# An anticancer effect of a new saponin component from *Gymnocladus chinensis Baillon* through inactivation of nuclear factor-κB

Yan-Xia Ma, Hong-Zheng Fu, Min Li, Wan Sun, Bo Xu and Jing-Rong Cui

***Gymnocladus chinensis Baillon*** **is widely distributed in China, and its fruits have been used in the treatment of rheumatism, furunculosis, soreness and swelling in traditional Chinese medicine for a long time. Few biological components were, however, isolated. In this study, a new triterpenoid saponin (GC-1) was extracted from the fruit of *Gymnocladus chinensis Baillon* and its biological actions were investigated. The results showed that GC-1 inhibited growth of a panel of human cancer cell lines *in vitro* by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide and sulforhodamine B assays. Furthermore, GC-1 was demonstrated to induce apoptosis in HL-60 cells in a dose-dependent manner. By using a reporter gene assay, nuclear factor-κB activity induced by tumor necrosis factor-α was decreased gradually by addition of increasing concentration of GC-1 (1–40 μmol/l). In parallel, the blockage of nuclear factor-κB translocation from cytoplasm to nucleus was determined by Western blotting. This is the first study investigating the link of antiproliferative action of the compound with the inhibition of nuclear factor-κB activation. The mechanism of the actions of GC-1 might be due to the interruption of nuclear factor-κB translocation in the signaling pathway, which contributes to the chemotherapy potential.** ***Anti-Cancer Drugs*** **18:41–46 © 2007 Lippincott Williams & Wilkins.**

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## Introduction

Saponins are a major family of secondary metabolites that are produced by many plant species. These molecules commonly have potent antifungal activity and their natural role in plants is likely to be protection against attack by pathogenic microbes. They are also very important bioactive substances with a variety of commercial applications including their use as drugs and medicines [1]. With the continuous development of isolation techniques and structural elucidation, saponin chemistry has developed fast and new bioactive substances can be found continuously. Saponins are divided into two groups: steroidal saponin and triterpenoidal saponin, and they have been demonstrated to possess various beneficial effects, such as anticancer and fungistatic properties [2,3]. On the basis of the various biological properties and unique structures, plant saponins were regarded as promising candidates for the development of pharmaceutical and/or nutraceutical agents in natural or synthetic forms [4].

*Gymnocladus chinensis Baillon* is widely distributed in Asian countries, especially in China, and its fruits have been used in the treatment of rheumatism, furunculosis, soreness and swelling in traditional Chinese medicine for a long time [5]. So far, only monoterpene glycosides [6] and a new triterpenoid saponin have been isolated from the fruit of *G. chinensis Baillon*. Their biological activities, however, remain to be determined.

The aim of the present study was to investigate the biological activities of a new triterpenoid saponin isolated from the fruit of *G. chinensis Baillon*, especially its anticancer property and possible molecular mechanisms. We demonstrated that the compound was able to suppress tumor cell proliferation in-vitro and induce apoptosis, which might link it to the inhibition of the nuclear factor (NF)-κB signaling pathway.

## Materials and methods

### Sample preparation

A new compound (128 mg) was isolated from the fruit of *G. chinensis Baillon* (4 kg) and its structure was determined as a triterpenoid saponin by extensive two-dimensional nuclear magnetic resonance data and mass spectrometry ($C_{88}H_{138}O_{40}$ 1834.5791, calculated 1834.88) (Fig. 1). The compound was named as 2β, 23-dihydroxy-3-*O*-α-L-rhamnopyranosyl-21-*O*-{(6s)-2-*trans*-2,6-dimethyl-6-[(4-*O*

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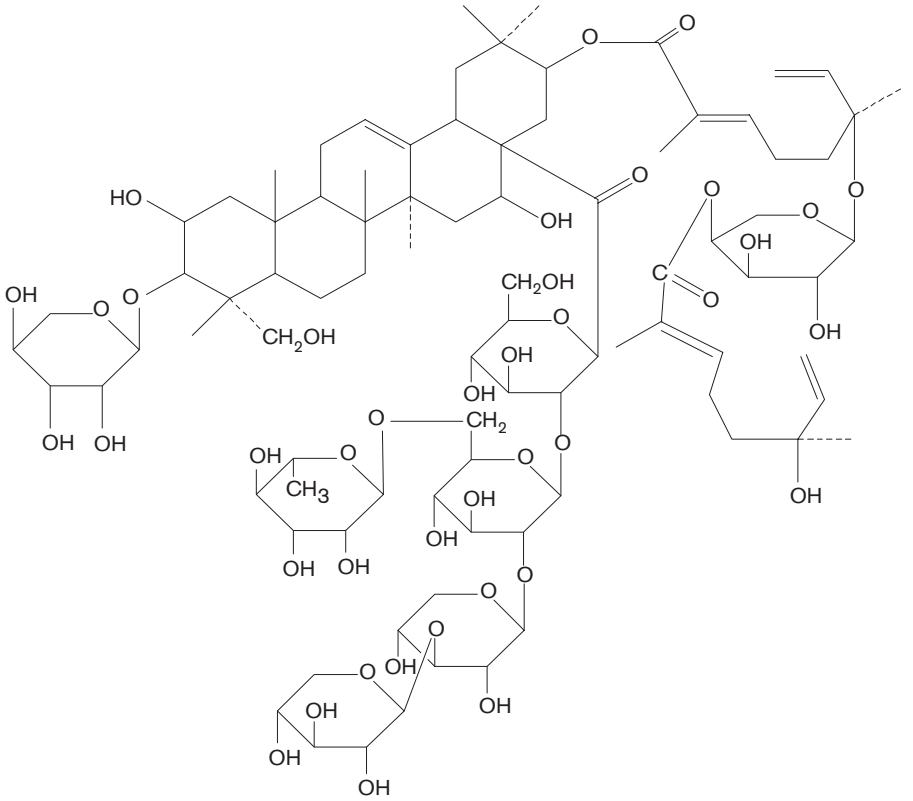
**Fig. 1**



Chemical structure of GC-1.

-(6s)-2-*trans*-2,6-dimethyl-6-hydroxy-2,7-octadienoate)-α-L-arabinopyranosyl]-2,7-octadienoyl acacic acid 28-*O*-β-D-xylopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1–6)]-β-D-glucopyranoside (GC-1). GC-1 was isolated and purified by D101 column, silica gel column and preparative high-pressure liquid chromatography (C-18), with purity higher than 97%. It was a white crystal, dissolved in 10% dimethylsulfoxide (DMSO) in phosphate-buffered saline (PBS) at a concentration of $10^{-2}$ mol/l as stock solution, and further diluted in PBS before use. The final concentration of DMSO in the solution was less than 0.1%.

### Chemicals and antibodies

Tumor necrosis factor (TNF)-α and antibiotic Zeocin were obtained from R&D, Invitrogen (Carlsbad, California, USA). Unless otherwise stated, all chemicals such as *p*-nitrophenyl phosphate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulphorhodamine B (SRB) and RNase were obtained from Sigma-Aldrich, (Sheboygen, Wisconsin, USA). Monoclonal antibodies against human NF-κB p65 protein and actin polyclonal antibody against Inhibitor of κBα (rabbit serum) and peroxidase-conjugated polyclonal anti-mouse or anti-rabbit antibodies were purchased from Sigma-Aldrich (Sheboygan, Wisconsin, USA).

### Cell lines and cell culture conditions

Human promyeloleukemic cell line (HL-60), cervical carcinoma (HeLa), hepatocarcinoma BEL-7402 cell line and gastric carcinoma BGC-823 cell line were maintained in RPMI 1640 medium (Gibco/BRL, Div. of Invitrogen, Germany), supplemented with 10% heat-inactived fetal calf serum and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml). Human embryonic kidney cell line (HEK293) was cultured in Dulbecco's modified Eagle's medium (Gibco/BRL) supplemented with 10% heat-inactived fetal bovine serum and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml). All cell lines were purchased from the American Type Culture Collection (University Boulevard, Manassas, Virginia, USA). Transfected cell line (HEK293/pNiFty-SEAP) was a gift from Dr. Davriche (INSERM 563, CHU Purpan, BP 3028, 31054 Toulouse Cédex, France), cultured in the same medium as HEK293 cells, and enriched with 200 μg/ml of Zeocin for selection and maintenance of stable transfected cells. Cell cultures were maintained in a 95% air and 5% $CO_2$ humidified atmosphere at 37°C.

### Cytotoxicity assay

Effect of GC-1 on cell proliferation was determined by a standard MTT or SRB-based colorimetric assay as previously described [7]. Briefly, different cell lines were seeded in 96-well microplates at $5 \times 10^4$ and $10^4$ cells/well for adherent and suspension cells, respectively. After 18–24 h, exponentially growing cells were exposed to increasing concentrations of GC-1 (1–80 μmol/l) for 48 h, before the addition of MTT or SRB. Plates were read at 570 or 540 nm for MTT or SRB staining, respectively, by using a FLUOstar OPTIMA microplate muti-detection reader (BMG Offenburg, Germany). Detection was followed by computerized data acquisition and processing. The experiments were run in triplicates.

### Apoptosis induction

HL-60 cells were plated at a density of $5 \times 10^5$ cells/ml. After 24 h, compound GC-1 (0.1–40 μmol/l) was added. After incubation for 12 h, cells were harvested by centrifugation at 1500 r.p.m., washed in PBS and fixed overnight in 70% ethanol at −20°C. Samples were centrifuged, washed and resuspended in PBS. Then they were stained with 0.5 mg/ml of propidium iodide. Proapoptotic effect was determined by analytical flow cytometry using a BD-LSR flow cytometer (BD Biosciences, with HQ in Franklin Lakes, New Jersey, USA) with an excitation/emission of 488/525 nm. All experiments were performed in triplicate and gave similar results.

### A gene reporter gene assay for nuclear factor-κB activity

NF-κB activities were measured by the secretory alkaline phosphates (SEAPs) reporter gene assay. Human embryonic kidney cells were transfected with pNiFty-SEAP (HEK293/pNiFty-SEAP), and they expressed SEAP reporter gene in response to NF-κB activation and secreted the SEAP enzyme into the culture medium. The stable transfected cells were plated onto 24-well plates, incubated for 24 h, given a fresh change of medium, pretreated with GC-1 at the indicated concentration or solvent (0.1% DMSO) for 30 min and then treated with TNF-α (0.2 ng/ml) for 24 h; 0.1% DMSO was used as solvent control. In terms of time-course effect of GC-1 on NF-κB, a control of TNF-α plus 0.1% DMSO was set at each indicated time. The supernatants were collected separately for the detection of NF-κB activity. In the reaction system, the product of the SEAP/substrate *p*-nitrophenyl phosphate presented in yellow and the absorbance was read at 405 nm. TNF-α (0.2 ng/ml) plus 0.1% DMSO and 0.1% DMSO alone were used as positive and solvent control, respectively. Dose effect of GC-1 on inhibition of NF-κB was analyzed by comparing absorbance values with those of controls. Inhibitory rate of GC-1 on NF-κB in each time point was calculated as follows: [the absorbance values at 405 nm (*A* values) of positive control (TNF-α plus 0.1% DMSO) minus the *A* values of sample treated with 20 μmol/l GC-1 plus TNF-α]/*A* values of positive control (TNF-α plus 0.1% DMSO) × 100%. The experiments were run in triplicate.

### Preparation of cell extracts and Western blotting

The HL-60 cells were seeded into incubation flask at $5 \times 10^5$ cells/ml; after incubation for 24 h, the culture medium was replaced by fresh medium and GC-1 (0.1–40 μmol/l) or solvent (0.1% DMSO) was added to the culture medium for 30 min, then incubated with TNF-α (50 ng/ml) for another 24 h; 0.1% DMSO alone was added to the medium as solvent control. Cytoplasmic and nuclear extracts were performed essentially as described [8]. The proteins were quantified by using a Coomassie Plus protein assay reagent (Pierce, Rockford, Illinois, USA).

Western blotting was used to analyze the expression of NF-κB p65 protein and IκBα in cytosolic and nuclear extracts. Equal amounts of proteins (20 μg) were diluted in 5 × Laemmli buffer, vortexed and heated at 100°C for 3 min. Samples were then separated by 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes (Hybond C; Amersham, Little Chalfont, UK). Immunoblots were stained with Ponceau red to visualize total proteins contained in each slot and probed alternatively with mouse monoclonal antibodies against p65 (1 : 500) and β-actin (1 : 2000), and polyclonal antibody against IκBα (1 : 250). Blots were revealed with peroxidase-conjugated polyclonal anti-mouse or anti-rabbit antibodies (1 : 5000) and ECL detection kit (Amersham).

### Statistical analysis

Statistical differences were calculated using Student's paired *t*-test at a significance level of $P < 0.05$.

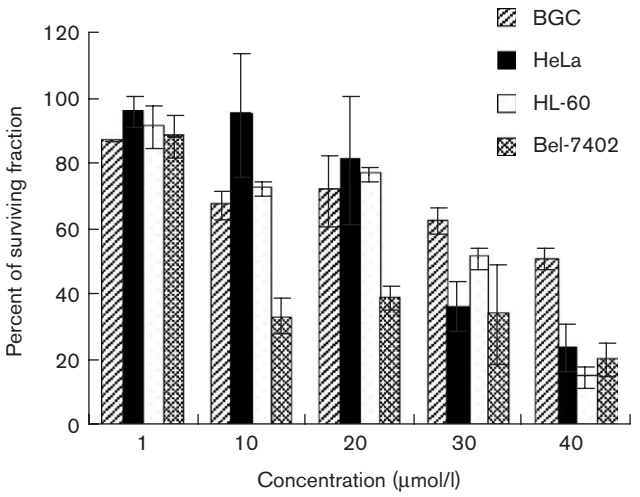
## Results

### Cytotoxic effect of compound GC-1

Several plant saponins have been shown to have anticancer effects [9–11]. Four tumorigenic human cell lines (HL-60, HeLa, BEL-7402, BGC-823) were chosen to determine the cytotoxic activity of compound GC-1. After a 48-h incubation, GC-1 showed different cytotoxicities with respect to the cell lines. The percentage of surviving fraction decreased gradually with the increase in GC-1 concentration (Fig. 2). The $IC_{50}$ values of GC-1 were determined to be 14.81, 18.43, 26.08 and 29.19 μmol/l for HL-60, Bel-7402, HeLa and BGC-823 cell lines, respectively. As the HL-60 cell line is the most sensitive to GC-1 in the tested cells, we chose HL-60 cells to elucidate the biological action of GC-1.

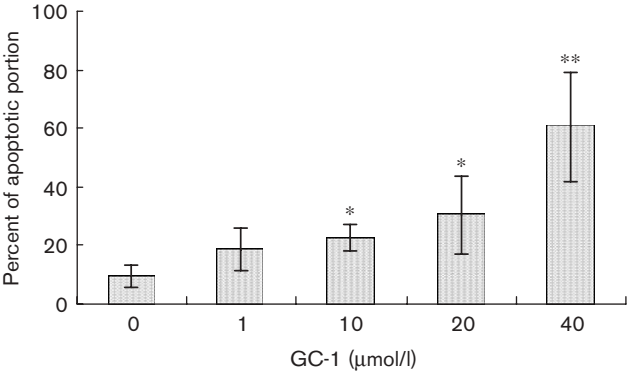
The ability of GC-1 to suppress the growth of different tumor cells provides interesting possibilities for the development of new anticancer strategies in humans. Elucidation of the mechanism involved in GC-1 cytotoxicity *in vitro* and tumor growth inhibition *in vivo* is an important step for a better understanding of the antitumor activity of saponin compounds.

**Fig. 2**



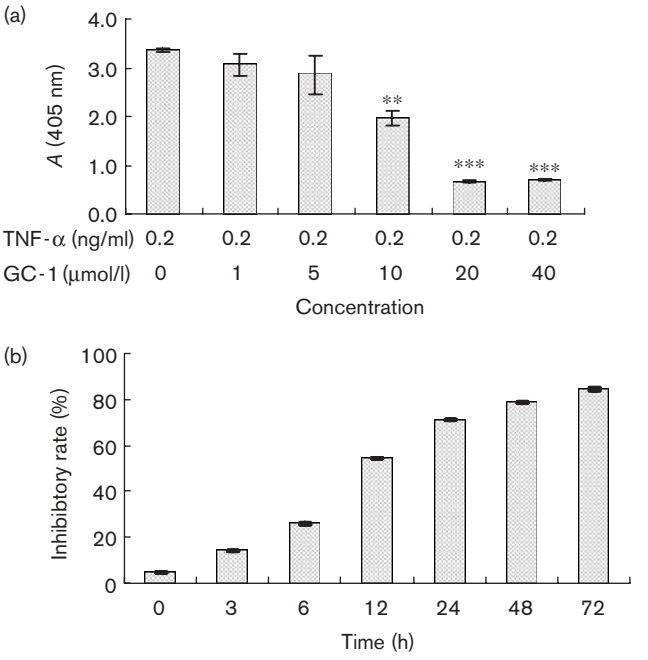

Cytotoxic activity of compound GC-1. Four cell lines, human promyeloleukemic (HL-60), human cervical carcinoma (HeLa), hepatocarcinoma BEL-7402 and gastric carcinoma BGC-823 cell line, were exposed to increasing concentrations (1–40 μmol/l) of GC-1 for 48 h. Surviving fraction was determined by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide and sulforhodamine B assay. Data are given as the mean of triplicate values ± SD.

**Fig. 3**




Proapoptototic effect of GC-1. HL-60 cells were exposed to a serial concentrations of GC-1 (1–40 μmol/l) for 12 h. '0' represents 0.1% dimethylsulfoxide medium (control). The experiments were run in triplicate. *$P<0.05$, **$P<0.01$.

**Fig. 4**




Inhibitory effect of GC-1 on nuclear factor (NF)-κB activation. Inhibitory effect of GC-1 on NF-κB activation induced by tumor necrosis factor (TNF)-α (0.2 ng/ml) was determined by the secretory alkaline phosphate reporter gene assay and the absorbance was measured at 405 nm. (a) Dose–effect results of HEK293/pNiFty-SEAP cells exposed to serial concentrations of GC-1 (1–40 μmol/l). The compound was added into the culture medium 30 min before TNF-α (0.2 ng/ml) and maintained for another 24 h. Inhibition effect of GC-1 on NF-κB activation was analyzed by comparing absorbance values with those of controls. (b) Time-course results of HEK293/pNiFty-SEAP cells treated with 20 μmol/l GC-1 and TNF-α (0.2 ng/ml) for different times (0–72 h). Inhibition effect of GC-1 on NF-κB activation was analyzed by comparing inhibitory rates of GC-1 on NF-κB activation at each time point. Experiments were run in triplicate. **$P<0.01$, ***$P<0.001$.

### Proapoptototic effect of compound GC-1 on HL-60 cells

Apoptosis is known to be a very important mechanism in the anticancer effects induced by chemopreventive and chemotherapeutic agents [12]. To assess whether GC-1 induced apoptosis, we checked the appearance of apoptotic portion (sub-$G_1$ population), a specific fraction for apoptosis by flow cytometry analysis. As illustrated in Fig. 3, exposure to a gradient concentration of GC-1 from 1 to 40 μmol/l increased the number of apoptotic cells from 18.59 to 60.60% versus 9.29% in the control, indicating the apoptotic induction of GC-1 in a dose-dependent manner, obviously after a treatment of GC-1 for 12 h.

### Effect of compound GC-1 on nuclear factor-κB activity

The ability of NF-κB to inhibit apoptosis, as well as to promote cell proliferation, makes it an attractive target for cancer therapy [13,14]. To analyze the molecular mechanism of GC-1 anticancer activity, transfected human embryonic kidney cell line (HEK293/pNiFty-SEAP) was used to test the effect of GC-1 on NF-κB. As the dosages of GC-1 (1–40 μmol/l) increased, NF-κB activity induced by TNF-α was reduced gradually and 10 μmol/l of GC-1 significantly decreased the SEAP activity. The treatment of 20 μmol/l GC-1 reached a maximal inhibitory effect (Fig. 4a). In parallel, the inhibitory rate of 20 μmol/l GC-1 on NF-κB activity was increased when the incubation time increased (Fig. 4b). The results indicated that GC-1 can inhibit NF-κB activity induced by TNF-α (0.2 ng/ml) in dose-dependent and time-dependent manners. It is interesting to investigate the effect of GC-1 on protein expression of NF-κB.

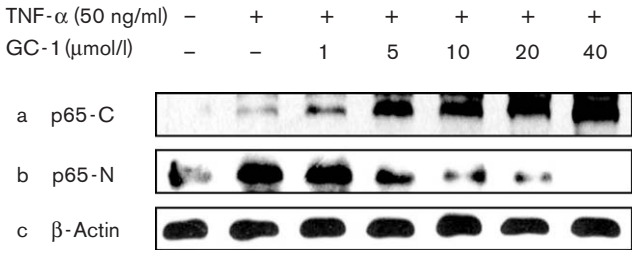
### Compound GC-1 restrains nuclear translocation of nuclear factor-κB

NF-κB activity is consistent with its nuclear translocation. A variety of extracellular stimuli alter gene expression in cells via pathways that involve nuclear translocation of the transcription factor NF-κB. GC-1 showed the effects on inducing apoptosis and cytotoxic activity, which might modulate this process. When HL-60 cells were exposed to increasing concentrations of GC-1 (1–40 μmol/l) that was added into the culture medium 30 min before TNF-α (50 ng/ml), TNF-α (50 ng/ml) plus 0.1% DMSO and the solvent control (0.1% DMSO) for 24 h, there was greater nuclear translocation of NF-κB in cells treated with 50 ng/ml TNF-α than those treated with GC-1 plus TNF-α (Fig. 5). NF-κB p65 protein in cytoplastic extracts gradually accumulated upon increasing GC-1 concentration, which was parallel with the disappearance of NF-κB p65 protein in nuclei extracts. The maximal effect of GC-1 on blocking nuclear translocation of NF-κB was observed at a dose higher than 40 μmol/l. The effect of GC-1 on protein p65 suggested the blocking of NF-κB translocation, as well as its action on the inhibition of NF-κB activity.

### Compound GC-1 inhibits IκBα degradation

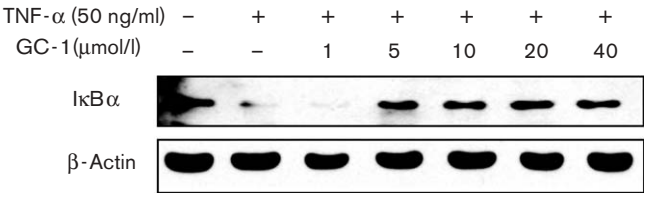
Activation of NF-κB is controlled by inhibitory proteins, IκB, including IκBα, IκBβ and IκBα. Under normal conditions, NF-κB is found in the cytoplasm in an inactive form bound to its inhibitor IκB. After an inflammatory stimulus, IκB is phosphorylated by IκB kinases, the phosphorylated IκB is then degraded, and NF-κB is released and translocated to the nucleus to induce transcription of genes related to the early onset of the inflammatory response and cancer development [13,15,16]. To examine whether IκBα has a role in the reduction of nuclear NF-κB levels by GC-1, the expression

**Fig. 5**




Effect of GC-1 on nuclear translocation of nuclear factor (NF)-κB. Cytoplasmic and nuclear proteins were extracted from HL-60 cells treated with GC-1 (1–40 μmol/l) that was added into the culture medium 30 min before tumor necrosis factor (TNF)-α (50 ng/ml) and maintained for another 24 h, and immunoblotted for NF-κB and β-actin. TNF-α (50 ng/ml) plus 0.1% dimethylsulfoxide (DMSO) and the solvent (0.1% DMSO) solely were used as controls. (a and b) Western blot image of NF-κB p65 protein in cytoplasmic extracts (p65-C) and nuclear (p65-N), respectively. (c) Western blot image of β-actin in cyoplasmic extracts.

**Fig. 6**




Effect of GC-1 on IκBα degradation. Cytoplasmic protein and Western blot analysis were performed as indicated in Fig. 4. Rabbit polyclonal antibody against IκBα was used to determine the expression of IκBα.

of IκBα in cytoplastic protein extracts from HL-60 cell line exposed to different concentrations of GC-1 with TNF-α (50 ng/ml) for 24 h was determined by Western blotting using an antibody that specifically recognizes the *N*-terminus of IκBα. TNF-α is known to enhance the IκBα degradation, resulting in NF-κB activation. The expression of IκBα in cytoplastic extracts was obviously decreasing after the treatment by TNF-α. Figure 6 shows a detectable increase in IκBα in cytoplastic extracts when cells were treated with TNF-α plus increasing concentration of GC-1. Addition of 5 μmol/l GC-1 before TNF-α almost blocked all IκBα degradation stimulated by TNF-α in cytoplasm.

## Discussion

In order to discover new anticancer compounds from natural products, we investigated the antiproliferative property and the possible mechanism of GC-1, a new triterpenoid saponin from the fruit of *G. chinensis Baillon*. Our results showed inhibition effect of GC-1 on several kinds of tumor cell proliferation and apoptosis induction of HL-60 cells in a dose-dependent manner, which might be due to the inactivation of NF-κB.

NF-κB is a crucial transcription factor, which shows a broad relationship with inflammatory, immune responses and cellular growth. The inhibition of NF-κB appears to be a promoting strategy for cancer therapy [17]. Many natural products acting on the NF-κB activation pathways were discovered by now, such as EGCG (Epigallocatechin gallate), a vital ingredient of tea polyphenols, cordyceps polysaccharide, norcantharidin, salicylate, soy isoflavone, genistein and curcumin [18]. Several diterpenoids, oridonin, ponicidin, xindognin A and B from *Isodon rubescens* were found to be potent inhibitors of NF-κB transcription activity as well as the expression of its downstream targets, cyclooxygenase-2 and inducible nitric oxide synthase [19]. Recently, soy isoflavone genistein was found to inactivate NF-κB both *in vivo* and *in vitro*, resulting in an increased apoptosis effect induced by chemotherapeutic agents in human cancer cells [20,21]. Interestingly, various experimental data also indicate that chemotherapeutic agents are known to induce NF-κB activity in tumor cells, resulting in lower

cell killing and drug resistance, and NF-κB constitutive activity is one of the most important reasons leading to the multidrug resistance of tumor cells [17,22]. Thus, inhibition of NF-κB resulted in increased sensitivity of tumor cells to cancer therapy [15]. GC-1 blocked NF-κB activities induced by TNF-α in a dose-dependent and time-dependent manner. Accordingly, the expression of NF-κB p65 protein in cytoplastic extracts was gradually increased upon the increase in GC-1 concentration, but decreased in nuclear extracts. Moreover, the disappearance of IκBα protein caused by TNF-α in cytoplastic extracts can be restored by addition of GC-1, resulting in an inhibition of NF-κB. It was reported that aspirin and arsenic were shown to prevent TNF-α-induced degradation of IκBα [23,24], which were consistent with our observation on GC-1. It was assumed that inactivation of NF-κB resulting from GC-1 treatment might be due to inhibition of IκBα degradation. More work concerning the effect of GC-1 on the regulation of the NF-κB activation pathway is necessary, so as to better understand the anticancer and/or anti-inflammatory properties of GC-1.

To determine whether the inhibition effect of GC-1 TNF-α-induced NF-κB activation was due to its surface active action on the cell membrane, we performed the cytotoxicity trial *in vitro* using the transfected cell line (HEK293/pNiFty-SEAP). The viability of cells treated by GC-1 at different concentrations during 24 h did not cause remarkable damage to the cells (data not shown). Therefore, the cytotoxicity of GC-1 preferably for human tumor cells should be further studied and developed.

It must be noted that the NF-κB activation pathways are relatively complicated. NF-κB exists as a homodimer or heterodimer composed of Rel family member proteins p50/105, p52/p100, p65, RelB and c-Rel, and these proteins are retained in the cytoplasm complexed with inhibitory proteins belonging to IκB family, which inhibit the translocation of NF-κB into the nucleus [10–12]. So, thorough molecular mechanisms of GC-1 that renders or regulates the complexity of oncologic and therapeutic outcomes that are controlled by NF-κB should be identified.

In conclusion, GC-1, a new triterpenoid saponin from the fruit of *G. chinensis Baillon*, takes on extensive biological activities that the saponin compounds have. We propose that the effects of GC-1 on cancer cell growth inhibition and apoptosis induction could be related to the blocking of the NF-κB signal pathway.

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