Supporting Information

Synthesis and Antitumor Activity of Ellagic Acid Peracetate

Yulin Ren,[†] Min Wei,[‡] Patrick C. Still,[†] Shunzong Yuan,[▽] Youcai Deng,[▽] Xiaozhuo Chen,[§,⊥,∥,○] Klaus Himmeldirk,[§] A. Douglas Kinghorn,[*,†,▽] and Jianhua Yu[*,▽,#]

[†]Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, and [‡]Department of Molecular Virology, Immunology, and Medical Genetics, The Ohio State University, Columbus, Ohio 43210, United States

[§]Department of Chemistry and Biochemistry, [⊥]Edison Biotechnology Institute, [∥]Department of Biomedical Sciences, and [○]Molecular and Cellular Biology Program, Ohio University, Athens, Ohio 45701, United States

[▽]Comprehensive Cancer Center and [#]Division of Hematology, College of Medicine, The Ohio State University, Columbus, Ohio 43210, United States

EXPERIMENTAL SECTION

General. NMR spectra were recorded at room temperature on a Bruker Avance DPX-300 or DRX-400 NMR spectrometer, with TMS as internal standard. ESIMS were recorded on a LCT-TOF mass spectrometer. Column chromatography was conducted using silica gel (70−230 mesh, Merck, Darmstadt, Germany). Analytical and preparative thin-layer chromatography (TLC) was performed on precoated silica gel 60 F254 plates (Sorbent Technologies, Atlanta, GA). For visualization of TLC plates, sulfuric acid reagent was used. All procedures were carried out using anhydrous solvents purchased from commercial sources and used without further purification. High-purity water (18.2 MΩ) was obtained using a Milli-Q system (Millipore, Bedford, MA). CD107a and IFN-γ (XMG1.2) were purchased from BD Pharmingen. All reactions were monitored by TLC using precoated silica gel plates. Yields refer to chromatographically and spectroscopically pure compounds.

HPLC Analysis. HPLC was performed on a Beckman (Beckman Coulter) HPLC instrument with a model 118 pump, model 7725i injector, and a Beckman 168 photodiode array detector. Chromatograms were recorded using System Gold 32 Karat software (Beckman). Solvent A of the mobile phase consisted of deionized water (Ultrapure Water, Dubuque, IA) and trifluoroacetic acid (Fisher Scientific) (999:1). Solvent B consisted of acetonitrile (Sigma-Alderich) and trifluoroacetic acid (999:1). A linear gradient was applied from 80:20 A/B to 50:50 A/B over 15 min at a flow rate of 1 mL/min for the analytical column. The detection wavelength was at 280 nm, and analyses were conducted at 20 °C.

Synthesis of 1. A mixture of 10 g (54.3 mmol) of methyl 3,4,5-trihydroxybenzoate, 4 g (24 mmol) of potassium iodide, and 44 g (318 mmol) of anhydrous powdered potassium carbonate in

500 mL of acetone was stirred at room temperature for 20 min. Then, 22 g (174 mmol) of benzyl chloride dissolved in 100 mL of acetone were added. The suspension was refluxed for 18 h, and the solid was filtered. The filtrate was evaporated, and the residue was taken up in 400 mL of dichloromethane. The suspension was filtered through Celite, and again the filtrate was evaporated. After the residue was dried for 1 h under a vacuum, methyl 3,4,5-tribenzyloxybenzoate was obtained and used for the next step without further purification.

Crude methyl 3,4,5-tribenzyloxybenzoate (26.52 g) was suspended in 500 mL of 95% ethanol. An amount of 3.54 g (88.5 mmol) of sodium hydroxide was added. After the mixture was refluxed for 2 h, the hot solution was poured into 525 mL of 0.6 M hydrochloric acid. A thick, voluminous suspension formed. The solid was filtered off, washed successively with 95% ethanol-water (1:1), water, 95% ethanol, methanol, and *tert*-butyl methyl ether, and dried overnight under vacuum to give 3,4,5-tribenzyloxybenzoic acid (22.6 g, 94%).

A suspension of 0.21 g (1.17 mmol) of D-glucose, 3.77 g (8.57 mmol) of 3,4,5-tribenzyloxybenzoic acid, 2.2 g (10.68 mmol) of dicyclohexylcarbodiimide (DCC), and 1.2 g (9.84 mmol) of *N*,*N*-(dimethylamino)pyridine (DMAP) in 135 mL of dry dichloromethane was refluxed for 18 h. After the mixture was cooled to room temperature, the urea byproduct was filtered and the filtrate was evaporated. The resulting residue was purified by column chromatography on silica gel, using a 75:25:1 mixture of dichloromethane, toluene, and ethyl acetate as the eluent. After evaporation, the residue of the product fraction was precipitated from toluene, and α (337 mg)- and β (319 mg)-D-glucopyranose pentakis[3,4,5-tris(phenylmethoxy)benzoate] were obtained.

A suspension of 332 mg (0.145 mmol) of α-D-glucopyranose pentakis[3,4,5-tris(phenylmethoxy)benzoate] and 31.8 mg (0.30 mmol) of palladium (10 wt % on activated

carbon) in 30 mL of dry THF was stirred at 40 °C under a hydrogen gas atmosphere for 16 h. The reaction mixture was cooled and filtered through Celite®, and the filtrate was evaporated. The residue was re-crystallized from water, and α-D-glucopyranose pentakis(3,4,5-trihydroxybenzoate) (α-PGG, 85.0 mg, 62.2%) was obtained. Using the same procedure, β-D-glucopyranose pentakis(3,4,5-trihydroxybenzoate) (β-PGG, 79.0 mg, 61.7%) was produced.

Crystalline α-PGG (80 mg, 0.085 mM) was dissolved in 15 mL of 5% Na_2CO_3 solution at 70 °C and cooled to room temperature. The mixture was stirred at room temperature for 6 h and filtered. The solid product was washed with water and then MeOH, and re-crystallized in pyridine to give ellagic acid (**1**, amorphous white powder, 32 mg, 50%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.57 (2H, s), 10.68 (4H, s). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 107.6, 110.2, 112.3, 136.4, 139.5, 148.1, 159.1. Positive ESIMS *m*/*z* 325.0 $[M + Na]^+$.

Using the same procedure, α-D-manopyranose pentakis(3,4,5-trihydroxybenzoate) (8 mg, 0.0085), α-D-allopyranose pentakis(3,4,5-trihydroxybenzoate) (8 mg, 0.0085), or α-D-galactopyranose pentakis(3,4,5-trihydroxybenzoate) (8 mg, 0.0085), were each dissolved in 2 mL of 5% Na_2CO_3 solution at 70 °C and cooled to room temperature. After the mixture was stirred at room temperature, no ellagic acid (**1**) was produced.

Acetylation of 1 to 2. An amount of 30 mg of ellagic acid (**1**, 0.1 mM) was dissolved in 5 mL of pyridine. A volume of 0.5 mL of acetic anhydride was transferred to the solution. The mixture was stirred at room temperature for 12 h, and TLC analysis of the reaction mixture showed that the starting material was completely changed to a product. The mixture was poured to 10 mL ice water, and a cream colored solid was filtered. The filtered solid material was powdered and washed with water and then methanol, followed by drying under an oil-pump vacuum overnight to yield **2** (amorphous white powder, 36 mg, 85%). ^{1}H NMR (400 MHz,

$CDCl_3$) δ 2.37 (3H, s), 2.45 (3H, s), 8.05 (3H, s). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 19.9, 20.4, 20.7, 21.0, 116.0, 119.3, 119.9, 134.3, 142.9, 156.5, 166.9, 168.0, 171.9. Positive ESIMS *m*/*z* 493.1 $[M + Na]^+$.

Mice. Eight- to twelve-week old C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used for evaluation of immunity-associated antitumor effects in this study. The mice were housed in laminar-flow cages at room temperature and a relative humidity of 50-60% under 12:12 h light-dark cycle with specific pathogen-free conditions and kept under such conditions for at least one week before being used. The animal work was approved by The Ohio State University Animal Care and Use Committee, and the mice were treated in accordance with the institutional guidelines for animal care.

Treatment regimen. Ellagic acid (**1**, purchased from Sigma-Aldrich, E2250-1G, 020M1106) and chromatographically and spectroscopically pure sample of ellagic acid percaetate (**2**, derived from commercial ellagic acid) were dissolved in DMSO at a concentration of 1 mg/mL. The solutions were diluted immediately to 5 μg/mL with a balanced salt solution containing 12% Tween 80 (pH 7.4), and the diluted solutions were subjected to an immediate use with a dose of 0.5 mg/kg daily by drinking water.

Antitumor Assay. After a one-week treatment, mice were injected in each flank with B16 melanoma cells (1×10^6), and treatments were continued for an additional two weeks. The mice were then sacrificed and the tumors were removed, weighed, and photographed. The kidneys, livers, and spleens of mice were inspected. The subcutaneous tumor were weighted, and in vivo antitumor activities were determined by comparison of the weights of solid tumors of treatment **1** or **2** with that of control group. All experiments were repeated at least once.

Immune Cell Quantity. After a one-week treatment, WBC in peripheral blood and immune cells enriched from BM and liver were counted by a Trypan Blue exclusion method. The numbers of WBC and immune cells of the test groups were compared with those of the control group. All experiments were repeated at least once.

NK Cell Degranulation Assay. After a one-week treatment, the spleens of mice were harvested, and splenocytes were processed immediately and co-cultured ex vivo with YAC-1 tumor cells for 4 h. Then, the cells were stained with NK 1.1, CD3 and CD107a mAbs. Histograms shown in Figure 5A represent the expression level of CD 107a of NK cells, which were gated on $NK1.1^{+}CD3^{-}$. The percentages were averaged from at least four mice and represent the mean percent positive cells.

NK Cell IFN-γ Intracellular Flow Assay. Following one week of treatment, the spleens of mice were harvested, and the splenocytes were immediately processed and stimulated with interleukin (IL)-12 and IL-18 overnight, followed by 4 h incubation with brefeldin A (BD Pharmingen) before flow cytometry. Surface staining was performed with anti-NK1.1 and anti-CD3 mAbs (BD Pharmingen), and the cells were fixed and permeabilized using Cytofix/Cytoperm reagent (BD Biosciences). Cells then underwent intracellular staining with an anti-mouse IFN-γ mAb or isotype control mAb (BD Pharmingen) and assessed on a FACS LSR II cytometer (BD Biosciences). The percentages were averaged from at least four mice and represent the mean percent of the maximum value.

Toxicity Assay. Following a three-week treatment, the whole body of each mouse was weighed. The mice were sacrificed, and the liver and spleen were inspected and weighed. The weights of the test groups were compared with those of the control group.

Cytotoxicity Assay. B16 melanoma cells (ATCC, 1×10^5/μL) were seeded in DMEM medium containing 10% fetal bovine serum. After 24 h incubation, the cells were washed with PBS buffer and cultured in fresh media. Then, the cells were treated with 10 μM ellagic acid (**1**), ellagic acid peracetate (**2**), or the vehicle control for 24, 48, 72, and 96 h, and counted in a Neubauer chamber using a trypan blue dye (Gibco) exclusion assay. All experiments were repeated twice in triplicate.

Annexin V Staining Method. B16 melanoma cells were treated with 10 μM ellagic acid (**1**), ellagic acid peracetate (**2**), or the vehicle control, for 72 h. The cells were washed with Annexin V binding buffer, centrifuged at 300×g for 10 min, and suspended (1×10^6) in 100 μL of 1× Annexin V binding buffer. Then, 10 μL of Annexin V fluorochrome was added to the suspension. After the suspension was mixed and incubated in a dark room at room temperature for 15 minutes, the cells were centrifuged, and the cell pellet was resuspended (1×10^6) in 100 μL of 1× Annexin V binding buffer. Next, 10 μL of Annexin V antibody were added, and the cells were incubated at 4–8 °C in a dark room for 10 min. The cells were centrifuged, and the cell pellet was resuspended (1×10^6) in 500 μL of 1× Annexin V Binding Buffer. After 1 μg/mL of PI solution was added in the suspension, a flow cytometric analysis was immediately performed.

Western Blotting. B16 melanoma cells were treated with 10 μM **1**, **2**, or the vehicle control for 72 h. The cells were harvested and lysed. Then, the BCL-2 levels were determined by Western blotting with mouse monoclonal antihuman Bcl-2 Ab (1:1,000, Santa Cruz, Santa Cruz, CA) as primary antibody using a previous procedure.[15]

Statistical Analysis. Data were compared by Student's two-tailed *t* test. A *p* value less than 0.05 was considered statistically significant.

Scheme S1. Summary of alternative approaches attempted for synthesis of ellagic acid (1) from analogues of α-PGG.