Immunomodulatory Activity of 3β,6β-Dihydroxyolean-12-en-27-oic Acid in Tumor-Bearing Mice

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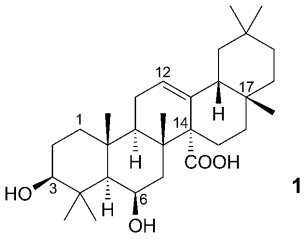
3β,6β-Dihydroxyolean-12-en-27-oic acid (**1**) is a pentacyclic triterpenoid isolated from the rhizomes of *Astilbe chinensis*. To evaluate the *in vivo* antitumor potential and to elucidate its immunological mechanisms, effect of **1** on the growth of mouse-transplantable tumors, and the immune response in naive and tumor-bearing mice were investigated. The mice inoculated with mouse tumor cell lines were orally treated with **1** at the doses of 40, 60, and 80 mg/kg for 10 days. The effects of **1** on the growth of mouse-transplantable S180 sarcoma and H22 hepatoma, splenocyte proliferation, cytotoxic T lymphocyte (CTL) activity, natural killer (NK) cell activity, and production of interleukin-2 (IL-2) from splenocytes in S180-bearing mice were measured. Furthermore, the effect of **1** on 2,4-dinitrofluorobenzene (DNFB)-induced delayed-type hypersensitivity (DTH) reactions and the sheep red blood cell (SRBC)-induced antibody response in naive mice were also studied. Compound **1** could not only significantly inhibit the growth of mouse transplantable S180 sarcoma and H22 hepatoma, increase splenocytes proliferation, CTL and NK cell activity, and the level of IL-2 secreted by splenocytes in tumor-bearing mice, but also remarkably promote the DTH reaction and enhance anti-SRBC antibody titers in naive mice. These results suggested that **1** could improve both cellular and humoral immune response, and could act as antitumor agent with immunomodulatory activity.

Introduction. – Malignant tumor is one of the most serious diseases that damage human health in the modern world and the second largest deadly disease just after heart disease. There exists a close relationship between the occurrence, growth, and decline of tumor and immune states. The low immune function of an organism may not only result in the generation and development of tumor, but also it may be one of the most important factors that prevent the tumor patients' recovery. Immunomodulation through natural or synthetic substances is considered an alternative for the prevention and cure of neoplastic diseases [1]. However, one of the major drawbacks of the current cancer therapeutic practices such as chemotherapy and radiation therapy is suppression of immune system [2]. The enhancement of host immune response has been recognized as a possible means of inhibiting tumor growth without harming the host. Therefore, it is very important to investigate novel antitumor substances with improving immunity.

Natural products have been shown to be excellent and reliable sources for the development of new drugs [3]. Triterpenoids are the major constituents of some medicinal herbs and are widely present in all parts of a variety of plants [4]. Thousands of structures have been reported, with hundreds of new derivatives being described

each year [5]. The biological activities of triterpenoids have recently attracted more attention in the biochemical and medical fields because of their immunomodulatory and antitumor effects [6]. Recently, some triterpenoids have been shown to promote specific and nonspecific immune response in different ways, and their antitumor activity might be achieved by improving immunity [7–12].

Astilbe chinensis (MAXIM.) FRANCH. et SAVAT. (Saxifragaceae) has been used for headache, arthralgia, chronic bronchitis, and stomachalgia in traditional Chinese medicine, and several forms of cancers in the folk medicine [13]. The triterpenoid fraction (ATF) from the rhizomes of *A. chinensis* has recently been reported to exhibit antineoplastic and immunopotentiating activities, as well as a protective effect against cyclophosphamide (CTX)-induced toxicity in tumor-bearing mice [14][15]. 3β,6β-Dihydroxyolean-12-en-27-oic acid (**1**) is a cytotoxic pentacyclic triterpenoid isolated from the rhizomes of *A. chinensis*, and its structure was established by a single-crystal X-ray diffraction analysis [16][17]. Compound **1** has been shown to possess the cytotoxic effect against human ovarian carcinoma HO-8910 cells, human cervical squamous carcinoma HeLa cells, and human leukemic HL60 cells [18]. We have also reported that **1** could significantly inhibit the proliferation of human colorectal carcinoma COLO 205 cells, arrest COLO 205 cells in G_0/G_1 phase, and induce cell apoptosis through down-regulating *Bcl-2* expression, up-regulating *Bax* expression, lowering $\Delta\psi$m, and activating the caspase-3 pathway [19]. In the present study, the antitumor effects of **1** and its immunomodulatory activities on the immune response in naive and tumor-bearing mice were investigated, and the relationships between them were discussed.



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Results. – 1. *Inhibition of* **1** *on the Growth of Transplantable Tumors in Mice.* The inhibition of **1** on the growth of mouse-transplantable tumors was shown in *Tables 1* and *2*. The growth of mouse-transplantable S180 sarcoma was significantly inhibited by **1** compared with the tumor-bearing control ($P < 0.001$), the inhibitory rate being 35.77, 51.11, and 47.94% for the doses of 40, 60, and 80 mg/kg, respectively. Compound **1** could also markedly inhibit the growth of mouse-transplantable H22 hepatoma ($P < 0.001$). The inhibitory rates of **1** against H22 hepatoma were 35.57, 48.84, and 41.10% for the above three doses, respectively. In addition, no signs of toxicity were observed in the **1**-treated mice on the basis of body weight (*Tables 1* and *2*) and microscopic examination of individual organs.

2. *Effects of* **1** *on Splenocyte Proliferation in S180-Bearing Mice.* The effects of **1** on mitogen-stimulated splenocyte proliferation in S180-bearing mice are shown in *Fig. 1*. Concanavalin A (Con A)-induced splenocyte proliferation in the tumor-bearing mice

Table 1. *Inhibitory Effect of* **1** *on the Growth of Transplantable S180 Sarcoma in Mice*[a])

Group	Dose [mg/kg]	Weight [g]		Tumor weight [g]	Inhibitory rate [%]
		Before treatment	After treatment		
Control	–	18.96 ± 1.58	31.19 ± 1.87	3.03 ± 0.54	–
CTX	50	19.34 ± 1.73	28.63 ± 1.36	1.08 ± 0.46***	64.43
1	40	18.90 ± 1.55	31.20 ± 2.79	1.95 ± 0.28***	35.77
	60	19.24 ± 1.92	31.30 ± 2.78	1.48 ± 0.49***	51.11
	80	19.15 ± 1.63	31.49 ± 2.84	1.58 ± 0.40***	47.97

[a]) The values are presented as means ± S.E. ($n = 10$). Significant differences with control group were designated as ***$P < 0.001$. CTX: cyclophosphamide (positive drug).

Table 2. *Inhibitory Effect of* **1** *on the Growth of Transplantable H22 Hepatoma in Mice*[a])

Group	Dose [mg/kg]	Weight [g]		Tumor weight [g]	Inhibitory rate [%]
		Before treatment	After treatment		
Control	–	18.34 ± 1.11	29.59 ± 3.74	3.25 ± 0.76	–
CTX	50	18.55 ± 0.99	25.78 ± 2.77	0.84 ± 0.16***	74.23
1	40	18.86 ± 1.20	29.34 ± 1.48	2.10 ± 0.25***	35.57
	60	18.85 ± 0.78	29.06 ± 2.19	1.66 ± 0.46***	48.84
	80	19.13 ± 1.21	30.04 ± 2.27	1.92 ± 0.33***	41.10

[a]) The values are presented as means ± S.E. ($n = 10$). Significant differences with control group were designated as ***$P < 0.001$. CTX: cyclophosphamide (positive drug).

was significantly enhanced by **1** at the doses of 40 and 60 mg/kg ($P < 0.05$ or $P < 0.01$). The cellular proliferation elicited by the B cell mitogen lipopolysaccharide (LPS) was also significantly increased by **1** at three doses ($P < 0.001$). However, Con A- and LPS-stimulated splenocyte proliferations in the cyclophosphamide (CTX)-treated mice were significantly lower than those of the control group ($P < 0.05$ or $P < 0.01$).

3. Effects of **1** *on Natural Killer* (NK) *Cell and Cytotoxic T Lymphocyte* (CTL) *Activity in S180-Bearing Mice.* Tumor-cell elimination is known to be mediated in part by the cytotoxic activity of NK cells and CTL. We, therefore, measured the cytotoxic activity of splenocytes against S180 cell and NK cell-sensitive YAC-1 cells. As shown in *Figs. 2* and *3*, **1** could significantly increase NK cell and CTL activity in the S180-bearing mice at the doses of 60 and 80 mg/kg ($P < 0.01$ or $P < 0.001$).

4. *Effects of* **1** *on Secretion of IL-2 from Splenocytes in S180-Bearing Mice.* Since cytokines play a prominent role in the development of immune response, we investigated the effects of **1** on the production of IL-2 from splenocytes in S180-bearing mice by MTT[1]) assay. As shown in *Fig. 4*, **1** markedly promoted IL-2 production from splenocytes in S180-bearing mice at the doses of 40 and 60 mg/kg ($P < 0.01$ or $P < 0.001$). However, the production of IL-2 was significantly decreased in CTX-treated mice ($P < 0.01$).

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[1]) MTT = 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium hydrobromide.

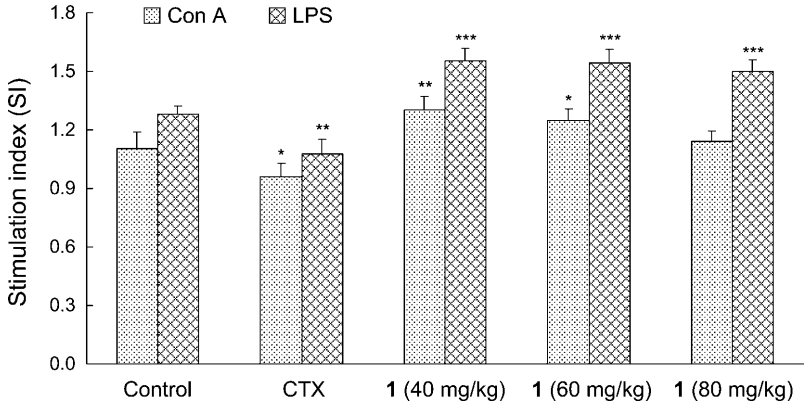

Fig. 1. *Effects of* **1** *on mitogen-stimulated splenocyte proliferation in S180-bearing mice.* Mice were inoculated with sarcoma S180 cells at the armpit, and administered with **1** at the doses of 40, 60, and 80 mg/kg for 10 d, or with CTX at a dose of 50 mg/kg for 2 d once daily. The control groups received the same volume of saline. On day 11, splenocytes were prepared and cultured with Con A, LPS, or *RPMI-1640* medium for 48 h. Splenocyte proliferation was measured by the MTT method as described in the text, and shown as a stimulation index. The values are presented as means ± S.E. ($n=5$). Significant differences compared to control group are designated as $*P<0.05$, $**P<0.01$, and $***P<0.001$.

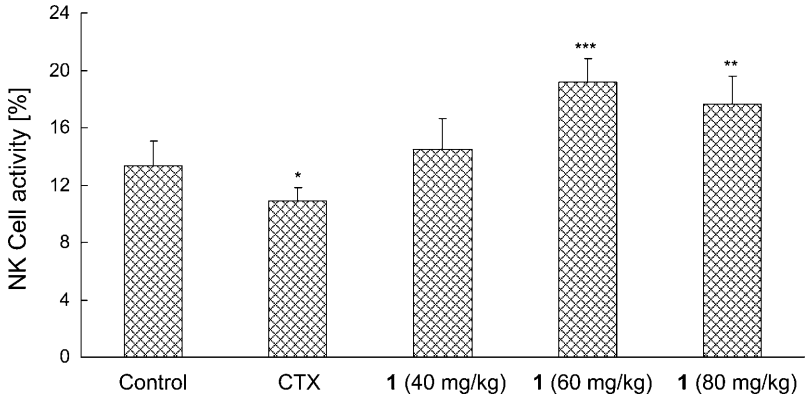

Fig. 2. *Effects of* **1** *on NK cell activity in S180-bearing mice.* Mice were inoculated with S180 sarcoma cells at the armpit, and administered with **1** at the doses of 40, 60, and 80 mg/kg for 10 d, or with CTX at a dose of 50 mg/kg for 2 d once daily. The control groups received the same volume of saline. On day 11, splenocytes were prepared and assayed for NK activity by the LDH release method as described in the text. The values are presented as means ± S.E. ($n=5$). Significant differences compared to control group are designated as $*P<0.05$, $**P<0.01$, and $***P<0.001$.

5. *Effects of* **1** *on 2,4-Dinitrofluorobenzene* (DNFB)*-Induced Delayed-Type Hypersensitivity* (DTH) *reactions in Mice.* The effects of **1** on DNFB-induced DTH reactions in naive mice was also measured, and the results were shown in *Fig. 5*. Compound **1** could significantly enhance DNFB-induced DTH reaction in mice in a dose-dependent

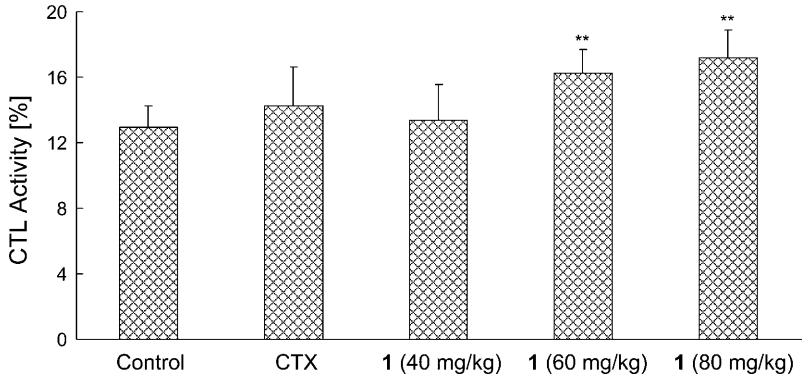

Fig. 3. *Effects of* **1** *on CTL activity in S180-bearing mice.* Mice were inoculated with S180 sarcoma cells at the armpit, and administered with **1** at the doses of 40, 60, and 80 mg/kg for 10 d, or with CTX at a dose of 50 mg/kg for 2 d once daily. The control groups received the same volume of saline. On day 11, splenocytes were prepared and assayed for CTL activity by the LDH release method as described in the text. The values are presented as means ± S.E. ($n=5$). Significant differences compared to control group are designated as $^{**}P<0.01$.

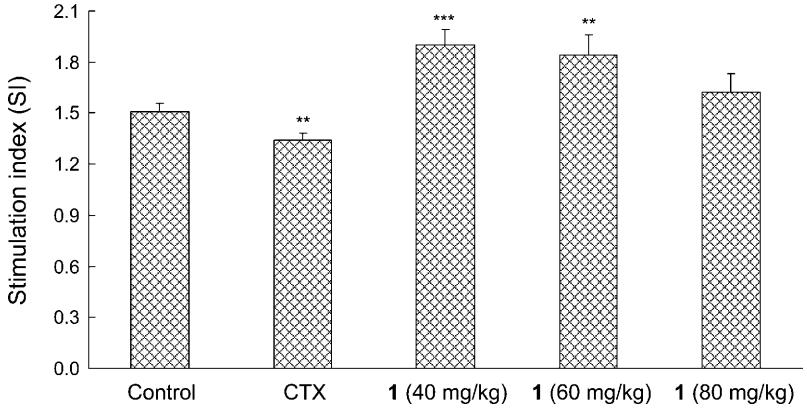

Fig. 4. *Effects of* **1** *on secretion of IL-2 from splenocytes in S180-bearing mice.* Mice were inoculated with S180 sarcoma cells at the armpit, and administered with **1** at the doses of 40, 60, and 80 mg/kg for 10 d, or with CTX at a dose of 50 mg/kg for 2 d once daily. The control groups received the same volume of saline. On day 11, splenocytes were prepared, and IL-2 level in the cultured supernatants was determined indirectly by the MTT method. The values are presented as means ± S.E. ($n=5$). Significant differences compared to control group are designated as $^{**}P<0.01$ and $^{***}P<0.001$.

manner ($P<0.05$, $P<0.01$ or $P<0.001$), while CTX could suppress DTH response ($P<0.01$), compared with the control.

6. *Effects of* **1** *on the Sheep Red Blood Cell* (SRBC)*-Induced Antibody Production in Mice.* Compound **1** was also assessed for its immunopotential activity on the production of SRBC-induced antibody. As shown in *Fig. 6*, the anti-SRBC antibody production in naive mice was significantly promoted by **1** in a dose-dependent manner

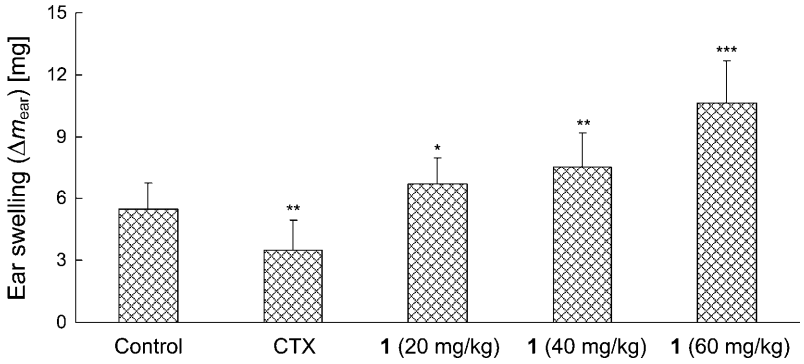

Fig. 5. *Effect of* **1** *on 2,4-dinitrofluorobenzene* (DNFB)*-induced delayed-type hypersensitivity* (DTH) *reaction in mice.* Mice were initially sensitized with DNFB on day 0, and administered with **1** at the doses of 20, 40, and 60 mg/kg for 5 d once daily, or with CTX at a dose of 100 mg/kg for 1 d. The control groups received the same volume of saline. Five days later, DTH reaction was elicited in ear by challenge with DNFB. The ear swelling (Δm_{ear}) was calculated as the difference between the weights of untreated and DNFB-treated ear punches 24 h after challenge. The values are presented as means ± S.E. ($n = 10$). Significant differences compared to control group are designated as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

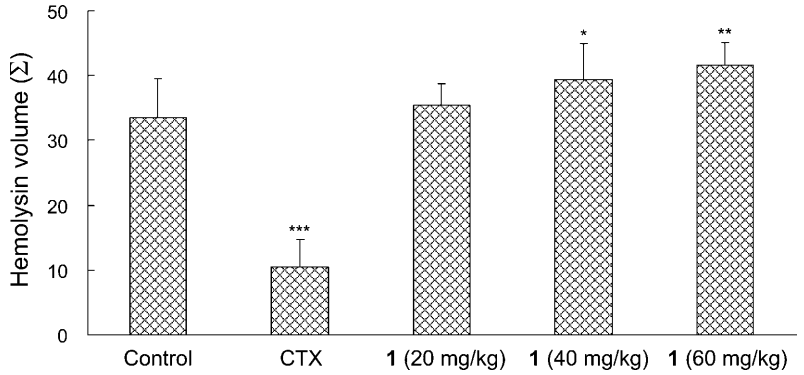

Fig. 6. *Effect of* **1** *on the sheep red blood cell* (SRBC)*-induced antibody production in mice.* Mice were immunized with 10^8 SRBC in 0.5 ml of PBS on day 0, and administered with **1** at the doses of 20, 40, and 60 mg/kg for 5 d, or with CTX at a dose of 50 mg/kg for 2 d once daily. The control groups received the same volume of saline. After 5 d, titers of hemagglutination against SRBC were tested for anti-SRBC antibody production. The values are presented as means ± S.E. ($n = 10$). Significant differences compared to the control group are designated as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

($P < 0.05$ or $P < 0.01$), while CTX significantly decreased the anti-SRBC antibody level ($P < 0.001$), compared with the control.

Discussion. – Clinical verification is being obtained, with a variety of different therapeutic approaches, for the concept that anticancer treatments based on exploiting

the host's own antitumor defense mechanism can be beneficial [2]. The relation between the occurrence, growth, and decline of tumor, and immune states is the essential problem of tumor immunology. The discovery and identification of new antitumor drugs, which can potentiate the immune function, has become an important goal of research in immunopharmacology and cancer therapeutics [20]. This study demonstrates the favorable antitumor and immunomodulatory activity of **1**, a triterpenoid from the rhizomes of *A. chinensis*. Compound **1** could significantly inhibit the growth of mouse-transplantable S180 sarcoma and H22 hepatoma after treatment for 10 d.

Immune responses of body include humoral immunity and cellular immunity. The humoral immune response by B cells is an antigen-specific antibody reaction. The antigen–antibody complex could neutralize toxins and prevent the infection induced by some pathogens. Cell-mediated immune defense was specifically mediated by T cells including nature killer cells. In addition to killing the tumor cells directly, T cells can produce many lymphocyte factors consisting of macrophage mobile factor, lymphotoxin, transfer factor, and interferon. Such factors can promote the proliferation and differentiation of immune cells, the phagocytosis of macrophages and the capacity of killing target cells, so that they play a role in preventing tumor [21]. The capacity to elicit an effective T- and B-cell immunity can be shown by the stimulation of lymphocyte proliferation response [22]. It is generally known that Con A stimulates T cells, and LPS stimulates B cell proliferation. As a first step towards understanding the immunomodulatory activity of **1**, we investigated its effect on lymphocyte proliferation. As shown in *Fig. 1*, **1** could significantly promote the Con A- and LPS-stimulated splenocyte proliferation in S180-bearing mice, while the positive drug CTX with high tumor inhibitory rate had immunosuppressive effect on splenocyte proliferation. The results indicated that **1** could significantly increase the activation potentials of T and B cells, and enhance the humoral immunity and cell-mediated immunity in tumor-bearing mice.

NK cells and $CD8^+$ CTL represent two major populations of cytotoxic lymphocytes [23][24], and are important in the defense against tumors and viruses [25][26]. These cells are able to kill autologous cells infected with intracellular pathogens, as well as tumor cells. NK Cells are functionally similar to CTL. Unlike CTL, however, the killing by NK Cells is nonspecific, and they do not need to recognize antigen/MHC on the target cell. NK Cells can react against and destroy another target cell without prior sensitization to it. NK-Cell and CTL activity assay is a routine method for analysis of a patient's cellular immune response *in vitro*, and can also be used to test the antitumor activities of possible drugs [27]. In this study, **1** significantly enhanced the NK cell and CTL activity in tumor-bearing mice, suggesting that **1** could enhance the nonspecific and specific cytolytic activities against autologous tumor cells.

Cytokines play a critical role in the induction of both humoral and cell-mediated immune responses. IL-2 is an important molecule excreted by the activated T cells and has many immunopotentiating effects, such as proliferation of T cells, B cells, NK cells, and monocytes, augmentation of cytotoxicities of T cells and NK cells, and *in vivo* generation of lymphokine-activated killer cells, which exhibit high cytolytic activities against autologous tumor cells [28]. As shown in *Fig. 4*, IL-2 level secreted by splenocyte in S180-bearing mice was significantly increased by **1**, which implied that IL-

2 may be involved in the immune response and induces NK cells production. The increase may also explain the antitumor properties of **1**.

The DTH reaction is a cell-mediated pathologic response involved in T-cell activation and the production of many cytokines [29][30]. SRBC-Induced antibody production assay is based on the principle that B lymphocytes are activated *in vivo* by SRBC and could produce anti-SRBC-specific antibody *ex vivo*. To confirm that **1** could promote the specific cellular and humoral immune response, we further evaluated the effect of **1** on DFNB-induced DTH reaction and SRBC-induced antibody production in naive mice. The results stated that **1** significantly enhanced DTH response and the anti-SRBC-antibody level in naive mice in a dose dependent manner (*Figs. 5* and *6*).

In conclusion, **1** could not only significantly inhibit the growth of transplantable tumor in mice, but also remarkably increase splenocytes proliferation, NK cell and CTL activity, and the level of IL-2 secreted by splenocytes in tumor-bearing mice, promote the DTH reaction, and enhance anti-SRBC-antibody level in naive mice, indicating that the **1** could improve both cellular and humoral immune responses. These results suggested that the antitumor activity of **1** might be achieved by improving immune response, and **1** could act as antitumor agent with immunomodulatory activity.

Experimental Part

General. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium hydrobromide (MTT), concanavalin A (Con A), and lipopolysaccharide (LPS) were obtained from *Sigma Chemical Co.* (St. Louis, MO, USA). *RPMI-1640* Medium and fetal bovine serum (FBS) were from *Gibco* (Grand Island, NY, USA). 2,4-Dinitrofluorobenzene (DNFB) was obtained from *BD Biosciences Pharmingen* (CA, USA). Cyclophosphamide (CTX) was provided by *Jiangsu Hengrui Company* (Jiangsu, P. R. China). Sheep red blood cells (SRBC) were delivered by the College of Animal Sciences, Zhejiang University (Hangzhou, P. R. China), and kept in *Alsever*'s medium and washed three times with PBS before use.

3β,6β-Dihydroxyolean-12-en-27-oic acid (**1**). $C_{30}H_{48}O_4$. M_r: 472.36. This compound has previously been isolated from the rhizomes of *A. chinensis*, and its structure was identified by spectroscopic analysis including two-dimensional NMR spectroscopy and HR-ESI-MS, and by single-crystal X-ray diffraction analysis [16][17]. Its purity was determined to be more than 98% by HPLC on a *Water 600E* HPLC instrument with a *Symmetry® C18* column (250 × 4.6 mm i.d.; 5 μm particle size) and a *Waters 2996 PDA* detector.

Mouse sarcoma S180 and hepatoma H22 cell lines were provided by Zhejiang Academy of Medical Sciences and reproduced in our laboratory. YAC-1 Cell lines obtained from Institute of Cell Biology, Chinese Academy of Sciences, were maintained in the logarithmic phase of growth in *RPMI-1640* medium supplemented with 2 mM L-glutamine (*Sigma*), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FBS at 37° under humidified air with 5% CO_2.

Experimental Animals. ICR Mice (Grade II, 5-weeks-old) weighing 18–22 g were purchased from Zhejiang Experimental Animal Center (Certificate No. 22-2001001, Hangzhou, P. R. China) and acclimatized for 1 week before use. Half of them were male, and the others were female. Rodent laboratory chow and tap water were provided *ad libitum*, and maintained under controlled conditions with a temp. of 24 ± 1°, humidity of 50 ± 10%, and a 12/12 h light/dark cycle. All the procedures were in strict accordance with the P. R. China legislation on the use and care of laboratory animals, and with the guidelines established by Institute for Experimental Animals of Zhejiang University and were approved by the university committee for animal experiments.

Treatment and Drug Administration. Ascites of the S180- or H22-bearing mice were drawn out under aseptic conditions and then diluted fourfold with aseptic saline. The diluted S180 or H22 cell suspension was subcutaneously (sc) inoculated into the armpit of ICR mice for 0.2 ml per mouse [31]. 24 h later, mice were divided into five groups, each consisting of ten mice. The inoculated mice were orally

administered with **1** at the doses of 40, 60, 80 mg/kg for 10 d or intraperitoneally (ip) injected with CTX at a dose of 50 mg/kg for 2 d once daily. The control groups received the same volume of saline. The dose volume was 0.2 ml/10 g body weight. On day 11, mice were weighed and sacrificed by cervical dislocation. The solid tumors and spleen were collected and weighed. Then, the inhibitory rate against the growth of tumor was calculated. The inhibitory rate [%] = $[(C-T)/C] \times 100$, where C is the average tumor weight of the control group; T is the average tumor weight of medicine groups.

Splenocyte-Proliferation Assay. Splenocytes collected from tumor-bearing mice were prepared as described in [32], and seeded into 4 wells of a 96-well flat-bottom microtiter plate at 5×10^6 cell/ml in 100 µl complete medium; thereafter, Con A (final concentration 5 µg/ml), LPS (final concentration 10 µg/ml), or *RPMI-1640* medium were added to give a final volume of 200 µl. The plates were incubated at 37° in a humid atmosphere with 5% CO_2. After 44 h, 50 µl of MTT soln. (2 mg/ml) were added to each well, and incubated for further 4 h. The plates were centrifuged (1400*g*, 5 min), and the untransformed MTT was removed carefully by pipetting. To each well, 150 µl of a DMSO working soln. (180 µl of DMSO with 20 µl of 1N HCl) were added, and the absorbance was evaluated in an ELISA reader (*Bio-Rad*, USA) at 570 nm after 15 min. The stimulation index (*SI*) was calculated based on the following formula: *SI* = the absorbance value for mitogen-cultures divided by the absorbance value for non-stimulated cultures.

Assay of Natural Killer (NK)-*Cell Activity.* The NK-cell activity from spleen was measured as described in [33]. Briefly, YAC-1 cells were used as target cells and seeded in 96-well U-bottom microtiter plate (Nunc) at 4×10^4 cells/well in *RPMI-1640* complete medium. Splenocytes prepared as described above were used as the effector cells, and were added at 2×10^6 cells/well to give E/T ratio 50 : 1. Each test was repeated four wells. The plate was centrifuged at 250*g* for 1 min to ensure effecter and target cell contact. After 4 h incubation at 37° in a humid atmosphere with 5% CO_2, the plate was again centrifuged at 250*g* for 4 min. Supernatants (100 µl) were collected from each well, and then transferred into the corresponding wells of a 96 flat-bottom microtiter plate. Lactate dehydrogenase (LDH) activity in the supernatants was measured by addition of the enzyme substrate and absorbance recording at 490 nm. Three kinds of control measurements were performed: a target-cell spontaneous release, a target cell maximum release, and an effector-cell spontaneous release. NK-Cell activity was calculated with the following equation: NK cell activity [%] = $(A_S - A_E - An)/(Am - An) \times 100$, where A_S, absorbance value of test samples; A_E, absorbance value of effector-cells control; An, absorbance value of target cells incubated with medium; Am, absorbance value of target cells incubated with 1% of NP-40.

Assays of Cytotoxic T-Lymphocyte (CTL) *Activity.* The CTL activity was analyzed using LDH release method as described above. S180 Cells and splenocytes were used as target cells and effector cells, respectively. The ratio of effector cells to target cells was 50 : 1. To determine the percentage of target cells killed, the following equation was used: % lysis = $(A_S - A_E - An)/(Am - An) \times 100$, where A_S, absorbance value of test samples; A_E, absorbance value of effector cells control; An and Am, as defined above.

Cytokine Levels in the Cultured Supernatants of Splenocytes. To analyze the effect of **1** on the levels of cytokine secreted by splenocytes of S180-bearing mice, splenocytes (5×10^6 cells/ml) prepared as described above were treated with Con A (5 µg /ml) for 48 h, and interleukin-2 (IL-2) in the supernatants were assayed. The stimulation index (*SI*) of the target cells was determined to measure IL-2 levels [34]. The splenocytes of the normal ICR mice, maintained in *RPMI-1640* complete medium at 37° for 48 h in a humid atmosphere with 5% CO_2, were used as the target cells. Splenocytes were washed with *RPMI-1640* medium, counted, and finally diluted to the concentration of 5×10^6/ml with the complete medium. The same volume of target lymphocytes and IL-2 supernatants were added to the wells of 96 flat-bottom microtiter plates. Each test was repeated three wells. After 20 h incubation, 50 µl of MTT (2 mg/ml) were added to each well. The plate was incubated for another 4 h, and then subjected to MTT assay. The target cells incubated with the same concentration of Con A and RPMI-1640 were used as the control. The SI was calculated based on the following formula: *SI* = the absorbance value for IL-2 cultures divided by the absorbance value for control cultures.

2,4-Dinitrofluorobenzene (DNFB)-*Induced Delayed-Type Hypersensitivity* (DTH) *Response.* Six-week-old female ICR mice were divided into five groups, each consisting of ten mice. Animals were initially sensitized with 50 µl of 1% DNFB dissolved in acetone/olive oil 1 : 1 on the shaved abdominal

skin of recipients. Beginning on the day of immunization, the immunized mice were orally administered with **1** at the doses of 20, 40, and 60 mg/kg for 5 d once daily or injected ip with CTX at a dose of 100 mg/kg for 1 d. The control groups received the same volume of saline. After 5 d, the DTH reaction was elicited by smearing 10 µl of 1% DNFB on both sides of the left ear. 24 h later, the DTH response to DNFB was evaluated by measuring the weight difference of right and left ear with an anal. balance [35].

Sheep Red Blood Cell (SRBC)-*Induced Antibody Production*. The effect of **1** on SRBC-induced antibody production was investigated as described in [36]. Six-week-old female ICR mice were divided into five groups, each consisting of ten mice. Mice were immunized ip with 10^8 SRBC in 0.5 ml of PBS. Beginning on the day of immunization, the immunized mice were orally administered with **1** at the doses of 20, 40, and 60 mg/kg for 5 d or injected ip with CTX at a dose of 50 mg/kg for 2 d once daily. The control groups received the same volume of saline. After 5 d, serum was collected, and titers of hemagglutination against SRBC were tested for anti-SRBC antibody production. Briefly, serum was diluted with saline according to multiple proportions. The diluted serum (100 µl) was transferred to hemagglutination microplate and blended with 5×10^6 SRBC in 100 µl of PBS. The degree of agglutinating was observed after incubation for 3 h at 37°. The serum hemolysin volume was calculated: $\Sigma = S_1 + 2S_2 + 3S_3 + \ldots\ldots nS_n$, where S is the degree of agglutinating, and n is the dilute rate.

Statistical Analysis. The data were expressed as mean ± standard errors (S.E.), and examined for their statistical significance of difference with standard's *t*-test.

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