Gambogic acid mediates apoptosis as a p53 inducer through down-regulation of mdm2 in wild-type p53-expressing cancer cells

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Abstract

Gambogic acid (GA) is a natural product with potent apoptotic activity. Here, we showed that GA broadly inhibited the growth of cancer cells that expressed wild-type p53 as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazol-iumbromide assay, ^{3}H-thymidine incorporation analysis, and an *in vivo* mouse xenograft model. GA induced massive cell apoptosis as judged by Annexin V and propidium iodide dual-staining experiments. Furthermore, we found that GA partially induced cancer cell growth inhibition in a p53-dependent manner because cell survival could be restored after endogenous p53 was attenuated by p53 transcriptional repressor pifithrin-α or p53 small interfering RNA. Interestingly, GA had no influence on p53 mRNA synthesis but dramatically enhanced its protein expression. This unique observation could be accounted for by the down-regulation of mdm2 at both mRNA and protein levels. It is concluded that GA enhances p53 protein level through inhibition of mdm2 expression and thereby hampers p53 harboring tumor growth. [Mol Cancer Ther 2008;7(10):3298–305]

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Introduction

Apoptosis is a biological process that removes cancer-infected or viral-infected cells and has been considered as a potential therapeutic approach to human tumor management. Two apoptotic pathways, mitochondria-dependent apoptosis and mitochondria-independent apoptosis, have been recognized. Mitochondria-dependent apoptosis mainly involves the activation of caspase-3 via bcl-2 family members, whereas mitochondria-independent apoptosis is associated with death ligands and receptors such as Fas ligand and receptor (1).

As one of the critical tumor suppressors, wild-type p53 modulates both apoptosis pathways and functions as a classic inducer of apoptotic programs in response to various stresses including DNA damage, hypoxia, and oncogenic activation (2, 3). p53 is also known to be associated with various biological functions such as cell cycle arrest, senescence (4), metastasis (5), metabolism (6), angiogenesis (3), and autophagy (7). Increasing lines of evidence support p53 as an attractive target in tumor therapy (8). However, mutated p53 is observed in ~50% of human cancer cells. Even in wild-type p53–expressing cancer cells, it is very common that the apoptosis pathway induced by p53 is blocked, leading to uncontrolled cell proliferation (8). Under non–stressed conditions, p53 is tightly controlled by mdm2 through a regulatory feedback loop (9, 10). p53 can up-regulate mdm2 expression which in turn leads to p53 degradation (11, 12).

Gambogic acid (GA; $C_{38}H_{44}O_8$) is one of the most active components isolated from gamboge, a brownish to orange resin extracted from the *Garcinia hanburyi* tree grown in Southeast Asia (13). Traditionally, gamboge was used as a coloring material and folk medicine due to its unique color and broad spectrum of cytotoxic activities (14, 15). Although the molecular structure of GA was established in 1966 (Fig. 1; ref. 16), the biological property of GA remained ambiguous until 1990 because of the lack of suitable protocols to obtain large quantities of this compound with high purity (15, 17, 18). Now GA having purity of >98% can be achieved according to a novel extraction method reported by Drs. Q.D. You and W.Y. Liu (19, 20).

It has been shown that GA inhibited the growth of different types of cancer including hepatocarcinoma, gastric carcinoma, lung carcinoma, and breast cancer (21, 22). Additionally, GA induced cancer cell G_2-M phase arrest and repressed telomerase reverse transcriptase (23, 24). Recently, Kasibhatla et al. showed that transferrin receptor (TfR or CD71) is the target protein of GA (25). All studies reported to date show that GA is a potent anticancer agent with diverse molecular targets through

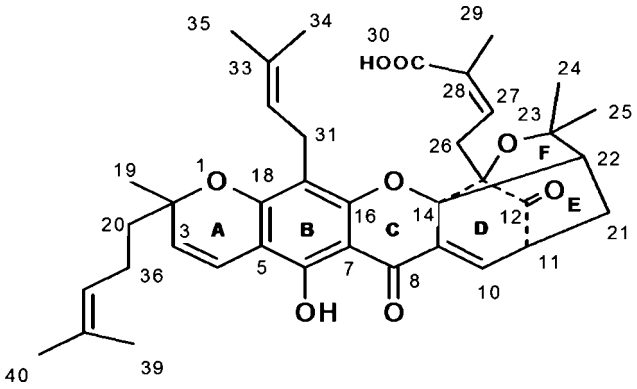

Figure 1. The molecular structure of GA and its atomic numbering scheme.

different mechanisms (26). Herein, we identify p53/mdm2 as new molecular targets which are modulated by GA to achieve its potent antitumor activity.

Materials and Methods

Reagents

The gamboge resin of *G. hanburyi* was purchased from Jiangsu Provincial Medicinal Materials Company, China. GA was isolated and purified according to the established methods (27). The purity of GA used in all experiments was 95% or higher (19). GA was solubilized in DMSO to a final stock concentration of 10 mmol/L and stored at −20°C. Doxorubicin, *N*-benzoyloxycarbonyl(*Z*)-Leu-Leu-Leu-al complex, and pifithrin-α were obtained from Sigma-Aldrich, Inc., and prepared according to the manufacturer's instructions. All drugs were diluted in the corresponding culture medium to the desired concentrations before use. DMSO was used as a solvent control.

Cell Lines and Tissue Culture

All cells adopted in this article were obtained from the cell bank of the Shanghai Institute of Biochemistry and Cell Biology, except for the human non–small cell lung H1299 cancer cell line, which was a generous gift from Dr. Caicun Zhou (Shanghai Pulmonary Hospital, Shanghai, China). All cancer cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Sijiqing Company, Ltd.) and sodium bicarbonate (2.2%, w/v), and incubated in a stable environment with 5% CO_2 at 37°C in a humidified incubator (310/Thermo, Forma Scientific, Inc.).

Mice

Male BALB/cA nude mice, 35 to 40 days old with body weights of 18 to 22 g, were supplied by Shanghai Institute of Materia Medica, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. The animals were kept at 22 ± 2°C and 55% to 65% humidity in stainless steel cages under controlled lights (12 h light/d) and were fed with standard laboratory food and water *ad libitum*. The procedures for animal care were in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals published by the NIH.

Cell Viability Assays

Cell viabilities were measured by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Roche, Ltd.) as described previously (22). Experiments were done in triplicate in a parallel manner for each concentration of GA used and the results were presented as mean ± SE. Control cells were given only culture media. After incubation for 48 h, absorbance (A) was measured at 570 nm. Survival ratio (%) was calculated using the following equation: survival ratio (%) = ($A_{\text{treatment}}$ / A_{control}) × 100%. IC_{50} was taken as the concentration that caused 50% inhibition of cell viabilities and calculated by the Logit method.

^{3}H-Thymidine Incorporation

Cells were inoculated in six-well plates to ensure 50,000 cells per well. After 24 h of incubation, serum-free medium were used for an additional 4 h. Four micromolar of GA was then added for different periods of time. At the end of each GA treatment, 1 μCi/mL of ^{3}H-thymidine was added to every well. Two hours later, growth media were decanted and cells were washed with 1 mL of ice-cold PBS. Cells were lysed with 5% trichloroacetic acid and 0.5 mol/L NaOH in the presence of 0.5% SDS. When lysis is completed, cells were counted by a liquid scintillation counter (LS8000, Beckman).

Annexin V and Propidium Iodide Dual-Staining Assay

Apoptotic cells were assayed according to a previously described method (28). Briefly, 1, 2, and 4 μmol/L of GA was exposed to HepG2 cells for 48 h. Cells were harvested, washed and resuspended with PBS buffer. Apoptotic or necrotic cells were identified by dual-staining with recombinant FITC-conjugated with Annexin V and propidium iodide (PI). The experiment was carried out according to the manufacturer's instructions (Becton Dickinson). Data acquisition and analysis were done in a Becton Dickinson FACSCalibur flow cytometer using CellQuest software.

Laser Scanning Confocal Microscopy

HepG2 cells were inoculated onto a glass coverslip in a six-well plate and cultured overnight. Cells were exposed to 4 μmol/L of GA for 0.5, 3, and 6 h. Immunofluorescent assay was carried out according to a previously described protocol (29) with monoclonal anti-p53 antibody (Ab-5, 1:100 dilution; Calbiochem) that specifically detects wild-type p53 followed by incubation with antimouse IgG-FITC (BD Biosciences) at 1:200 dilution. Cell nucleus was stained by 4′,6-diamidino-2-phenylindole (Sigma). Images were observed and captured by a Bio-Rad MRC 1024 laser scanning confocal microscope. In all cases, negative controls were treated in the same way, except that the primary antibody was replaced by a PBS buffer.

Plasmids and Transfection

The plasmids pcMV-Neo-Bam containing the complete p53 (human wt-p53) or mdm2 sequence were generous gifts from Professor Moshe Oren (The Weizmann Institute of Science, Rehovot, Israel). Mdm2 gene was subcloned into pcDNA3.1 His/HA plasmid by PCR. The mdm2 primers

were 5′-TACGGAATTCACCATGGGCAATACCAACATGTCTG-3′ (forward) and 5′-TACGCTCGAGGGGGAAATAAGTTAGCACAATC-3′ (reverse) and the annealing temperature was 55°C. p53 small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology, Inc. Both transient and siRNA transfections were done according to the manufacturer's instructions using Lipofectamine 2000 reagent (Invitrogen). After that, cells were exposed to different concentrations of GA. Lysates were prepared and Western blotting analysis was done as described below.

Reverse Transcription-PCR

Total RNA was extracted according to the manufacturer's instructions using Tripure isolation reagent (Roche, Ltd.). The primers were synthesized by Sanggon. Reverse transcription-PCR kit was purchased from TaKaRa Biotechnology, Co., Ltd. The amplified PCR products were identified by electrophoresis on a 2% agarose gel containing ethidium bromide. The sequences of PCR primers were p53, 5′-CTCCTCAGCATCTTATCCG-3′ (forward), 5′-AGCCTGGGCATCCTTG-3′ (reverse), and annealing temperature was 52°C; mdm2, 5′-CCTACTGATGGTGCTGTAAC-3′ (forward), 5′-TGATTCCTGCTGATTGACT-3′ (reverse), and the annealing temperature was 50°C; β-actin, 5′-CTGTCCCTGTATGCCTCTG-3′ (forward), 5′-ATGTCACGCACGATTTCC-3′ (reverse).

Western Blotting Assay

Western blotting was done as previously reported (23) with anti-p53 (Ab-6) monoclonal (Calbiochem), anti-CD71, and β-actin monoclonal antibodies (Santa Cruz Biotechnology) followed by IRDye 800 conjugated with anti-mouse IgG antibody (LI-COR Biosciences) incubation and visualized by an Odyssey IR imaging system (LI-COR Biosciences).

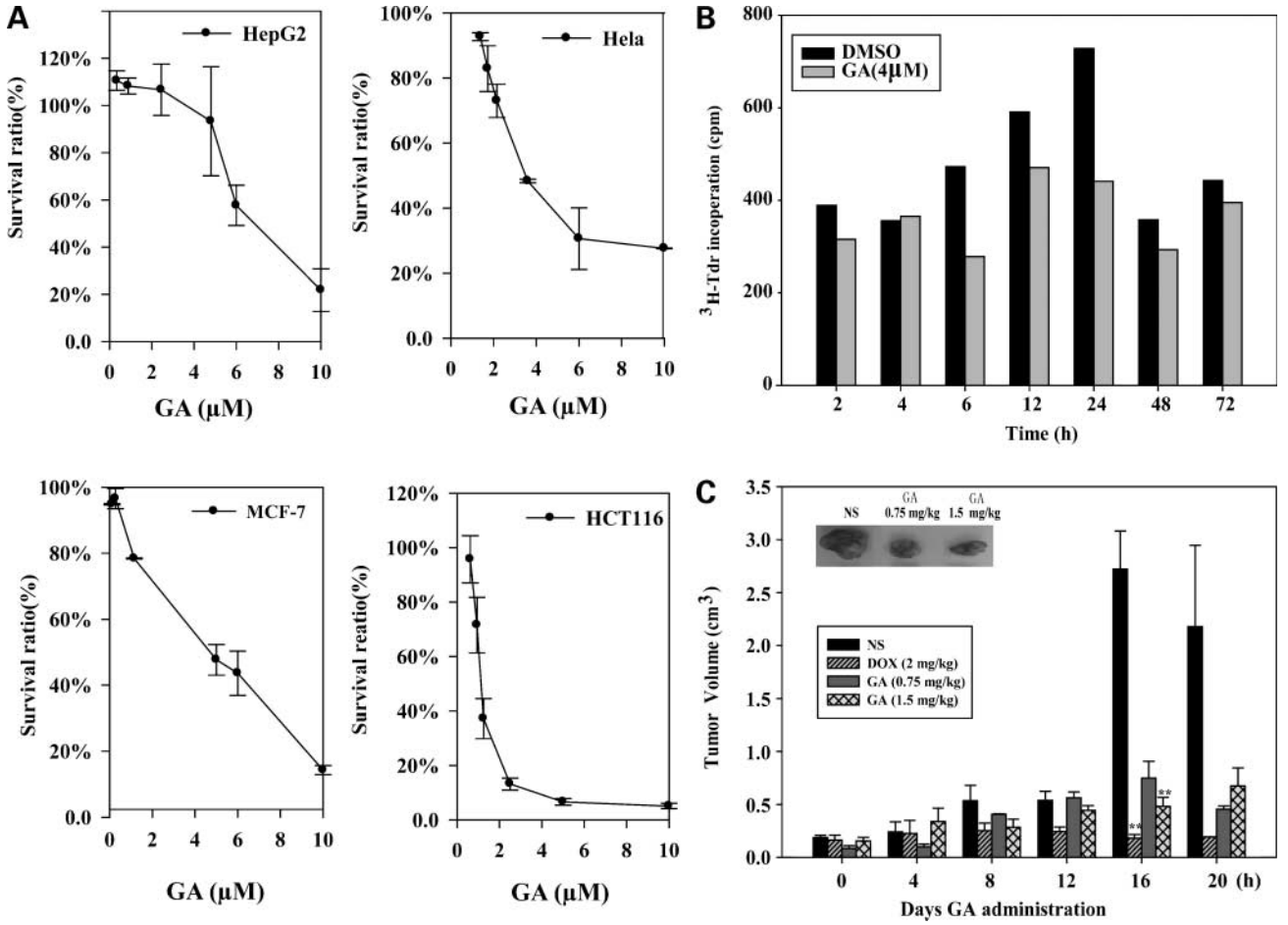

Figure 2. GA broadly inhibited cancer cell growth *in vitro* and *in vivo*. **A,** HepG2, MCF-7, HeLa, and HCT116 cells were treated with the indicated concentrations of GA for 48 h and viability was determined by MTT assay. The IC_{50} was calculated by the Logit method. *Points,* mean; *bars,* SE ($n = 3$). **B, B,** GA repressed DNA synthesis. MCF-7 cell treatment with or without 4 μmol/L of GA were added with ^{3}H-thymidine as described in Materials and Methods. DMSO was used as a negative control. Data shown were representative of three independent experiments. **C,** HepG2 tumor cells were inoculated in the right flank region of BALB/cA nude mice. Each group was treated with 2 mg/kg of doxorubicin, 1.5 and 0.75 mg/kg of GA, and 0.9% NaCl (vehicle) given correspondingly i.p. once every 4 d. *Inset,* picture of tumors obtained from the nude mice treated with 0.75 and 1.5 mg/kg of GA and 0.9% NaCl. *, $P < 0.05$; **, $P < 0.01$. *Points,* mean; *bars,* SE ($n = 3$).

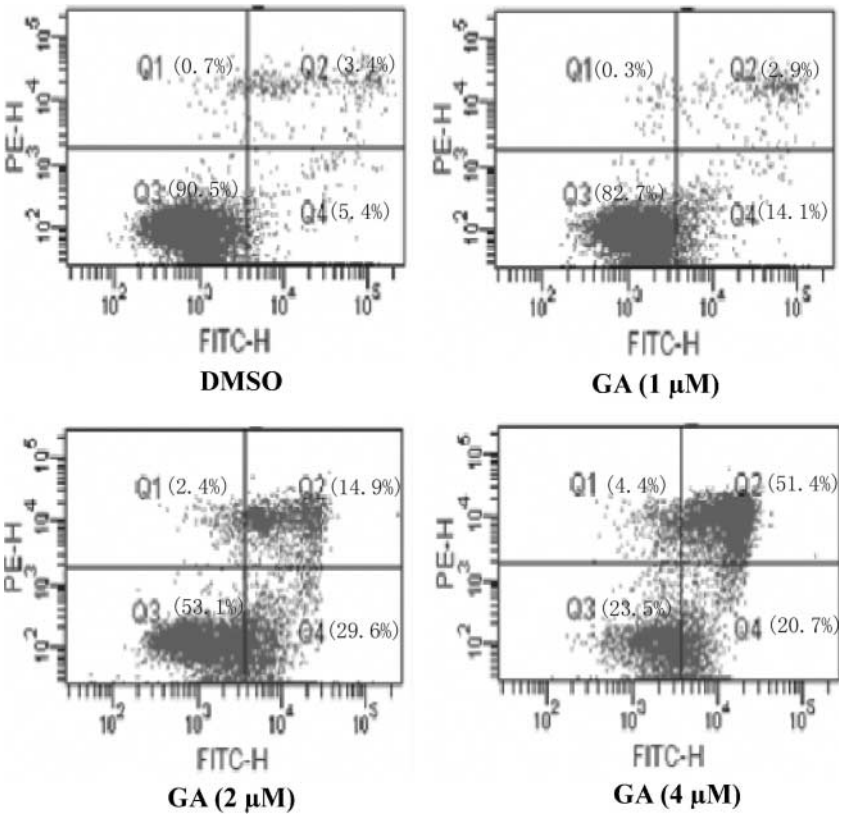

Figure 3. GA induced cell apoptosis by Annexin V-PI dual-staining assay. HepG2 cells were exposed to 1, 2, and 4 μmol/L of GA for 48 h. According to the manual of the Annexin V-FITC apoptosis detection kit, apoptotic cells were identified by dual-staining with recombinant FITC-conjugated Annexin V-PI. *Q1–4,* cell debris, cells in the end stage of apoptosis, surviving cells, and apoptotic cells, respectively.

Immunoprecipitation

MCF-7 cells in 100-mm dishes were treated with GA for 6 h before harvest. Cells were lysed in the ice-cold lysis buffer as described previously (23). The lysate was centrifuged and the supernatant was used directly for immunoprecipitation. Proteins were precipitated from the supernatant by the addition of p53 (Ab-5) antibody for 1 h at 4°C. Beads (20 μL/group; Santa Cruz Biotechnology) were then added for a further incubation at 4°C overnight. The bead-antigen complexes were collected by centrifugation at 5,000 × *g* for 10 min. Then, samples were denatured at 95°C for 8 min. Samples were stored at −20°C for Western blotting assay. Mdm2 (Ab-3) antibody (Calbiochem) was used and the secondary antibody and band detection were operated as described above.

Animal Studies

HepG2 cells (5×10^6) were implanted subcutaneously in the right flank region of BALB/cA nude mice following protocols described in the literature (30). Briefly, when tumor size increased to 100 to 200 mm^3, the mice were randomly divided into four groups consisting of equal numbers (six) of animals. Each group was treated with 2 mg/kg of doxorubicin, 0.75 and 1.5 mg/kg of GA, and 0.9% NaCl (vehicle), respectively. The drugs or equal volumes of the vehicle were administered by i.p. injection once every 4 days, whereas tumor size was measured and converted to tumor volume using the formula: $a \times b \times b / 2$, where a and b refer to the length (a) and width (b) of the solid tumor ($a \geq b$; ref. 31).

Statistical Analysis

All data were expressed as mean ± SD or mean ± SE as indicated (32), and statistically compared by one-way ANOVA. $P < 0.05$ was taken as statistically significant and $P < 0.01$ was considered as dramatically significant. All the experiments in this study were repeated at least twice.

Results

GA Broadly Inhibited Cancer Cell Growth *In vitro* and *In vivo*

Cancer cells of different origins (HepG2, MCF-7, HeLa, and HCT116 harboring p53) were used to test the effect of GA on the cells containing endogenous p53 expression. MTT assay revealed that GA inhibited these cell growth in a concentration-dependent (Fig. 2A) and time-dependent manner (data not shown). The IC_{50} values of HCT116, HeLa, HepG2, and MCF-7 cells were 1.24, 3.53, 3.80, and 4.11 μmol/L, respectively. ^{3}H-thymidine incorporation assay further confirmed that GA inhibited DNA synthesis in a time-dependent manner (Fig. 2B). Four micromolar of GA significantly blocked DNA synthesis at 6 hours ($P < 0.05$), exerted its maximum effect at 24 hours ($P < 0.01$), and sustained activity up to 48 hours ($P < 0.05$). Hence, we decided to use the 6-hour reaction time in

the following experiments as the minimum time required for GA to take effect.

Furthermore, a mouse xenograft model was used to determine the effect of GA on tumor growth. As shown in Fig. 2C, 1.5 mg/kg of GA did not substantially inhibit tumor growth until 16 days of treatment, similar to that of 2 mg/kg of doxorubicin. A reduction of ~67.6% in HepG2 tumor weight was observed in mice treated with GA at 1.5 mg/kg (data not shown).

GA Induced Cancer Cell Apoptosis

Our data showed that GA significantly inhibited cell growth *in vitro* and *in vivo*. Annexin V-PI dual-staining assay was then done to ensure whether this growth inhibition resulted from cell apoptosis. Cells undergoing apoptosis would stain positive for Annexin V-FITC and negative for PI (quadrant 4, Q4). Cells which stained positive for both Annexin V-FITC and PI (Q2) are either at the end stage of apoptosis or undergoing necrosis, and those which stained negative for both Annexin V-FITC and PI (Q3) were alive or undergoing undetectable apoptosis. In contrast, cell debris stained only for PI (Q1). As shown in Fig. 3, 1, 2, and 4 μmol/L of GA induced ~17%, 44.5%, and 72.1% (Q2 + Q4) cell apoptosis, respectively, whereas fewer apoptotic cells (8.8%) were observed in vehicle, confirming that cell growth inhibition mediated by GA was due to increased cell apoptosis.

GA Up-regulated p53 at a Posttranscriptional Level

Results of reverse transcription-PCR showed that no induction of p53 gene was observed even in cells treated with toxic concentrations of GA (8 μmol/L), as shown in Fig. 4A. Instead, up-regulation of p53 protein was readily detected in HepG2, MCF-7, HeLa, and HCT116 cells after 6 hours of GA treatment (Fig. 4B). Essentially identical results were obtained in H1299 cells (p53 null) that were transfected with exogenous p53. The expression of p53 protein was enhanced approximately 10-fold in the presence of 4 or 8 μmol/L of GA compared with the vehicle (Fig. 4C). Doxorubicin was used as a positive control which had been shown as a p53 enhancer. Immunofluorescent assay confirmed the results from Western blotting experiments. Compared with untreated cells, GA treatment increased the amount of p53, especially

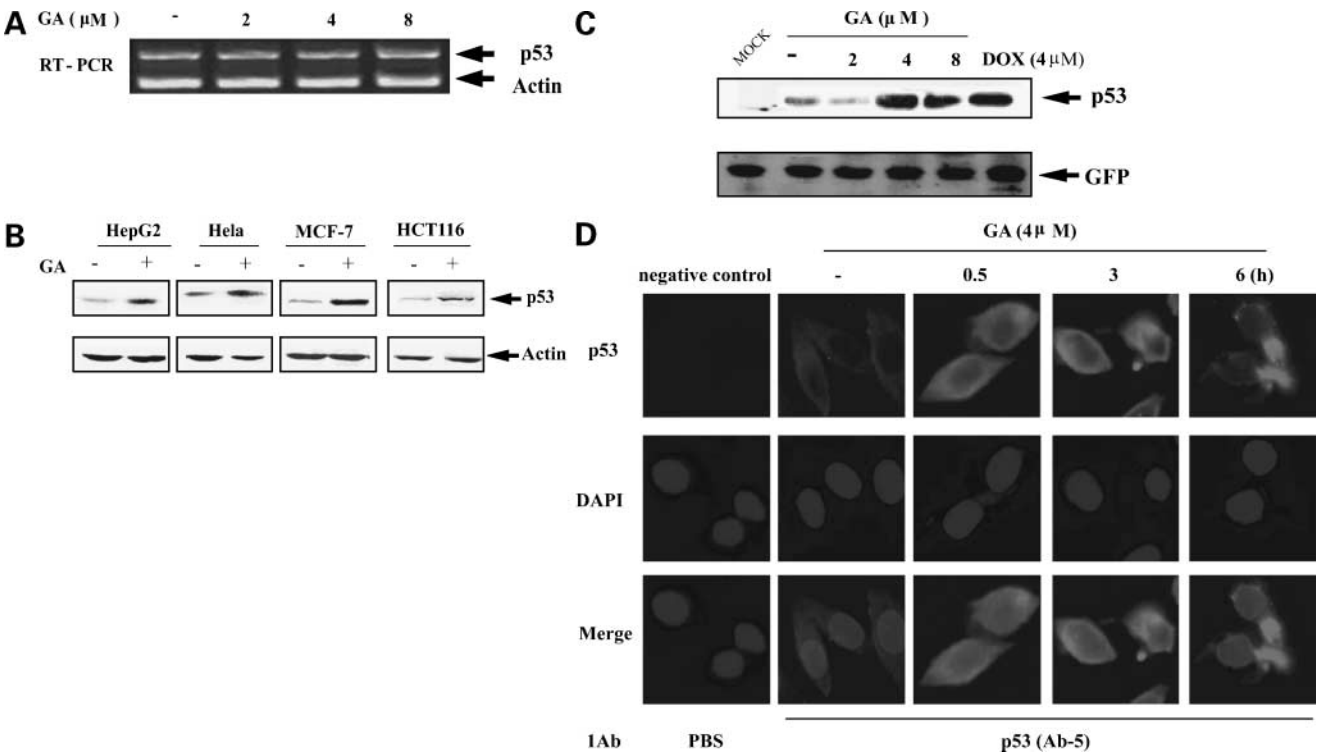

Figure 4. GA affected p53 at the posttranscriptional level. **A,** HepG2 cells were administered with 2, 4, and 8 μmol/L of GA. Total RNA was then extracted with Tripure isolation reagent and reverse transcription-PCR was done according to the manufacturer's instructions. β-Actin was used as the loading control. **B,** HepG2, MCF-7, HeLa, and HCT116 cells were treated with or without GA (4 μmol/L) and the expression of endogenous p53 protein was observed by Western blotting. **C,** H1299 cells were overexpressed with exogenous p53 and treated with 2, 4, and 8 μmol/L of GA. Cell lysates were immunoblotted with anti-p53 (Ab-6) and β-actin monoclonal antibody followed by IRDye 800 conjugated with antimouse IgG antibody. The membrane was visualized by Odyssey IR imaging system. Green fluorescent protein was used as the measurement of transfection efficiency and doxorubicin was used as the positive control. **D,** HepG2 cells were inoculated onto glass coverslips in six-well plates overnight and then exposed to 4 μmol/L of GA for 0.5, 3, and 6 h. Cells were incubated with monoclonal anti-p53 antibody (Ab-5) followed by antimouse IgG-FITC. The cell nucleus was stained with 4′,6-diamidino-2-phenylindole. Images were observed and captured using Bio-Rad MRC 1024 laser scanning confocal microscope. No primary antibody was used in the negative control.

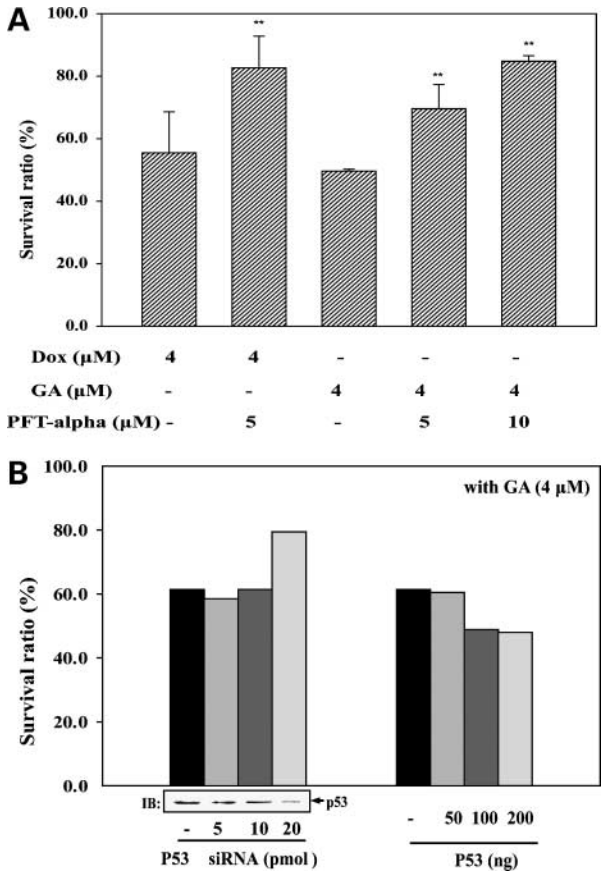

Figure 5. GA induced apoptosis partially through up-regulation of p53. **A,** HepG2 cancer cells were cotreated with pifithrin-α and GA or treated with GA alone for 6 h and cell viabilities were determined by MTT assay. Doxorubicin was used here as the positive control. *Columns,* mean; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$. **B,** HepG2 cells transfected with different concentrations of p53 siRNA (*left*) and p53-null H1299 (*right*) transfected with different amounts of p53 were treated with 4 μmol/L of GA for 6 h, and then MTT assay was done and the survival ratio was calculated. Data shown are representative of three independent experiments.

in the cytoplasm, as shown in Fig. 4D. All of these data strongly suggested a posttranscriptional regulation of p53 by GA.

GA Induced Apoptosis Partially through Up-regulation of p53

Pifithrin-α, a p53 transactivation blocker, and doxorubicin were used to assess the effect of GA on cell survival (33). As shown in Fig. 5A, cell growth inhibition induced by GA was as similar to that of doxorubicin. However, this inhibitory effect could be restored by pifithrin-α. Cell survival ratio was significantly increased after 6 hours of treatment with 5 or 10 μmol/L of pifithrin-α, compared with that treated with GA alone.

To confirm this finding, p53 was knocked down by p53 siRNA transfection into HepG2 cells. Consistent with pifithrin-α experiments, cell survival was significantly restored after p53 knockdown, whereas obvious growth inhibition was detected in the cells overexpressing p53 (Fig. 5B). All these results showed that p53 played an important role in GA-triggered apoptosis.

GA Induced p53 Up-regulation via Down-regulation of mdm2 Transcription

Because mdm2 is assumed to target p53 for proteasome-mediated protein degradation, we examined the involvement of mdm2 in GA mediated p53 up-regulation. Reverse transcription-PCR and Western blotting results showed that GA repressed mdm2 transcription and protein expression significantly (Fig. 6A). Furthermore, as shown in Fig. 6B, the p53-mdm2 interaction was decreased after GA treatment in the presence of *N*-benzoyloxycarbonyl(*Z*)-Leu-Leu-Leu-al complex, an inhibitor of proteasomal proteolysis used to prevent the degradation of the p53-mdm2 complex.

Discussion

GA has been shown to be a potent apoptosis inducer that significantly inhibits the growth of a variety of tumors. In this study, we showed that GA substantially inhibits p53 harboring cancer cell growth both *in vitro* and *in vivo*. This effect was a result of cell apoptosis induced by GA. We also found that GA-induced cell apoptosis is related to p53 expression. When p53 was knocked down by siRNA or blocked by pifithrin-α, cell growth was significantly increased. Surprisingly, GA enhanced p53 at the protein level only, without any observable effect on its mRNA level. As

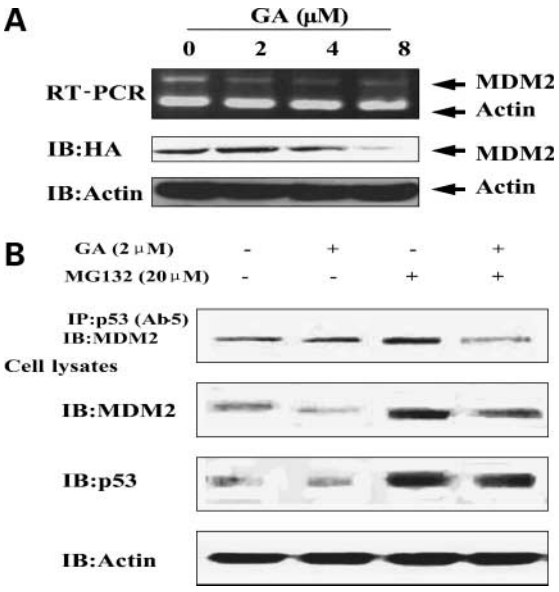

Figure 6. GA induced p53 up-regulation through down-regulation of mdm2 transcription. **A,** HepG2 cells were treated with indicated concentrations of GA for 6 h. Cells were harvested and RNA was extracted as described in the text. Cell lysates were immunoblotted by anti-MDM2 (Ab-3) antibody. **B,** MCF-7 cells were treated with vehicle or 4 μmol/L of GA in the presence or absence of 20 μmol/L of *N*-benzoyloxycarbonyl(*Z*)-Leu-Leu-Leu-al complex for 6 h. Cell lysates of MCF-7 were divided into two portions. One part was used for immunoprecipitation with anti-p53 and immunoblotted with anti-mdm2, the other part was used for Western blotting analysis. Immunoprecipitation was done as described in Materials and Methods.

mdm2 is the critical protein that regulates p53 degradation, we examined the effect of GA on mdm2 and found that GA decreased mdm2 at both the mRNA and protein levels. Immunoprecipitation assay further indicated that GA inhibited p53 binding to mdm2 after cotreatment with an *N*-benzoyloxycarbonyl(*Z*)-Leu-Leu-Leu-al complex, a proteasome inhibitor. Therefore, tumor growth inhibition induced by GA is achieved via regulation of mdm2/p53.

Our data strongly support that GA up-regulated p53 protein expression through down-regulation of mdm2 and thereby triggered the occurrence of apoptosis, but we do not think that mdm2/p53 is the only target of GA. In a xenograft tumor study, although tumor cells expressing wild-type p53 were hypersensitive to GA, it turns out that GA has a weaker effect on p53-null tumor growth (data not shown). This result suggests the presence of other targets aside from p53. In addition, a recent study from Kasibhatla et al. (25) and Pandey et al. (34) showed that transferrin receptor (CD71), an iron-transport protein that was highly expressed in tumor cell membrane, was the ligand of GA, leading to mitochondria-dependent and mitochondria-independent apoptosis pathway activation by GA. Therefore, we examine the involvement of CD71 using siRNA to knock down CD71. However, our data suggested that GA-induced up-regulation of p53 protein is independent of CD71 (data not shown). Moreover, other GA targets have been reported including bcl-2 (30), telomerase hTERT (35), survivin (36), topoisomerase IIα (37), and so on. All these studies show that GA has been a multimechanism anticancer agent with low toxicity in normal cells (26). In this article, we report p53/mdm2 as one of the mechanisms through which GA inhibits tumor cell growth. To figure out the other mechanisms, the effects of GA on p53-null tumors or tumors with p53 mutation are still in progress. These studies will help us illustrate other potential therapeutic targets. We will also examine the mdm2 expression in tumors to detect whether GA's effect is positively associated with down-regulation of mdm2 and test the selectivity of GA on these tumors.

In terms of antitumor effect, our previous studies showed that a high dosage of GA effectively inhibited tumor growth with less toxic effects compared with doxorubicin, an anticancer drug now used in current clinical cancer treatment. This is very important for clinical application in the tumor patients because most therapeutic failures are due to the intolerable toxic side effects from antitumor drugs. In China, GA is currently under phase II clinical trials. Therefore, we believe that GA could be a very promising antitumor agent in the near future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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