SUPPLEMENTARY MATERIAL

Antiproliferative effect of 2-Hydroxy-6-tridecylbenzoic acid from ginkgo biloba sarcotestas through the aryl hydrocarbon receptor pathway in triple-negative breast cancer cells

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Abstract

This study aims to isolate the potential antiproliferative and cytotoxic compounds from ginkgo biloba sarcotestas and investigates the underlying mechanism in human MDA-MB-231 and mouse 4T-1 triple-negative breast cancer cells. Our results showed that 2-Hydroxy-6-tridecylbenzoic acid was isolated by cytotoxicity-guided fractionation where different fractions were assessed using MTT assay against MDA-MB-231 and 4T-1 cells. Colony formation assay showed that 2-Hydroxy-6-tridecylbenzoic acid significantly inhibited cell proliferation. The inhibition was associated with the enhancement of cytochrome P450 (CYP) 1B1 expression in a dose- and time-dependent manner and no significant change of CYP1A1 expression by qPCR and Western blot assays in MDA-MB-231 and 4T-1 cells. The molecular mechanism was further demonstrated by the activation of aryl hydrocarbon receptor (AhR) pathway with the upregulation of AhR, AhR nuclear translocator (ARNT) and AhR-dependent xenobiotic response elements (XRE) activity. These findings may have implications for development of anticancer agents containing 2-Hydroxy-6-tridecylbenzoic acid as functional additives.

Keywords: Breast cancer; Ginkgo biloba sarcotestas; 2-Hydroxy-6-tridecylbenzoic acid; Antiproliferation; CYP1B1; AhR

Experimental

General experimental procedures

Column chromatography was performed over silica gel (200–300 mesh, Qingdao Haiyang Chemical Co., Ltd., Qingdao, China). Semi-preparative HPLC was performed using a Waters LC-1525 HPLC system (Milford, MA, USA) equipped with an UV detector.LC/MS analysis was performed on an Agilent 1100 Series HPLC system (Agilent Technologies) equipped with a diode array detector and Series ESI-MS using an analytical Kinetex C_{18} 100 Å column (2.1 × 150 mm, 5 μm). NMR spectra were recorded on a Bruker DMX 500 NMR spectrometer operating at 250 MHz (^{1}H), with chemical shifts given in ppm (δ).

Plant material

The fresh GBS were collected from Shenyang Agricultural University campus, Liaoning province, China in December 2016 and identified according to the application standard of Pharmacopeia of China. A voucher specimen (SW 2016-207) has been deposited in the college of Bioscience and Biotechnology, Shenyang Agricultural University, Shenyang, China

Extraction and isolation procedures

The fresh sarcotestas (1kg, water content 52.82%) were carefully cleaned, minced and macerated in 10L ethanol at room temperature for 2 days. The dried EtOH extract (24.18g) was suspended in water and partitioned successively in increasing order of polarity by petroleum ether, ethyl acetate and n-butanol, consecutively (twice for each solvent) yielding 2.21g, 3.13g and 2.14g of residue, respectively. The petroleum ether fraction showed the higher cytotoxic effect in MDA-MB-231 and 4T-1 cells. A portion of the petroleum ether extract (2.0g) was subjected to purification by silica gel column (10×500mm, 200 mesh), and eluted with an increasingly polar gradient of petroleum ether/chloroform (1:0, 2:1, 1:1, 1:2, 0:1, each 240mL) and chloroform/acetone (1:0, 2:1, 1:1, 1:2, 0:1, each 240mL), to yield nine Sub-fractions (Subf 1-Subf 9). Of these, the higher cytotoxic effect in both cells of Subf.4 (87.44mg) was further divided into eight fractions (Subf4-1-Subf4-8) using semi-preparative C_{18} HPLC (250×20mm,5μm, Waters, USA) with a gradient of MeOH/H_2O (90:10-100:0, 3% acetic acid, flow rate of 2 mL/min) for 100min to yield compound (t_R 38.0 min, 5.13mg), which exhibited the highest cytotoxic effect in MDA-MB-231 and 4T-1 cells.

Chemicals and reagents

HPLC-grade methanol was purchased from TEDIA (Fairfield, USA). All other solvents were of analytical grade. Column chromatography was performed over silica gel (200–300 mesh, Qingdao Haiyang Chemical Co., Ltd., Qingdao, China). 3-(4,5-dimethylthiaxolone-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), CH223191, aminoflavone, dimethyl sulfoxide (DMSO), RPMI1640 medium, fetal bovine serum, penicillin/streptomycin, sodium pyruvate, Trypsin-EDTA were purchased from Sigma-Aldrich (St. Louis, MO, USA).The primary antibodies for anti-CYP1A1, anti-CYP1B1, anti-ARNT and anti-AhR were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-β-actin and other secondary antibodies were obtained from En Jing (Beijing, China).

Cell lines and culture conditions

The MDA-MB-231 and 4T-1 breast cancer cells were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). The cells were grown and maintained in RPMI-1640 cell culture medium supplemented with 10% fetal bovine serum and 1% antibiotics in a tissue culture apparatus with atmosphere at 37˚C containing 5% CO_2. For experiments, cells were plated at an appropriate density, according to each experimental design.

Measurement of cytotoxicity

The cytotoxic effects of fractions, subfractions and 2-Hydroxy-6-tridecylbenzoic acid were measured using MTT. Cells were cultured in sterile 96 well plates (1×10^5/well) and treatment with samples containing different concentrations. 0.6 μM doxorubicin (positive control), 0.1% DMSO (solvent control) and no treated cells (negative control) were used in the experiment. After the plate was incubated for 24h, 10μL/well MTT reagents were added to each well and incubated for 4h.The medium was carefully removed and formazan crystals were dissolved in 110μL of DMSO. The optical density was determined at 490 nm with SpectraMax190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).The 50% inhibitory concentration (IC_{50}) was defined as the effective cytotoxicity.

Colony formation Assay

The antiproliferative activity was measured by the colony formation assay. Cells were cultured in sterile 6 well plates (1×10^3/well). Treatment with 2-Hydroxy-6-tridecylbenzoic acid containing various concentrations for 24h, the media was replaced and cells were further grown for one week allowed

colonies to form. Afterwards, the cells were fixed in 10% formaldehyde solution and stained with 0.4% trypan blue solution (Sigma). The number of colonies was counted on inverted microscope (Eclipse 90i, Nikon, Japan). The results of proliferative activity were expressed in terms of surviving fraction determined as percentage of treated cells compared to the solvent control cells.

Quantification of reverse transcription polymerase chain reaction (qPCR)

Total RNA was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and reverse transcription into the first-strand complementary DNA (cDNA) using the reverse transcriptase kit (Ta Ka Ra, China) according to the instructions. Primer pairs for CYP1A1 and CYP1B1 are listed (Table S1). The PCR reactions were performed using a Bio-Rad iQ5 (Bio‑Rad Laboratories, Inc., Hercules, CA, USA). The PCR amplification was performed in a total reaction volume of 20μL, containing 1.0μL of cDNA sample, 2.0μL(10μM) of each primer, 10μl 2 × All-in-One qPCR Mix, 4.6μL diethylpyrocarbonateH2O and 0.4 μl 50×ROX Reference Dye (Trans Gen Biotech, Beijing, China).The cycling parameters were: initial denaturation at 94˚C for 10 min, followed by 36 cycles of denaturation at 94˚C for 30 sec, annealing at 58˚C for 20 sec and a final extension at 72˚C for 20 sec. Light Cycler 480 analysis software (Roche Light Cycler 480, Hoffmann La Roche, Ltd., Basel, Switzerland) was used to obtain the Ct values. The △△CT method was used to analyse the relative expression of CYP1A1 and CYP1B1. Reactions were run in three independent experiments.

Western blot assay

After treatment with indicated concentrations of 2-Hydroxy-6-tridecylbenzoic acid, cells were harvested according to the manufacturer's instructions and lysed with RIPA buffer containing 1 mM PMSF and protease inhibitor cocktail. The proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE gel was transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Germany). The membranes were blocked by 5% skim milk and incubated with different primary antibodies, followed with secondary antibody (horseradish peroxidase conjugated anti-rabbit IgG).Antibody binding was detected by chemoluminescence regent.Bands were scanned and quantified by automatic chemiluminescence image analysis system (Tanon Science and Technology Co., Ltd., Shanghai, China).

AhR Reporter Luciferase Assay.

Luciferase assay was carried out using XRE Reporter Assay Kit (Qiagen, Germany). Briefly, cells were transfected with the XRE Reporter (including an AhR-regulated luciferase construct and expressing *Renilla* luciferase constitutively) and positive or negative controls. At 20h after transfection, the fresh medium was changed to assay medium. Cells were treated with 2-Hydroxy-6-tridecylbenzoic acid with concentrations for 24h. The Dual Luciferase Reporter Assay System was performed using the Glomax Explorer Luminescence microplate reader (Promega).

RNA interference studies

RNA interference was performed by transfection of small interfering RNA (siRNA) targeting CYP1B1 and AhR (Qiagen, Germany). Cells were transfected with CYP1B1 and AhR siRNA and a non-specific negative control siRNA using Lipofectamine 3000 (Invitrogen), according to the manufacturer's instructions. Briefly, cells were plated into 96-well plate (1×10^5/well) and appropriate amounts of siRNA and Lipofectamine 3000 transfection reagent were added to each well. After 6-8h of incubation, transfection media were replaced with fresh media and cells were incubated with 2-Hydroxy-6-tridecylbenzoic acid for growth inhibition analysis.

Statistical analysis

Data were expressed as the means ± standard deviation (SD) of three replications and were evaluated by one-way analysis of variance (ANOVA). Statistical analysis and bar graphs were performed by the GraphPad Prism version 5.0 (Graph PadTM Software, SanDiego, CA, USA). *P <0.05 and **P <0.01 were regarded as significant.

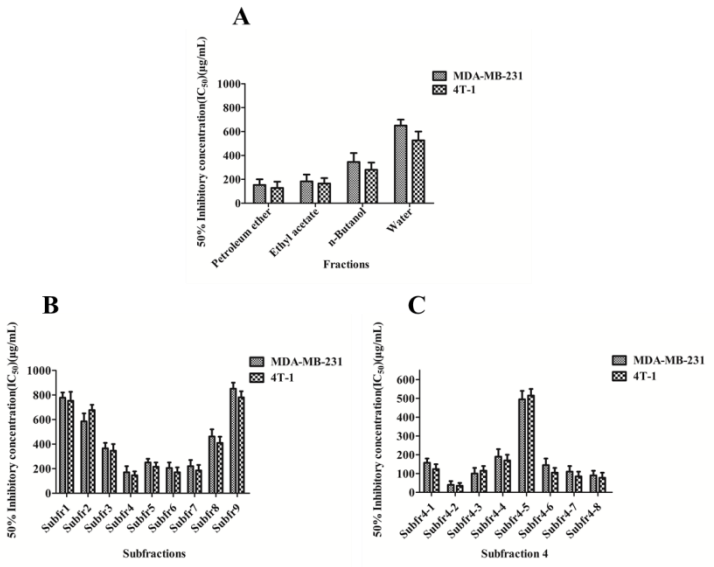

Figure S1. The comparison of 50% inhibitory concentration (IC_{50}) of (A) Fractions, (B) Subfractions and (C) Subfraction4 were based on MTT assay in MDA-MB-231 and 4T-1 cells. Cells were incubated with different concentrations of samples (0-800μg/mL) for 24h. The data were means ± SD for three replicates per treatment.

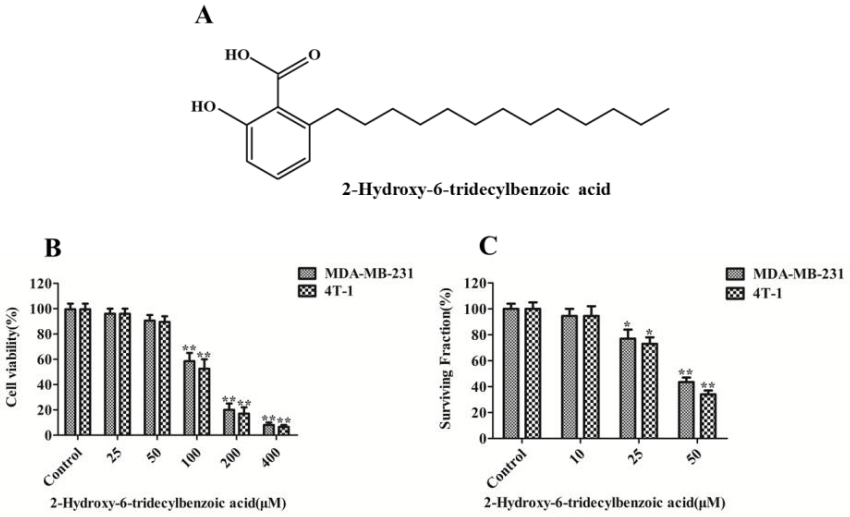

Figure S2. 2-Hydroxy-6-tridecylbenzoic acid exhibits cytotoxicity and antiproliferation in MDA-MB-231 and 4T-1 cells. (A) Chemical structure of 2-Hydroxy-6-tridecylbenzoic acid. (B) Cells exposed to 2-Hydroxy-6-tridecylbenzoic acid with concentrations (0-400μM) for 24h. Cell viability was detected by MTT assay. (C) The antiproliferation of colony formation assay performed on both cells. Surviving fraction was expressed as a percentage of the control (DMSO). The data were means ± SD for three replicates per treatment. *P < 0.05 and **P < 0.01 versus 0.1% DMSO control.

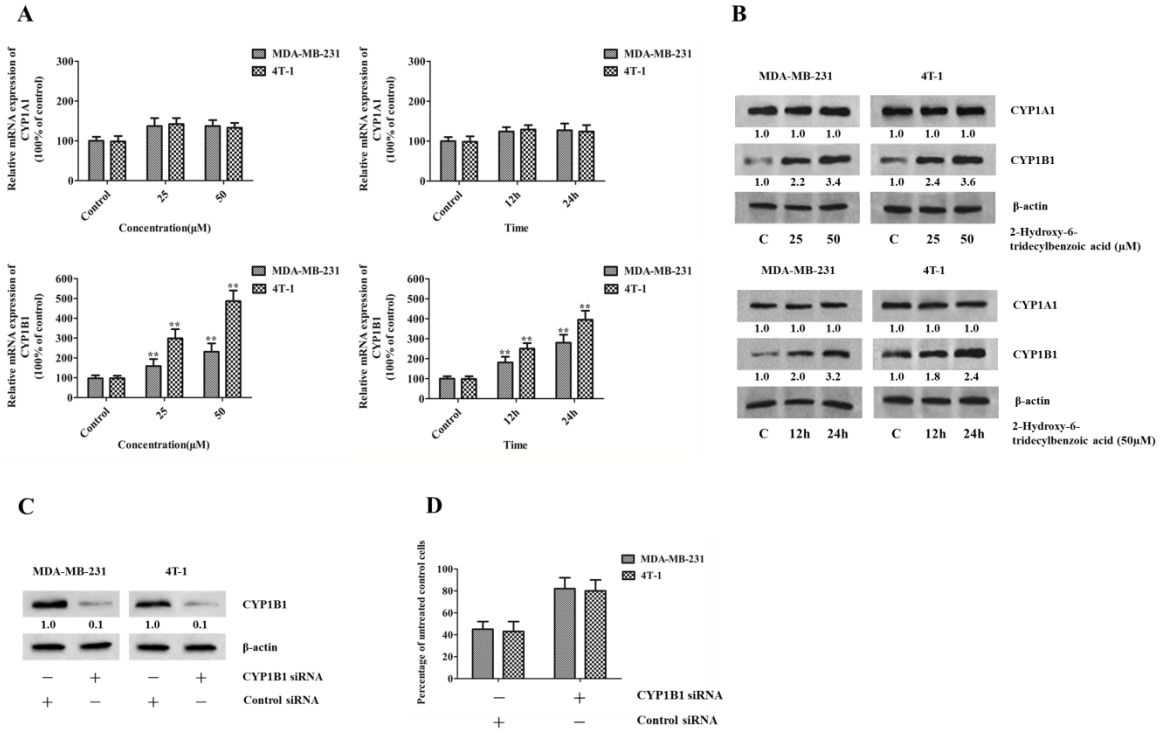

Figure S3. 2-Hydroxy-6-tridecylbenzoic acid activates the CYP1 members in MDA-MB-231 and 4T-1 cells. Cells were treated with 2-Hydroxy-6-tridecylbenzoic acid at concentrations (0-50μM) for 0-24h. (A), (B) The expressions of CYP1A1 and CYP1B1 were examined by qPCR and western blot. (C) Effect of siRNA for CYP1B1 on CYP1B1 protein. (D) The antiproliferation of the presence of CYP1B1 siRNA and control siRNA after treated with 2-Hydroxy-6-tridecylbenzoic acid (50μM, 24h). The data were means ± SD for three replicates per treatment. **P < 0.01 versus 0.1% DMSO control.

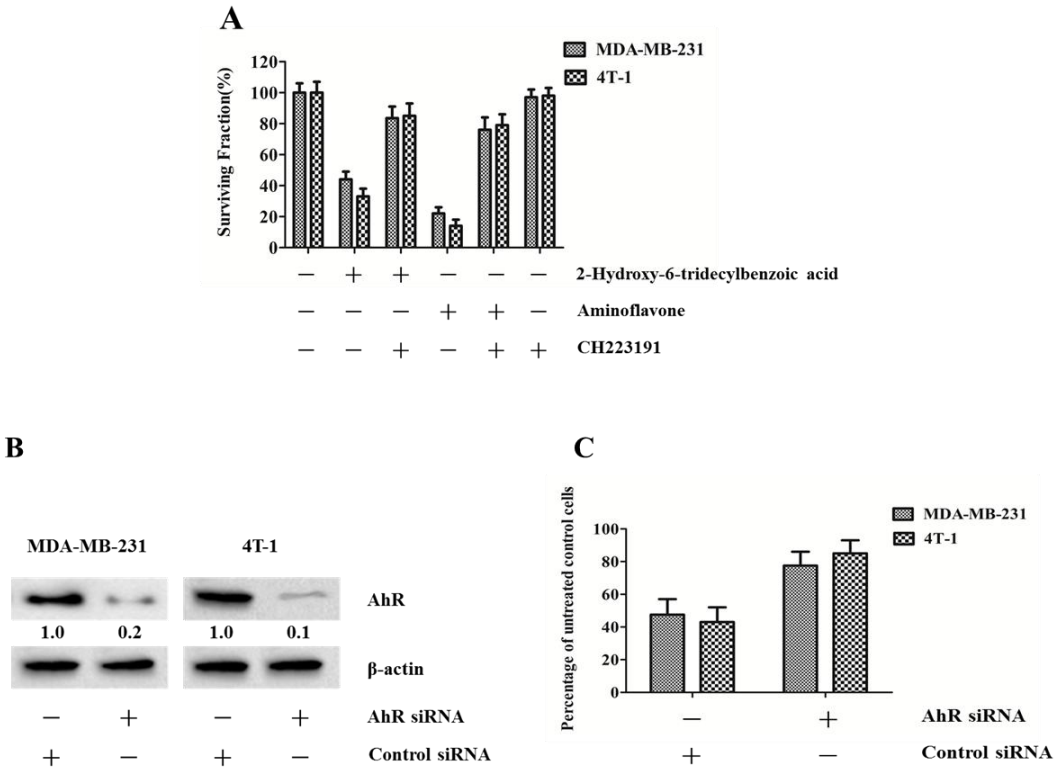

Figure S4. Inhibition of the AhR pathway reduces the antiproliferation of 2-Hydroxy-6-tridecylbenzoic acid in MDA-MB-231 and 4T-1 cells. (A) After treated with 2-Hydroxy-6-tridecylbenzoic acid (50μM) or aminoflavone (30μM) for 24h, the surviving fraction of cells in the presence and absence of the AhR antagonist CH223191 (5 μM). (B) Effect of siRNA for AhR on AhR protein. (C) The antiproliferation of the presence of AhR siRNA and control siRNA after treated with 2-Hydroxy-6-tridecylbenzoic acid (50μM) for 24h. The data were means ± SD for three replicates per treatment.

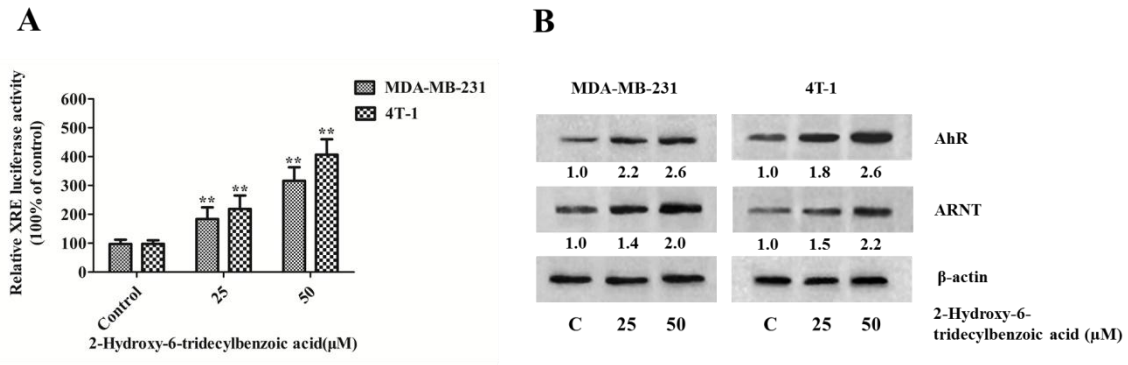

Figure S5. 2-Hydroxy-6-tridecylbenzoic acid activates the AhR pathway in MDA-MB-231 and 4T-1 cells. After treated with 2-Hydroxy-6-tridecylbenzoic acid (0-50μM) for 24h, (A) the induction of XRE activity using a reporter assay. (B) Western blot analysis was detected for the expressions of AhR and ARNT. The data were means ± SD for three replicates per treatment. **P < 0.01 versus 0.1% DMSO control.

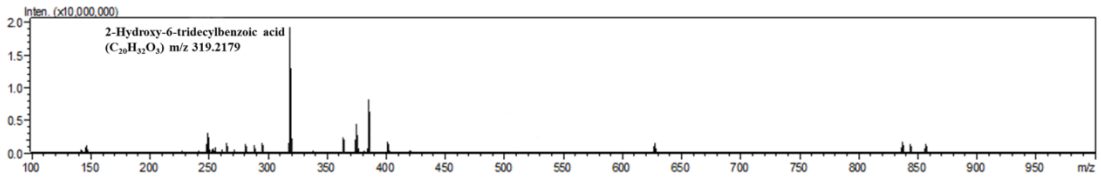

Figure S6. The chromatogram of 2-Hydroxy-6-tridecylbenzoic acid was analysed by ESI-MS.

Table S1. Sequences of primer pairs used in qPCR

Primer name	Squence (5'—3')	Organism
CYP1A1-For	TGGATGAGAACGCCAATGTC	human
CYP1A1-Rev	TGGGTTGACCCATAGCTTCT	
CYP1B1-For	GGCTGGATTTGGAGAACGTA	human
CYP1B1-Rev	GTCCTTGGGAATGTGGTAGC	
CYP1A1-For	TCCTGTCCTCCGTTACCTGC	mouse
CYP1A1-Rev	ACCTGCCACTGGTTCACAAA	
CYP1B1-For	GATGTGCCTGCCACTATTACGG	mouse
CYP1B1-Rev	GCACACAGAGACTATCGCACT	