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Synthesis and Antitumor Activity of Ellagic Acid Peracetate

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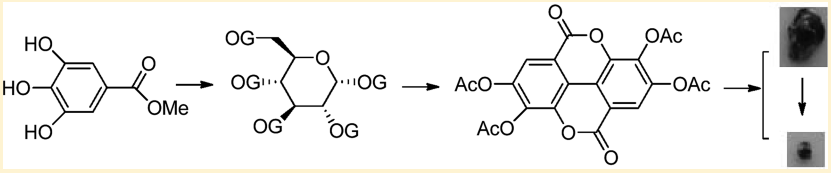
Supporting Information



ABSTRACT: Ellagic acid (**1**) was synthesized for the first time from methyl gallate through α-pentagalloylglucose (α-PGG), and ellagic acid peracetate (3,4,3′,4′-tetra-*O*-acetylellagic acid, **2**) was derived from **1** by acetylation. Oral administration of **2** suppressed melanoma growth significantly in C7BL/6 immunocompetent mice without having any effect on natural killer (NK) cell activity. Comparison of the immunoenhancing activities of **1** and **2** indicated that the latter compound increased white blood cell quantities in peripheral blood and immune cells enriched from the bone marrow and liver of mice. Therefore, both the antitumor efficacy and the immunity enhancement by **2** were greater than those by **1**. In addition, on oral administration, neither **1** nor **2** resulted in whole body, liver, or spleen weight changes of normal, tumor-free mice, indicating that these compounds are potentially nontoxic to mice. It was shown that ellagic acid peracetate (**2**) inhibits B16 melanoma cell growth in vitro and induces B16 cell apoptosis, corresponding to BCL-2 down-regulation. Collectively, the present data imply that **2** can suppress tumor growth by enhancing mouse immunity and inducing tumor cell apoptosis without apparent side effects.

KEYWORDS: *ellagic acid, ellagic acid peracetate, antitumor efficacy, enhancement of immunity, induction of apoptosis, in vivo, BCL-2 down-regulation*

Cancer is a life-threatening disease, and the development of promising novel agents to treat this condition is therefore an urgent need. One of the undesired side effects of current chemotherapy is the appearance of reduced levels of total white blood cells in some patients,[1] and another is the induction of a second cancer by the primary cancer treatment.[2] Natural products and their semisynthetic derivatives are used widely in cancer chemotherapy,[3] and the discovery of novel agents of natural or synthetic origin to selectively suppress tumor growth with enhancement of human immunity and without apparent adverse effects is highly desired.

Ellagic acid (**1**, Figure 1), commonly found in many fruits of the human diet, has been reported previously as a potential antitumor agent. This compound exhibited cytotoxicity toward T24 human bladder cancer cells by induction of p53/p21 expression, G1 arrest, and apoptosis, and the tumor incidence in mouse lung explants was suppressed by ellagic acid through inhibition of benzo(α)pyrene and benzo(α)pyrene-*trans*-7,8-diol metabolism and DNA binding.[4,5] Compound **1** inhibited methylbenzylnitrosamine-induced formation of esophageal O^6-methylguanine in rats, and this in vivo anticarcinogenic efficacy was mediated by modulation of oxidative stress-regulated genes.[6,7] Furthermore, **1** has been reported as a stimulator of immune functions, and it has been proposed that coadministration of this compound is supportive of vinorelbine and estramustine phosphate chemotherapy for prostate cancer patients.[8,9]

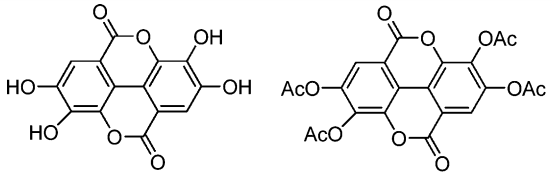


Figure 1. Structures of **1** and **2**.

Previous work has indicated that ellagic acid peracetate (3,4,3′,4′-tetra-*O*-acetylellagic acid, **2**) exhibited more potent bioactivity in vitro than **1**.[10] For example, **2** was more potent than **1** in preventing aflatoxin B_1 (AFB_1)-induced genotoxicity

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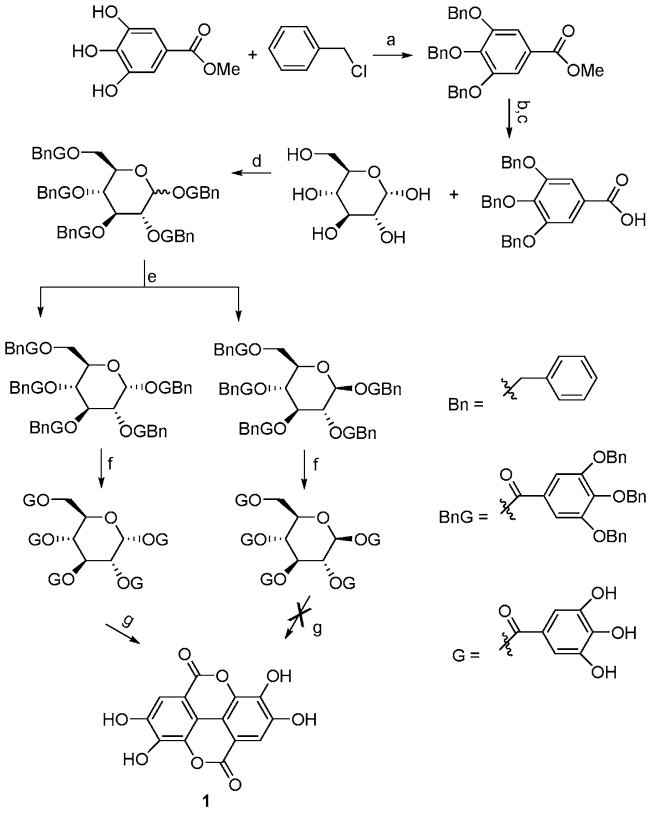
in bone marrow and lung cells[10] and in the inhibition of cytochrome P450 (CYP450)-linked mixed function oxidases (MFOs) and benzene-induced genotoxicity mediated by the action of calreticulin transacetylase.[11] However, the in vivo antitumor and immune modulatory efficacies of **2** have not been reported. In the present study, the synthesis of **1** and **2** is presented, and their comparative in vivo antitumor efficacy, immunity stimulation, natural killer (NK) cell modulation, toxicity determination, and preliminary mechanism of action characterization are described.

Compound **1** has been synthesized from gallic acid using oxidative coupling.[12] Following this synthetic procedure, several methylated analogues of **1** were produced by a series of methods, including intermolecular Suzuki cross-coupling, intramolecular Heck type coupling, and intramolecular Ullmann coupling.[13] Ellagic acid also can be obtained by the hydrolysis of ellagitannins.[14,15] Because ellagitannins are rather inaccessible starting materials, the close analogue pentagalloylglucose (PGG) was used instead. As demonstrated in a previous study,[16] PGG can be prepared easily on a multigram scale. Following this earlier work, a new strategy using glucose as an aid in the aryl coupling of gallic acid molecules was established for the synthesis of **1**.

As shown in Scheme 1, the α- and β-isomers of PGG were synthesized from methyl gallate, which was transformed to

Scheme 1. Synthetic Scheme for 1[a]



[a]Reagents and conditions: (a) KI, K_2CO_3, acetone, reflux, 18 h. (b) NaOH, ethanol, reflux, 2 h. (c) HCl, water. (d) DCC, DMAP, CH_2Cl_2, reflux, 18 h. (e) Silica gel, dichloromethane−toluene−ethyl acetate (75:25:1). (f) H_2, 10% Pd/C, THF, 40 °C, 16 h. (g) 5% Na_2CO_3, rt, 6 h.

3,4,5-tribenzyloxybenzoic acid. A mixture of the α- and β-anomers of D-glucopyranose pentakis[3,4,5-tris(phenylmethoxy)benzoate] was obtained by esterification of D-glucose in the presence of dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)-pyridine (DMAP). Two isomers were separated and hydrogenolized to α- and β-PGG, respectively.[16] Upon treatment of α-PGG with a 5% Na_2CO_3 solution at room temperature for 6 h, **1** was obtained (this condition is milder than that used in the literature[12]). When α-PGG was treated with 1 N HCl at room temperature for 6 h, no change was observed. However, when α-PGG was treated with 1 N NaOH at room temperature for the same period, it was totally decomposed without **1** being produced (Scheme S1 in the Supporting Information). This indicates that 5% Na_2CO_3 solution is necessary for this reaction. Interestingly, when β-PGG (Scheme 1), α-pentagalloylmanose, α-pentagalloylallose, or α-pentagalloylgalactose (obtained in a previous study[16]) was treated with 5% Na_2CO_3 solution at room temperature for 6 h, **1** was not produced (Scheme S1 in the Supporting Information), indicating that the stereochemistry of C-1, C-2, C-3, and C-4 of the PGG isomeric forms is important in this synthesis.

Molecular models of α- and β-PGGs show that the galloyl groups linked to the C-2, C-3, C-4, and C-5 positions of α-PGG can arrange in one plane. In contrast, for β-PGG, the galloyl groups are located less favorably to effect an aryl−aryl coupling reaction (Figure 2). The models suggest that α-PGG is better suited than β-PGG for an intramolecular coupling reaction to occur between the aromatic rings of two galloyl groups. The conversion can produce an ellagitannin intermediate,[14,15] which was not afforded when α-PGG was reacted with Dess-Martin reagent (Scheme S1 in the Supporting Information) and converted further to **1**. This indicates that as a new synthetic strategy, the glucose core may be used as a scaffold to synthesize **1**-related natural products.

A subcutaneous B16 melanoma tumor model using C57BL/6 immunocompetent mice was used to compare the antitumor efficacy of **1** and **2**. Eight- to 12-week-old C57BL/6 mice were fed daily with test compounds or the vehicle control in the drinking water with a dose of 0.5 mg/kg of each for a week.[17−19] The B16 melanoma cells were then inoculated, and treatment was continued for an additional 2 weeks. The mice were sacrificed, the tumors were removed, and their kidneys, livers, and spleens were inspected. The tumors were weighed, compared with the control group,[20] and summarized in Figure 3. The results showed that when compared with the control treatment group, the tumor weights decreased around 70% in the treatment group with a dose of 0.5 mg/kg of **2**, but no significant change was observed for **1**. No overt toxicity was observed in the mice for either treatment group.

To characterize the possible role of immune modulation in mediating the antitumor activity of the test compounds, the effects of **1** and **2** on white blood cells (WBCs) in peripheral blood and immune cells enriched from the bone marrow (BM) and liver of the tumor-free normal mice were tested. After a 1 week of treatment with **1**, **2**, or the vehicle control, the WBCs in peripheral blood and immune cells enriched from BM and liver were counted by a Trypan Blue exclusion method, and the data obtained were summarized in Figure 4. As compared to the vehicle group, the WBCs in peripheral blood were increased significantly by 70%, and immune cells enriched from the BM or liver were increased by 50 and 200%, respectively, in the group treated by **2** ($p < 0.05$). However, no change was observed for immune cells enriched from the BM or liver in the group treated by **1**. The percentages of each immune subset

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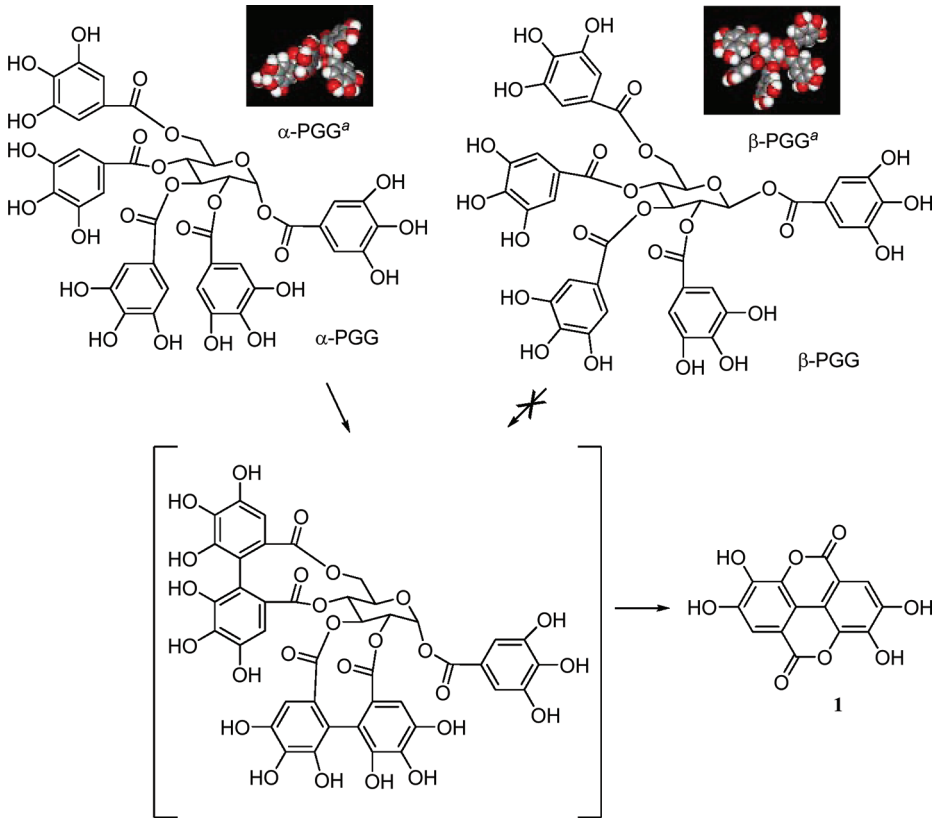

Figure 2. Reaction mechanism of hydrolysis of α-PGG to **1** ([a]CPK model colored by different atoms).

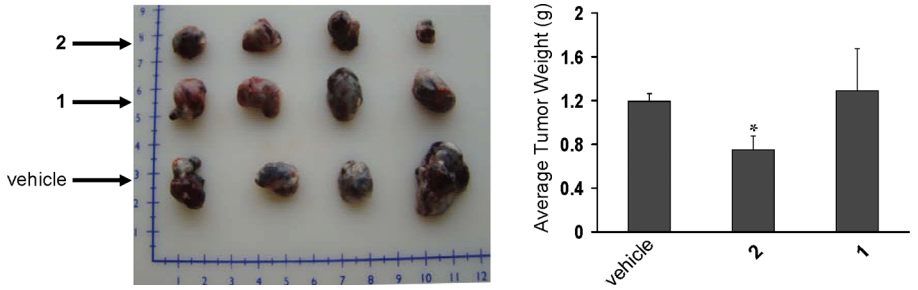

Figure 3. Inhibition of melanoma tumor growth in mice by **1** and **2** [columns, mean in each group ($n \geq 4$); bars, SE; *$p \leq 0.05$].

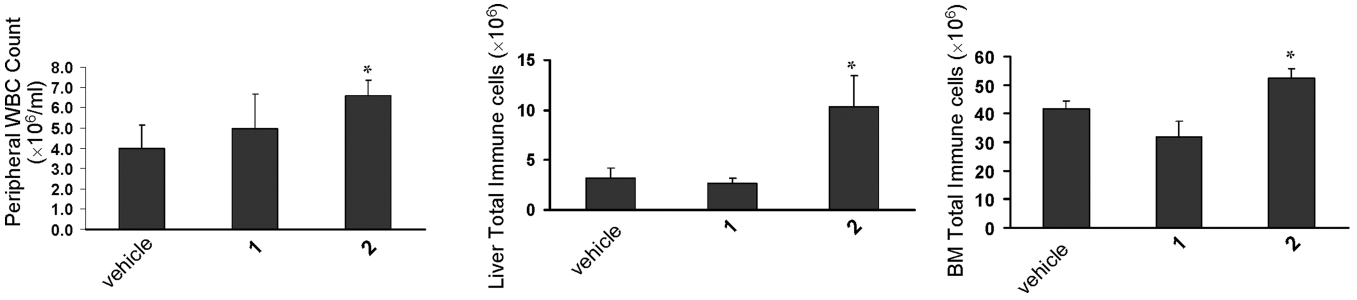

Figure 4. Enhancement of immune cell quantity by **1** and **2** [columns, mean in each group ($n \geq 4$); bars, SE; *$p \leq 0.05$].

were also measured by flow cytometry, but no significant changes were observed. These results suggest that **2** is capable of enhancing mouse immunity through increasing the total number of immune cells rather than individual cell subsets, and such an enhancement may contribute to its antitumor property, consistent with other natural products showing both immunomodulatory and antitumor activities.[21,22]

NK cells play an important role in the innate immune response to tumors and infections,[23] and lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) is a sensitive marker to measure NK cell degranulation, which correlates with NK cell cytotoxicity.[24] To determine if the antitumor activity observed for **2** is associated with its NK cell stimulation, the expression of CD107a in NK cells was tested. After a 1 week

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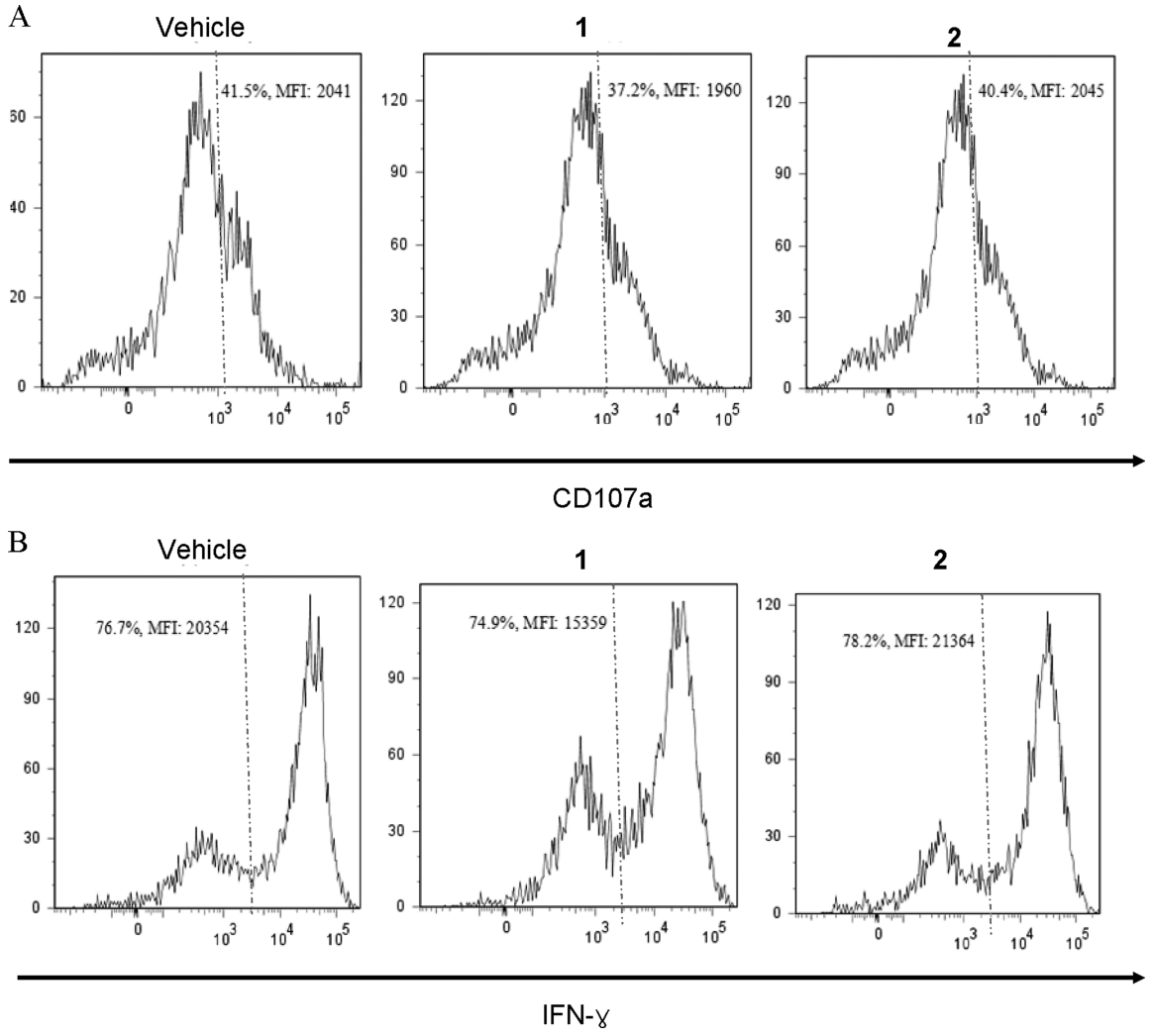

Figure 5. Modulation of NK cell by **1** or **2**. (A) Characterization of surface marker expression of CD107a. (B) Characterization of production of IFN-γ by mouse NK cells.

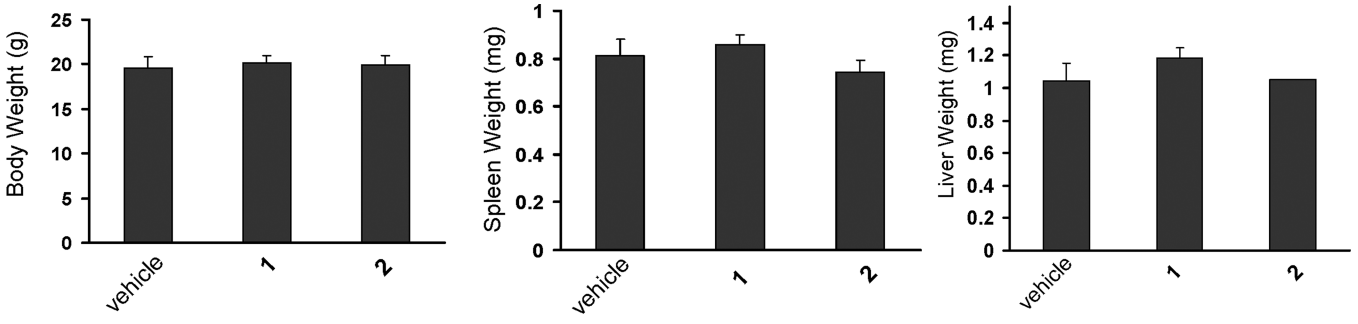

Figure 6. Evaluation of acute toxicity of **1** and **2** [columns, mean in each group ($n \geq 4$); bars, SE].

treatment, the spleens of mice were harvested, and the splenocytes were processed immediately. A flow cytometric analysis was used to determine CD107a expression of NK cells, which were defined as NK1.1+CD3+. The data showed that when compared to the vehicle control, no change of CD107a expression was observed in mice treated with **2** (Figure 5A). Negative results in this assay were also obtained for the vehicle control- and **1**-treated mice.

Interferon-γ (IFN-γ) produced by NK cells is essential for innate and adaptive immune responses in the clearance of intracellular pathogens and for the host defense against malignant transformation.[18] The modulation of IFN-γ production by NK cells was explored for **1** and **2**. After a 1 week of treatment, spleens were harvested, and splenocytes were processed immediately and cultured with brefeldin A. Cell surfaces were stained by NK1.1 and CD3 mAbs, and the cells were fixed, permeabilized, and underwent intracellular staining with an antimouse IFN-γ mAb or its isotype control. A flow cytometric analysis was conducted to determine the level of IFN-γ production by NK (NK1.1+CD3−) cells (Figure 5B). The results

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showed that both compounds did not modulate IFN-γ production by NK cells.

To test the diverse effects of **1** and **2**, the potential oral acute toxicity to the host mice was evaluated. After normal mice were treated daily with **1**, **2**, or the vehicle control for 3 weeks, the body, spleen, and liver of mice were inspected and weighed. As shown in Figure 6, no significant differences were observed in the three treatment groups.

The cytotoxicity toward B16 mouse melanoma cells of **1** and **2** was tested using an in vitro assay.[23] The results showed that **2** significantly suppressed the B16 cell growth, when compared with the vehicle control, and was more potent than **1** (Figure 7).

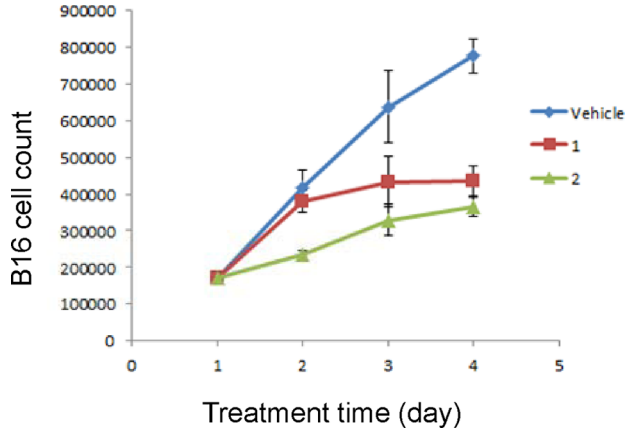

Figure 7. Inhibition of B16 melanoma tumor cell growth by **1** and **2**.

This result is consistent with that showing the in vivo antitumor efficacy of **2**.

A mechanistic study demonstrated that **2** induced B16 cell apoptosis, as evaluated by an annexin V staining method.[25] Treatment of **2** resulted in 6.99% early apoptosis of B16 cells, while the analogous values for the vehicle control and **1** treatment were 2.28 and 4.33%, respectively (Figure 8A). Also, **2** induced 9.78% of B16 late apoptosis/necrosis, but the vehicle control and **1** induced 2.48 and 6.84%, respectively (Figure 8A). The percentages of the viable B16 cells with **1**, **2**, and the vehicle control treatment were 83.9, 71.4, and 90.0%, respectively. These data were consistent with down-regulation of BCL-2, an antiapoptotic protein, by **1** and **2** in comparison to their vehicle control (Figure 8B).[26]

Compound **1** is well documented in terms of its antitumor activity, but similar information concerning its analogue, **2**, is limited. The present study showed that **2** possesses potential antitumor efficacy superior to that of **1**, when evaluated in a B16 melanoma-inoculated C57BL/6 mouse model. In addition, **2** showed significant immunity enhancement and cytotoxicity toward B16 melanoma cells, accompanied by apoptosis induction. Thus, **2** has the potential for further investigation as an immune stimulatory anticancer drug candidate, although this may be hindered by its poor solubility, for which new approaches will be required to overcome.

■ ASSOCIATED CONTENT

Supporting Information

Description of synthetic procedures, biological methods, and Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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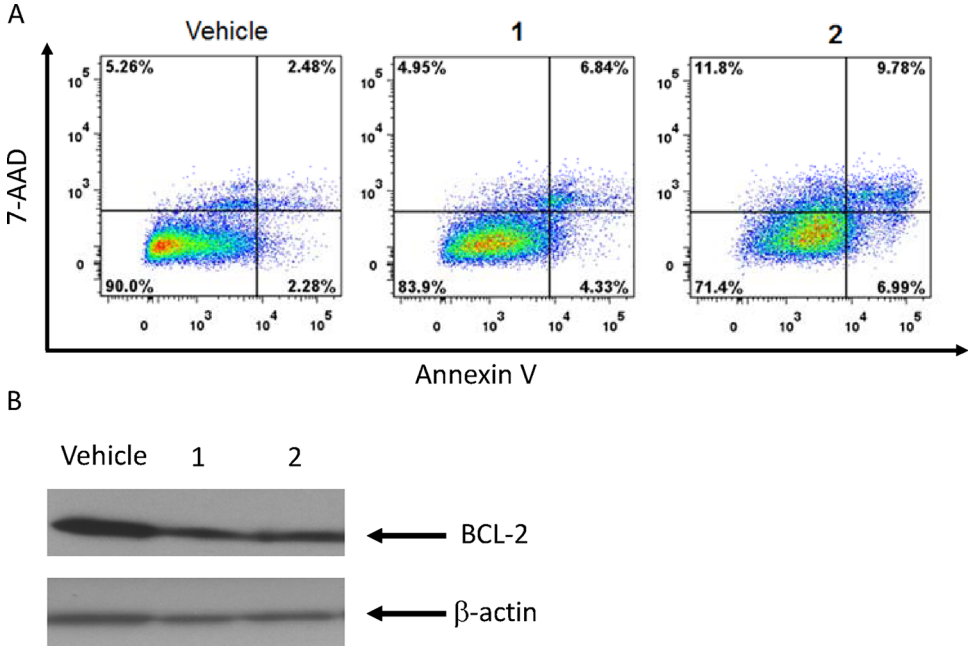

Figure 8. B16 cell apoptosis induction and BCL-2 down-regulation by **1** and **2**. (A) Data are representative of at least three experiments. Lower left quadrant, the percentage of viable cells; lower right quadrant, of early-stage apoptotic cells; and upper right quadrant, the percentage of the late-stage apoptotic cells or dead cells. Data are representative of at least three experiments. (B) Determination of the BCL-2 protein level by Western blotting.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PGG, pentagalloylglucopyranose; NK, natural killer; AFB_1, aflatoxin B_1; CYP450, cytochrome P450; MFO, mixed function oxidase; DCC, dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; WBC, white blood cell; BM, bone marrow; LAMP-1 or CD107a, lysosomal-associated membrane protein-1; IFN-γ, interferon-γ

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