has been searching for potential anti-hepatoma compounds from TCM in recent years [8,9]. In this investigation, two 8-*O*-4′-type neolignan epimers *erythro*-guaiacylglycerol-β-coniferyl aldehyde ether (**1**) and *threo*-guaiacylglycerol-β-coniferyl aldehyde ether (**2**) were isolated from the stem of *P. quassioides*, and further chiral separation of them gave two pairs of enantiomers **1a**/**1b** and **2a**/**2b**, respectively. Although guaiacylglycerol-β-coniferyl aldehyde ether was reported to show cytotoxicity on hepatocellular carcinoma (HCC) cells [10], the anti-tumor effect of its pure stereoisomers has not been studied yet. The

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present study aims to evaluate the cytotoxicity of **1a**/**1b**-**2a**/**2b** as well as the underlying mechanism in HCC cells.

2. Materials and methods

2.1. General experimental procedures

Optical rotations were measured on a JASCO DIP-370 digital polarimeter. NMR spectra were obtained on Bruker ARX-400 spectrometers. The CD spectra were obtained using MOS 450 detector from Bio-Logic. HR-ESI-MS data was measured on a Bruker Micro Q-TOF spectrometer. Silica gel (100–200 and 200–300 mesh, Qingdao Marine Chemical, Inc., Qingdao, People's Republic of China) and ODS gel (40–63 μm, YMC Co. Ltd., Kyoto, Japan) were used for column chromatography. Preparative HPLC was conducted on a Shimadzu SPD-20A with a ultraviolet–visible (UV–vis) detector and an LC-6AD series pump equipped with a reversed-phase (RP) C_{18} column (10 × 250 mm, 5 μm) and a CHIRALPAK IC column (4.6 × 250 mm, 5 μm).

2.2. Plant material

The stems of *Picrasma quassioides* Benn were collected from Anhui Province, China in September 2015, and were identified by Professor Jin-Cai Lu (Department of Natural Products Chemistry, Shenyang Pharmaceutical University, PR China.). A voucher specimen (20151101) has been deposited in the Nature Products Laboratory, Shenyang Pharmaceutical University, Liaoning, PR China.

2.3. Extraction and isolation

The stems of *Picrasma quassioides* Benn (30 kg) were crushed and refluxed with 95% EtOH (3 × 50 L) for three times (3 h each). The crude residue (1200 g) was chromatographed on a macroporous resin column eluted with EtOH-H_2O (from 0:100 to 90:10) and obtained four fractions (Fractions I-IV). The fraction I portion was subjected to silica gel CC by using CH_2Cl_2-MeOH to afford four sub-fractions (III_a-III_d). Among them, III_a (30 g) was further subjected to ODS CC eluted with EtOH-H_2O (from 10:90 to 70:30) to yield five fractions (A–E). The fraction B (5.0 g) was separated by silica gel CC using CH_2Cl_2-MeOH (from 90:10 to 50:50) and afford four sub-fractions (A1–A4). Fr.A1 was further subjected to preparative HPLC (eluted with 25% MeOH/H_2O, v/v) to afford four fractions (A1-1–A1-4). Fr.A1-1 was subsequently purified by semipreparative HPLC eluted with 20% MeCN/H_2O (v/v) to give compound **1** (13.1 mg). Fr.A3 was separated with preparative HPLC (eluted with 25% MeOH/H_2O, v/v) followed by purification with semipreparative HPLC to obtain compound **2** (20.0 mg). Chiral resolution of **1** and **2** were performed on a Daicel Chiralpak IC column (eluted with *n*-hexane-2-propanol, v/v, 66:34, flow rate 0.7 mL/min) to obtain **1a** (4.8 mg) and **1b** (4.4 mg), **2a** (7.6 mg) and **2b** (6.8 mg).

(**1**): colorless gum; HR-ESIMS at *m/z* 397.1309 $[M+Na]^+$ (calcd for $C_{20}H_{22}NaO_7$, 397.1308); ^1H and ^{13}C NMR, see Supporting Information Table S1.

(+)-(7*S*, 8*R*)-guaiacylglycerol-*β*-coniferyl aldehyde ether (**1a**): $[\alpha]_D^{20}$ + 10 (c 0.1, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 242 (−4.68), 339 (+2.12) nm.

(−)-(7*R*, 8*S*)-guaiacylglycerol-*β*-coniferyl aldehyde ether (**1b**): $[\alpha]_D^{20}$ − 11 (c 0.1, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 242 (+5.14), 342 (−3.24) nm.

(**2**): colorless gum; HR-ESIMS at *m/z* 397.1368 $[M+Na]^+$ (calcd for $C_{20}H_{22}NaO_7$, 397.1369); ^1H and ^{13}C NMR, see Supporting Information Table S1.

(−)-(7*R*, 8*R*)-guaiacylglycerol-*β*-coniferyl aldehyde ether (**2a**): $[\alpha]_D^{20}$ − 25 (c 0.1, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 230 (−10.20), 279 (−2.01), 341 (−1.51) nm.

(+)-(7*S*, 8*S*)-guaiacylglycerol-*β*-coniferyl aldehyde ether (**2b**): $[\alpha]_D^{20}$ + 26 (c 0.1, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 228 (+7.49), 280 (+1.55), 341(+0.95) nm.

2.4. ECD calculations

The absolute configurations of compounds **1** and **2** were determined by comparison between the experimental electronic circular dichroism (ECD) and the calculated ECD using time-dependent density functional theory (TDDFT) [11]. Conformational searches were performed using the MMFF94 force field to obtain energy minimization by CONFLEX [12]. Conformers whose energy was no more 3 kcal/mol higher than the lowest energy (99 conformations for compound **1** and 101 conformations for compound **2**) were then optimized at the B3LYP/6-31G(d) level using Gaussian 09. The quantum chemical calculations of ECD were carried out using TDDFT at B3LYP/6-311 + +G(2d, p) level with the CPCM model in methanol solution. The calculated ECD curves was produced by SpecDis 1.51 [13].

2.5. Cell culture

Hepatocellular carcinoma Hep3B and HepG2 cells were purchased from American Type Culture Collection (ATCC, Manassas, USA). The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 10 mg/mL streptomycin, 100 U/mL penicillin and 0.03% L-glutamine and maintained at 37 °C with 5% CO_2 at a humidified atmosphere. Logarithmically growing cells were used in all the experiments.

2.6. Cytotoxicity assay

The cells were seeded in 96-well cell culture clusters (Corning, NY, USA) at a density of 5×10^3 cells per well. After 12 h incubation, 6.25–100 μM compounds incubated with the cells. After treatment with 48 h, 20 μL of MTT (5 mg/mL) was added to each well and incubated for another 4 h at 37 °C. The absorbance was measured at 490 nm using a microplate reader (Thermo, CA, USA). The IC_{50} (half maximal inhibitory concentration) values and cell growth inhibition curves were calculated by GraphPad Prism 6.0 (San Diego, CA, USA). 5-Fluorouracil (Sigma, MO, USA) was used as a positive control.

To investigate the role of PI3K/AKT and MEK/ERK pathway in compound-treated cells, the specific inhibitors were used in this study. The cells were treated with or without LY294002, U0126 (Selleck, Houston, USA) at given concentrations for 1 h and subsequently treated with **1a**/**1b**-**2a**/**2b**.

2.7. Morphological observation

The cellular morphological changes of Hep3B cells were observed by using a phase contrast microscopy (Olympus, Tokyo, Japan).

For the assessment of apoptotic nuclear morphology, cells were incubated with compounds **1a**/**1b**-**2a**/**2b**, the fixed cells were washed with PBS and then stained with Hoechst 33258 (Beyotime, Shanghai, China) at room temperature for 20 min. The cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

2.8. Apoptotic ratio detection

Hep3B cells were cultured in 6-well cell culture plates at a density of 2×10^5 cells per well overnight. After 48 h treatment of compounds at same concentrations (50 μM), cells were harvested and stained with Annexin V-FITC/PI (Bimake, Houston, USA) for 15 min at room temperature in the dark and then analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, USA).

2.9. The examination of reactive oxygen species

To measure the level of reactive oxygen species (ROS), the cells