Cancer Letters 284 (2009) 102–112

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Gambogic acid down-regulates MDM2 oncogene and induces p21$^{Waf1/CIP1}$ expression independent of p53

Jing-Jing Rong, Rong Hu [1], Qi Qi [1], Hong-Yan Gu, Qing Zhao, Jia Wang, Rong Mu, Qi-Dong You, Qing-Long Guo *

Jiangsu Key Laboratory of Carcinogenesis and Intervention, China Pharmaceutical University, Nanjing 210009, China

ARTICLE INFO

Article history:
Received 28 February 2009
Received in revised form 8 April 2009
Accepted 9 April 2009

Keywords:
Gambogic acid
Anti-tumor
MDM2
p53
p21$^{Waf1/CIP1}$

ABSTRACT

Gambogic acid (GA), the natural compound extracted from gamboges, has recently been established as a potent anti-tumor agent. Although it was proved that GA enhances p53 protein level through inhibition of MDM2 in p53 wild-type cancer cells, the mechanisms of MDM2 inhibition especially with the absence of p53 are not fully understood. Herein we further studied the MDM2 regulation by GA and propose novel explanations of its unrecognized mechanism. Regardless of p53 status, GA reduced MDM2 expression in a concentration- and time-dependent manner. Moreover, the inhibitory effects were exhibited at both transcriptional and posttranslational levels. We found that P1 and P2 promoter of MDM2 were both responsive to GA, resulting in decreased *Mdm2* RNA level. At the post-translational level, GA promoted the autoubiquitination of MDM2, followed by proteasome-mediated degradation. Additionally, GA increased p21$^{Waf1/CIP1}$ expression in p53 null cancer cells, which was associated with GA-mediated impairing of the interaction between MDM2 and p21$^{Waf1/CIP1}$. Furthermore, the apoptosis, cytotoxicity and G2/M cell cycle arrest induced by GA were detected in both p53 wild-type and p53 null cancer cells. *In vivo* anti-tumor activity of GA was also confirmed in H1299 xenografts. It is concluded that GA down-regulates the MDM2 oncogene and exerts the anti-tumor activity independent of p53, and therefore provide more evidences for its therapeutic application.

1. Introduction

The MDM2 gene was originally identified by virtue of its abnormal amplification in spontaneously transformed mouse BABL/c cell line. Subsequently, the MDM2 protein was observed to bind to both mutant and wild-type p53 protein [1,2]. Overexpression of human homolog oncogene MDM (also called HDM2) was also found in variety of human cancer cells, typically in approximate one third of osteosarcomas with retained wild-type p53 [3]. The relationship between p53 and MDM2 has been extensively investigated. Under the transcriptional control of p53, MDM2 acts as the negative regulator of p53 in a feed-back auto-regulatory loop, inactivating the apoptotic and cell cycle arrest functions of p53 [4–6]. In the nucleus, MDM2 blocks p53-mediated transactivation; in the cytoplasm, MDM2 functions as an E3 ubiquitin ligase to degrade p53 protein [7].

Although MDM2 has been characterized as the regulator of p53, there is considerable evidence that MDM2 has p53-independent functions in cancer etiology and progression [8]. MDM2 interacts with various cellular proteins, including Rb, E2F1, p300, ARF, Numb, MTBP, and others, which involve in cell cycle control, differentiation, basal

Abbreviations: CHX, cycloheximide; CS, calf serum; CTX, cyclophosphamide; DAPI, 4′,6-diamidino-2-phenylindole; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GA, gambogic acid; KD, knockdown; MG132, N-benzoyloxycarbonyl(Z)-Leu-Leu-Leu-al complex; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazol-iumbromide; PI, propidium iodide; WT, wild-type.

* Corresponding author. Address: P.O. Box 209, 24 Tongjia Xiang, China Pharmaceutical University, Nanjing 210009, China. Tel./fax: +86 25 83271055.
E-mail address: anticancer_drug@yahoo.com.cn (Q.-L. Guo).

[1] These authors contributed equally to this work.

0304-3835/$ - see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2009.04.011

transcription or cell fate determination [9–14]. It is also demonstrated that MDM2 displays evident tumorigenesis under the $p53^{-/-}$ background *in vivo* [15]. So to inhibit the oncogenetic functions of MDM2, a potential molecular target for cancer therapy, increasing studies are searching for new therapeutic strategies and agents. Possibilities focus on inhibiting the expression of cellular MDM2, repressing MDM2-mediated ubiquitination, blocking the interactions between MDM2 and its target proteins [16–18].

Gambogic acid (GA, $C_{38}H_{44}O_8$), a compound extracted from natural resin gamboge, has been proved to own potent anti-tumor effects on different types of cancer cells [19,20]. Its *in vitro* and/or *in vivo* activities include induction of apoptosis and cell cycle arrest, inhibition of telomerase activities, antagonism of angiogenesis, and suppression of invasion and metastasis [21–25]. Moreover, transferring receptor (TfR or CD71) was considered as one target, which mediated the pro-apoptotic activation induced by GA [26]. Nevertheless, as a promising anti-tumor agent, it also performs its anticancer effect under other mechanisms which are not well detected. It has been demonstrated that GA decreased the expression of MDM2 in p53 wild-type cancer cells, resulting in the stabilization of p53 followed by stimulating p53-dependent apoptosis [27]. What remains to be addressed is whether the regulation of MDM2 by GA is p53-dependent.

The results of the present study indicate that the cellular level of MDM2 can be down-regulated by GA p53-independently at both transcriptional and posttranslational levels, which may contribute to the GA-mediated anti-tumor effects. We further established that GA elevated the expression of $p21^{Waf1/CIP1}$ independent of p53 *in vitro*, associated with GA-mediated inhibition on the binding of MDM2 to $p21^{Waf1/CIP1}$, thus leading to G2/M cell cycle arrest. These provide more mechanistic insights into GA's anti-tumor activities and render that GA could be served as a potential therapeutic candidate for treatment of cancer.

2. Materials and methods

2.1. Medicine and reagents

GA was isolated and purified according to the established methods [28]. The purity of GA used in all experiments was 95% or higher [29]. It was dissolved in PBS containing arginine to a concentration of 10 mM as the primary stock solution and stored at −20 °C. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazol-iumbromide (MTT), Cycloheximide (CHX), and N-benzoyloxycarbonyl(Z)-Leu-Leu-Leu-al complex (MG132) were purchased from Sigma (USA). All drugs were diluted in the corresponding culture medium to desired concentrations before use.

2.2. Cell culture

Human breast cancer cell MCF-7 was obtained from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, and cultured in Dulbecco's Modified Eagle's Medium (DMEM) contained 10% fetal bovine serum (FBS). Human non-small cell lung cancer H1299 cell line was a generous gift from Dr. Cai-Cun Zhou (Shanghai Pulmonary Hospital, Shanghai, China), which was cultured in RPMI-1640 medium contained 10% calf serum (CS). All culture media was added with 100 U/mL penicillin, and 100 mg/L streptomycin. All the cells were incubated in a stable environment with 5% CO_2 at 37 °C in a humidified incubator (Thermo, Forma Scientific, Inc., USA).

2.3. Plasmids and transfection

The Plasmids pCMV-Neo-Bam containing the complete *Mdm2* cDNA sequence was kindly gifted from Professor Moshe Oren (The Weiamann Institute of Science, Rehovot, Israel). The *Mdm2* cDNA insert was subcloned into pcDNA3.1 His/HA at sites of KpnI and XhoI and confirmed by sequencing as reported before [27]. The *Mdm2* P1 promoter reporter was provided by Dr. Wang Meilin (Nanjing Medical University, China). To construct the *Mdm2* P2 promoter reporter, a genetic fragment covering −599 to +220 sites of P2 promotor amplified from genome of human cancer cells was cloned into pGL3-basic plasmid as usual cloning protocol. And the plasmid pcDNA3-His-*Ubi* for expression of human ubiquitin was provided by Professor Chang Zhijie (Tsinghua University, China). The p53 siRNA was purchased from Santa Cruz Biotechnology, Inc. Both transient plasmid and siRNA transfections were performed according to the manuals of Lipofectamine™ 2000 regent (Invitrogen, Carlsbad, CA).

2.4. Reverse transcription PCR

Total RNA was extracted using the Trizol reagent from Invitrogen, quantified by UV spectrophotometry, and used to create cDNA with the RT-PCR kit from Takara (Dalian, China). The amplification program included an initial denaturation step at 94 °C for 3 min, followed by denaturation at 94 °C for 30 s, annealing for 20 s at 55 °C and extension at 72 °C for 45 s, for 30 cycles. The primers for cDNA fragment amplification were: *Mdm2*, 5′CTTGATGCTGGTGTAAGT3′ (forward), and 5′GTTGATGGCTGAGAATAG3′ (reverse); $p21^{Waf1/CIP1}$, 5′CCCGTGAGCGATGGAACT3′ (forward), and 5′CGAGGCACAAGGGTACAAGA3′ (reverse); beta-*Actin*, 5′CTGTCCCTGTATGCCTCTG3′ (forward), and 5′ATGTCACGCACGATTTCC3′ (reverse).

2.5. Luciferase reporter assay

Cells were cotransfected with *Mdm2* P1/P2 promoter vectors with Renilla luciferase reporter (as internal control) for 40 h followed by incubation with various concentrations of GA for 6 h. The luciferase activity of cell lysate was determined with the Dual-Luciferase Reporter kit (Beyotime, China) according to the provided protocol. Luciferase signals were collected by Dual-Luciferase Assay system (Thermo, USA) and all assays were repeated at least three times.

2.6. Western blotting

Protein extraction from cell cultures or tumor homogenates was performed as standard protocols. For western

blotting assay, equal amounts of total cellular protein (60 μg) were denatured in 2× sample buffer and subjected to SDS–PAGE. The separated proteins were transferred to nitrocellulose membranes followed by blocking with 5% nonfat milk powder (w/v) in TBS (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature. Then membranes were, respectively, incubated with anti-p53 (Ab-6) monoclonal antibody (Calbiochem, CA), anti-MDM2 antibodies (Santa Cruz, CA), and anti-p21 (Cell Signaling, CA) followed by IRDye™ 800 conjugated with anti-mouse or anti-rabbit IgG antibody (LI-COR Biosciences, Nebraska, USA) incubation and visualized by an Odyssey infrared imaging system (LI-COR Biosciences).

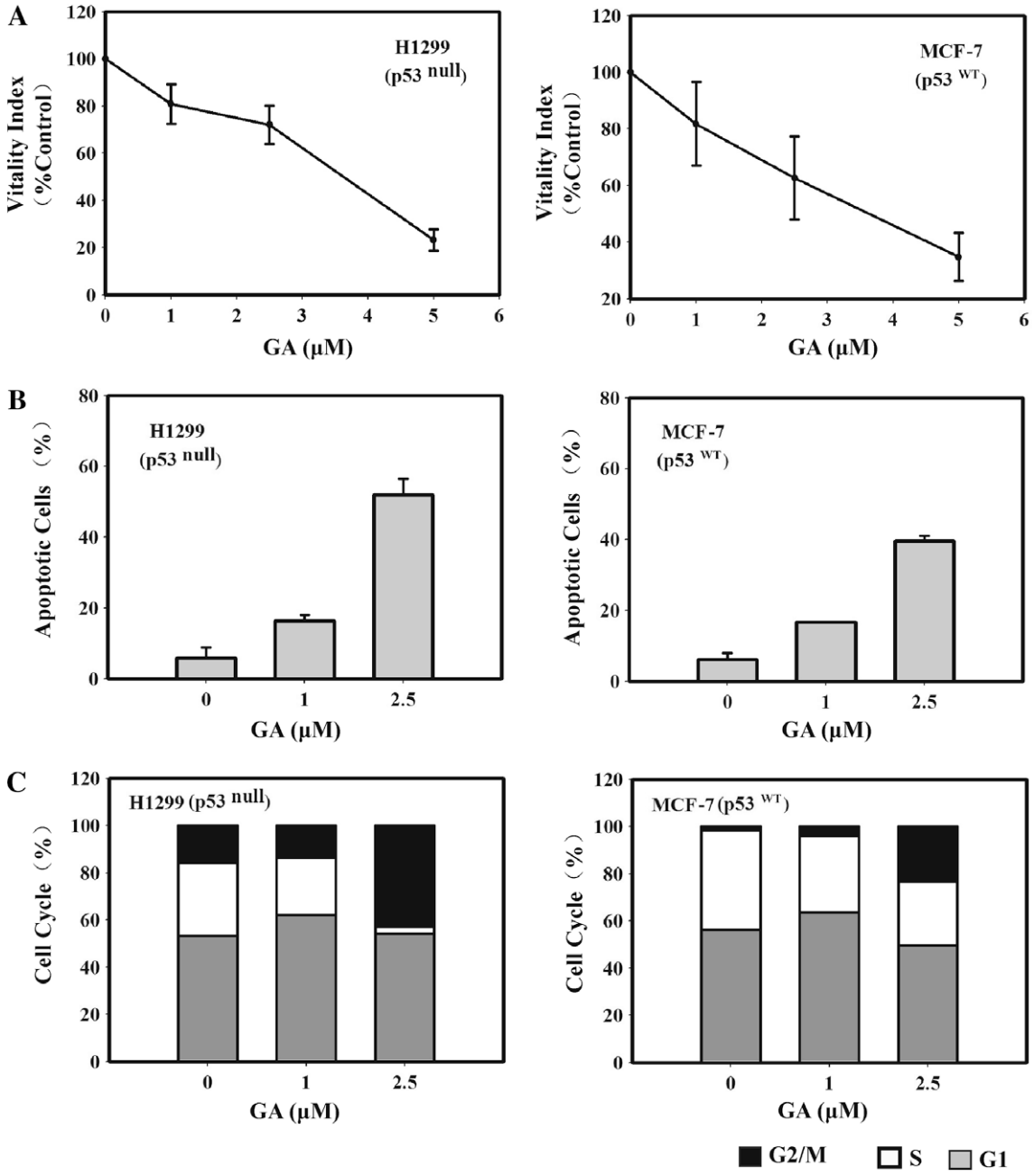

Fig. 1. GA induces apoptosis and cell cycle arrest regardless of p53 status. (A) GA inhibits cell growth independent of p53. H1299 cells (p53 null) and MCF-7 cells (p53 WT) were treated with various concentrations of GA as indicated for 24 h and cell viability was determined by MTT assay. The IC50 was calculated by Logit method. (B) GA induces apoptosis independent of p53. H1299 cells and MCF-7 cells were treated as described above. 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 and PI. Bars, ±SD. (C) GA induces cell cycle arrest in G2/M phase independent of p53. H1299 and MCF-7 cells treated with GA as above for 24 h. Cell cycle distribution was determined by PI staining. The cell population in each cell cycle phase is the mean percentage of the total events counted. All assays were done in triplicate.

2.7. Immunofluorescence

Cells were inoculated onto glass coverslips in a 6-well plate and cultured overnight. Then they were exposed to diverse concentrations of GA for 6 h. Immunofluorescent assay was carried out according to the protocol described previously [30] with anti-MDM2 antibody followed by incubation with anti-rabbit IgG-FITC (BD Biosciences, San Diego, CA) at 1:200 dilution. Cell nucleus was stained by 4′, 6′-Diamidino-2-phenylindole (DAPI, Sigma). Images were observed and captured by fluorescence microscope (Olympus, JP).

2.8. Immunoprecipitation (IP)

Cells were treated with diverse concentrations of GA for 6 h before harvest in all IP assays. For ubiquitination detection, H1299 cells were cotransfected with pcDNA3-His-*Ubi* and pCMV-Neo-Bam-*Mdm2* for 40 h, followed by incubation with 25 mM MG132 for 8 h. The cell lysate was centrifuged and the supernatant was used directly for immunoprecipitation. Proteins were precipitated from the supernatant by addition of anti-MDM2 antibody for 4 h at 4 °C. Protein A/G plus-agarose beads (20 μL per group) (Santa Cruz) were then added for a further incubation at 4 °C overnight. The beads-antigen complexes were collected by centrifugation at 5000×*g* for 10 min. Anti-p21 or anti-ubiquitin antibody was used as secondary antibody and Western blotting detection was operated as described above.

2.9. Assays for cell viability, apoptosis, and cell cycle distribution

Cells viabilities were measured by a colorimetric assay using MTT as described previously [20]. Triplicate experiments were performed in a parallel manner for each concentration of GA used and the results were presented as

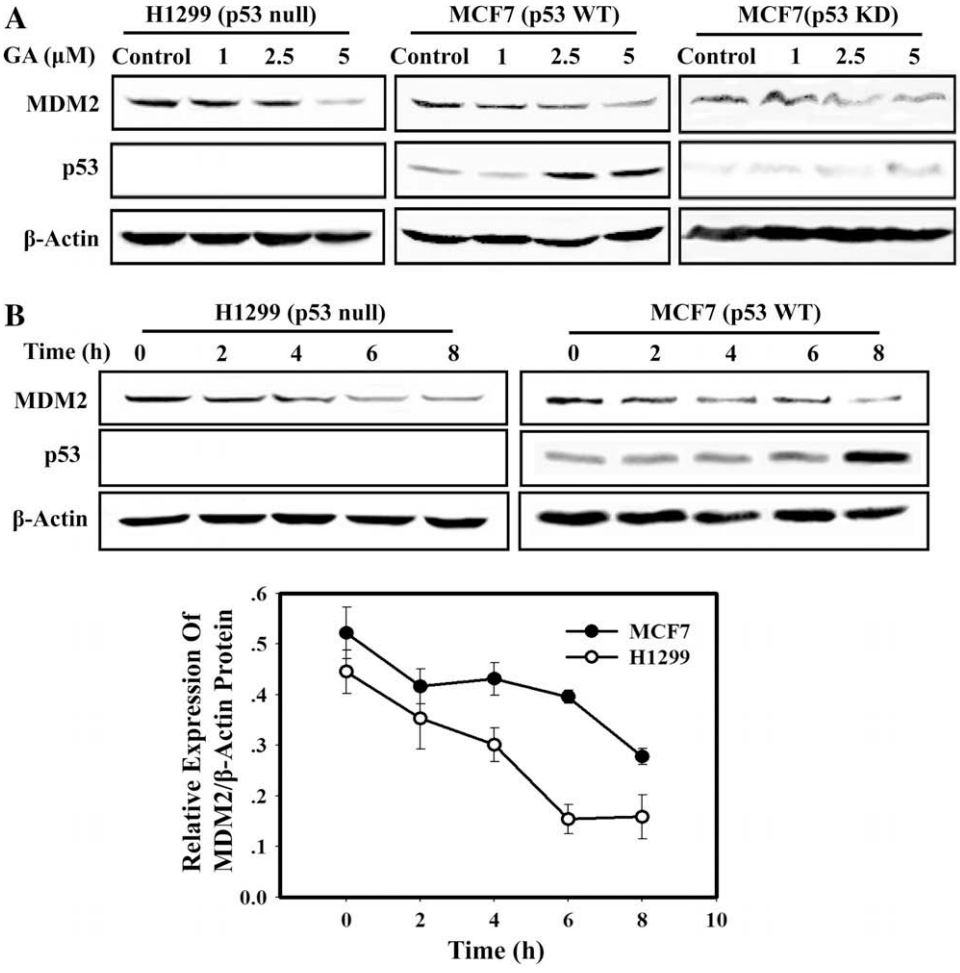

Fig. 2. GA inhibits MDM2 expression independent of p53. (A) MDM2 protein levels were decreased by GA concentration-dependently. H1299 cells (p53 null), MCF-7 (p53 WT) and MCF-7 cells (p53 KD) were treated with various concentrations (0, 1, 2.5, and 5 μM) of GA for 6 h. Cells were lysed, normalized for protein sample, and subjected to western blotting assay with anti-MDM2 antibody. Cellular p53 was knocked down in MCF-7 cells, named with MCF-7 (KD), via transient transfection with p53 siRNA for 40 h, followed by GA treatment for another 6 h. (B) GA down-regulates MDM2 expressing in a time-dependent manner. H1299 and MCF-7 cells were treated with 5 μM GA for various hours as indicated, and protein were extracted from cell cultures. Protein levels of MDM2 and p53 were detected by western blotting.

means ± SD. Vitality Index (%) was calculated using the following equation: Vitality Index (%) = (A treatment/A control) × 100%. Apoptotic cells were assayed according to the method described in a previous report [31]. Apoptotic or necrotic cells were identified by dual staining with recombinant fluorescein isothiocyanate (FITC)-conjugated with Annexin-V and propidium iodide (PI) (Sigma). The experiment was carried out according to manufacturer's instructions (Becton Dickinson, USA). For cell cycle distribution assay, cells were trypsinized, washed with PBS, and fixed in 1.5 mL 95% ethanol at 4 °C overnight followed by incubation with RNase and staining by propidium iodide (PI). Data acquisition was performed with a Becton Dickinson FACS Calibur flow cytometer.

2.10. Xenograft model

Male BALB/cA nude mice, 35–40 days old with body weight of 18–22 g were supplied by Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Cultured H1299 cells were washed with and resuspended in serum-free medium. Portions of the suspension (5×10^6 cells in 0.2 mL/mouse) were then injected into the right flank region of each mouse. When tumor size increased to 100–200 mm^3, the mice were randomly divided into five groups, with six mice each. Each group was treated with 20 mg/kg Cyclophosphamide (CTX), 2, 4, and 8 mg/kg GA, and 0.9% NaCl (vehicle), respectively. The drugs or equal volumes of the vehicle were administered by tail veil injection (i.v.) once every 2 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 ($a \geqslant b$). Relative tumor volume was calculated as V_t/V_0 (V_t, tumor volume at n days after treatment; V_0, tumor volume at initiation).

3. Results

3.1. GA induces apoptosis and cell cycle arrest regardless of p53 status

Our previous study has revealed that GA induced apoptosis and growth inhibition in a panel of cells expressing wild-type p53 [27], and here we investigated whether GA promoted apoptosis in cells not expressing p53. H1299 cells (p53 null), accompanied with MCF-7 cells (p53 WT) were employed to test the cytotoxic and proapoptotic effects of GA. Results of the MTT uptake method showed that GA exhibited cytotoxic effect in a concentration-dependent manner, with IC_{50} value of 3.5 μM in H1299 cells similar to that obtained in MCF-7 cells (Fig. 1A). According to the results of AnnexinV-PI dual staining, GA concentration dependently promoted the apoptosis in H1299 cells from 5% to 55%, and from 6% to 40% in MCF-7 cells (Fig. 1B). Little difference was observed between the sensitivity of H1299 cells (p53 null) and MCF-7 cells (p53 WT) when treated with GA.

In addition, the cell cycle distribution of MCF-7 and H1299 cells were analyzed by PI staining. As shown in Fig. 1C, in both MCF-7 (p53 WT) and H1299 (p53 null) cells, the population of G2/M phase cells was increased by over 20% after the treatment of GA. These results together indicated that GA exerts apoptosis and cell cycle arrest inducing effects regardless of the p53 status.

3.2. GA inhibits MDM2 expression independent of p53

We have reported that GA stimulates the activation of p53 via suppressing the negative regulator MDM2 in cells expressing wild-type p53; furthermore, whether GA-mediated the inhibitory effect of MDM2 occurs p53-independently was tested. To study the direct effect of GA on MDM2 regulation, the endogenous expression of p53 in MCF-7 cells was knocked down by transient transfecting with p53 siRNA. H1299 (p53 null), MCF-7 (p53 WT), and MCF-7 (p53 KD) cells were, respectively, treated with different doses of GA for 6 h, and subjected to western blotting with anti-MDM2 antibody. As shown in Fig. 2A, there was a striking concentration-dependent decrease in the amount of MDM2 protein, regardless of p53 status. Similar results were observed time-dependently, when cells were treated with 5 μM GA for various time (Fig. 2B). As early as 6 h after treatment, GA caused 2-fold decrease of MDM2 levels in H1299 cells, compared with control. These results consist with our previous reports that MDM2 oncoprotein could be down-regulated by

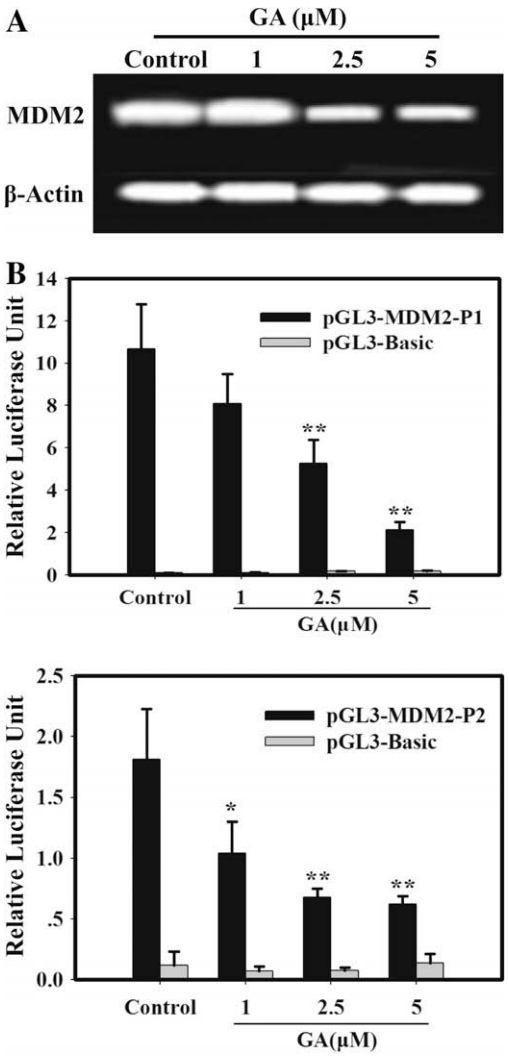

Fig. 3. GA down-regulates MDM2 expression at transcriptional level. (A) MDM2 RNA level was decreased by GA. H1299 cells were treated with various concentrations of GA for 6 h followed by extraction of total RNA. cDNA fragments of *Mdm2* was amplified by RT-PCR, using the primers indicated in material. (B) GA down-regulates MDM2 expression at transcriptional level via P1/P2 promoters. H1299 cells were cotransfected with the MDM2 reporter pGL3-*Mdm2*-P1 or pGL3-*Mdm2*-P2 and corresponding empty vector (pGL3-Basic) for 40 h followed by incubation with various concentrations of GA for an additional 6 h, before luciferase activities were quantified. Data were shown as means ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$. Each group was repeated for at least three times.

GA, and importantly, these data are the first to show this inhibitory effect is independent of p53.

3.3. GA down-regulates MDM2 expression at transcriptional level

Based on the results shown above, it was of great interest to further explore the underlying mechanism of MDM2 inhibition by GA. Firstly, we focused on the possible regulation at transcriptional level, by examining the effect of GA on the expression of MDM2 mRNA levels. H1299 cells were treated with various concentrations of GA for 6 h, and there was an evident GA-induced decrease in MDM2 mRNA levels in a concentration-dependent way, as shown in Fig. 3A.

To verify the results above, we further performed luciferase reporter assay in the cells. As well known, there are two promoters regulating the transcription of MDM2 gene, so we, respectively, transfected MDM2-P1 and MDM2-P2 into H1299 cells for 40 h and then detected the reporter activity following GA treatment for another 6 h. The luciferase activity of the MDM2-P1 reporter was dramatically decreased by over

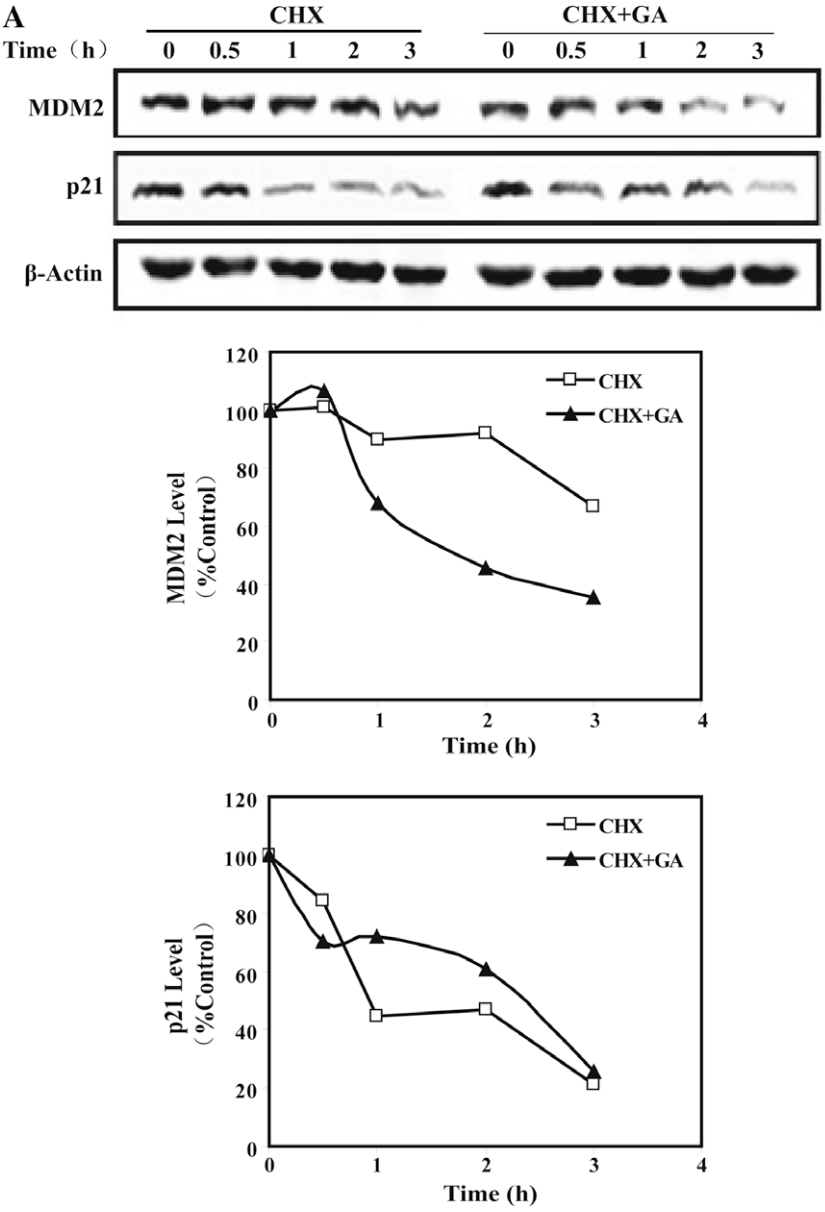

Fig. 4. GA down-regulates MDM2 expression at posttranslational level. (A) GA accelerates the degradation of MDM2. MDM2 and p21 protein levels in H1299 cells, treated with or without 5 μM GA for 6 h, were detected by western blotting at different time points after the protein synthesis inhibitor cycloheximide (10 μg/mL) was added. The quantification of data showed as bottom was carried out by Photoshop grey value analysis. (B) GA promotes the autoubiquitination of MDM2. H1299 cells were cotransfected with pCMV-*Mdm2* and a ubiquitin expressing plasmid pcDNA3-*Ubi* for 40 h, and then were treated with various concentrations of GA and MG132 (25 μmol/L) for 6 h. Cells were lysed and the lysates were subjected to immunoprecipitation with anti-MDM2 antibody. Ubiquitinated MDM2 was detected by anti-ubiquitin antibody. (C) GA mainly promotes the degradation of cytoplastic MDM2. H1299 cells or MCF-7 cells were inoculated in 6-well plate overnight and then exposed to various concentrations of GA for 6 h. Cells were incubated with monoclonal anti-MDM2 antibody followed by incubation of anti-rabbit IgG-FITC. The cell nucleus was stained with DAPI. Images were observed and captured using fluorescence microscope.

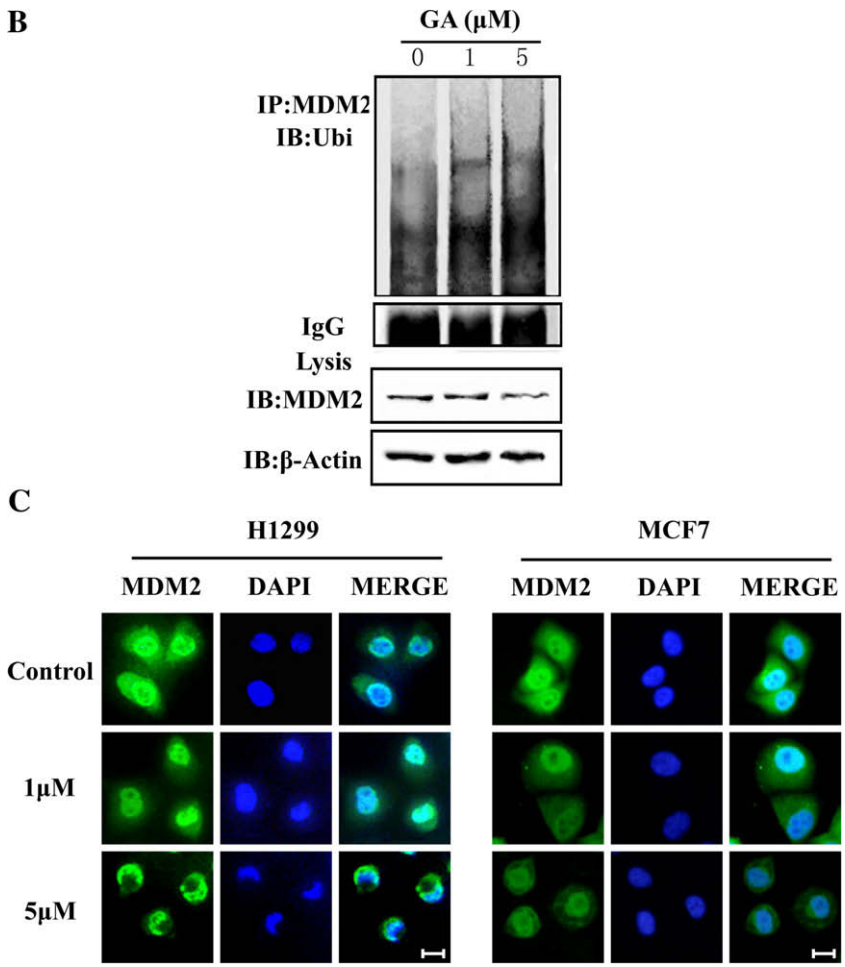

Fig. 4. (*continued*)

70% when cells were treated with 5 μM GA; meanwhile there were no apparent changes in the cells transfected with the corresponding empty vector reporter (Fig. 3B). In comparison, the luciferase activity of the MDM2-P2 reporter was not so vital in H1299 cells, whereas still dropped down by around 50% (Fig. 3B). Overall, these data clearly showed that the decrease in MDM2 protein was partially due to GA-induced inhibition of the transcriptional activation of gene MDM2.

3.4. GA down-regulates MDM2 expression at posttranslational level

MDM2 functions as an E3 ubiquitin ligase responsible for the ubiquitination of many targets followed by proteome-mediated degradation, including MDM2 itself. Therefore we proceeded to test whether GA promotes the degradation of MDM2 protein independent of p53 at posttranslational level. The degradation rate of MDM2 protein in H1299 cells was determined at different time points after the protein synthesis inhibitor cycloheximide (CHX) (10 μg/mL) was added. After 3-h exposure to CHX, the amount of celluar MDM2 protein was decreased by ~40% with the treatment of GA, versus only ~70% decrease in the absence of GA (Fig. 3A).

Furthermore, H1299 cells were cotransfected with pCMV-*Mdm2* and a plasmid expressing human ubiquitin, and then exposed to GA plus the proteasome inhibitor MG132 (25 μM) for total 6 h. Ubiquitinated MDM2 protein from cell lysates was isolated by immunoprecipitation with anti-MDM2 antibody, followed by western blotting assay with anti-ubiquitin antibody. As shown in Fig. 4B, GA promoted the ubiquitination of exogenous MDM2 concentration-dependently, providing another envidence for GA-induced degradation of MDM2.

Moreover, since the degradation of MDM2 was considered to take place in both cellular cytoplasm and nucleus, we further carried out immunofluorescent assay to test where GA-mediated MDM2 degradation mainly occurs. Treatment of both H1299 and MCF-7 cells with GA dramatically decreased the amount of MDM2 in cytoplasm as shown in Fig. 4C. According to our previous work, GA stabilized cytoplasmic p53 [27], consistent with the result of reducing MDM2 expression in cytoplasm observed here. These results taken together support the hypothesis that the inhibition of MDM2 by GA at posttranslational level is through acceleration of MDM2 degradation via autoubiquitination pathway.

3.5. GA elevates expressing of $p21^{Waf1/CIP1}$ independent of p53 but not mRNA level

As described above, GA induces G2/M cell cycle arrest p53 independently; the other hand, GA has been shown previously to cause cell cycle arrest triggered by $p21^{Waf1/CIP1}$ activation, a crucial CDK inhibitor in cell cycle regulation [22]. Thus, whether the activation of $p21^{Waf1/CIP1}$ by GA involves with MDM2 inhibition was investigated here. Firstly, MCF-7 (p53 WT), MCF-7 (p53 KD), and H1299 (p53 null) cells were treated with GA as above, and employed to test the protein level of $p21^{Waf1/CIP1}$ in different context of p53 expressing by western blotting. As shown in Fig. 5A, regardless of cellular p53 status, GA increases the protein level of $p21^{Waf1/CIP1}$ concentration dependently, as well as time dependently (Fig. 5B).

Nevertheless, in the RT-PCR assay, the mRNA level of $p21^{Waf1/CIP1}$ in H1299 cells was not affected by the treatment of GA (Fig. 5C). In addition, the degradation rate of protein $p21^{Waf1/CIP1}$ was indeed decreased by GA

in our tests (Fig. 4A). These results suggest that GA-induced up-regulation of $p21^{Waf1/CIP1}$ in H1299 cells may happen at posttranscriptional level.

3.6. GA attenuates the physical interaction between MDM2 and $p21^{Waf1/CIP1}$

It is proved that MDM2 mediates the proteolytic degradation of $p21^{Waf1/CIP1}$, thus reducing $p21^{Waf1/CIP1}$ protein stability [41]. Thereby, considering the results above, we next tested whether GA-mediated MDM2 inhibition affects the interaction between MDM2 and $p21^{Waf1/CIP1}$. After H1299 cells treated with GA as described above, we performed immunoprecipitation isolation using anti-MDM2 antibody, followed by immumoblotting assay with anti-p21 antibody. As shown in Fig. 6, the interaction between MDM2 and $p21^{Waf1/CIP1}$ was impaired by GA, as the amount of endogenous $p21^{Waf1/CIP1}$ binding to MDM2 was remarkably decreased in a concentration-dependent manner, while the expression of $p21^{Waf1/CIP1}$ in cell lysate was increased. The results suggest that GA-mediated the inhibition of MDM2 weakens the access of MDM2 to $p21^{Waf1/CIP1}$, contributing to the $p21^{Waf1/CIP1}$ stabilization.

3.7. GA inhibits tumor growth and MDM2 expression in vivo

As we have identified p53-independent anti-tumor effects of GA *in vitro*, H1299 xenograft model was constructed to determine the *in vivo* effect of GA on tumor growth with the absence of p53. Mice were given GA i.v. at 2, 4, and 8 mg/kg once every 2 days for total 18 days before sacrifice. Cyclophosphamide (CTX) has been shown to inhibit tumor inhibition in variety of xenografts and therefore used as positive control. As shown in Fig. 7A, 8 mg/kg of GA did not substantially inhibit tumor growth until 8 days of treatment similar to that of 20 mg/kg of CTX. At the end of the experiment, approximately 60% reduction in tumor volume was observed in mice treated with GA at 4 mg/kg, and 75% reduction in mice treated with GA at 8 mg/kg.

Tumor tissues were retrieved after the final administration of GA and analyzed by western blotting to determine the change in MDM2 and $p21^{Waf1/CIP1}$ protein levels. Administration of 4 mg/kg GA produced remarkable inhibition of MDM2 and induction of $p21^{Waf1/CIP1}$ compared with vehicle control, whereas little change was observed in the CTX treat-

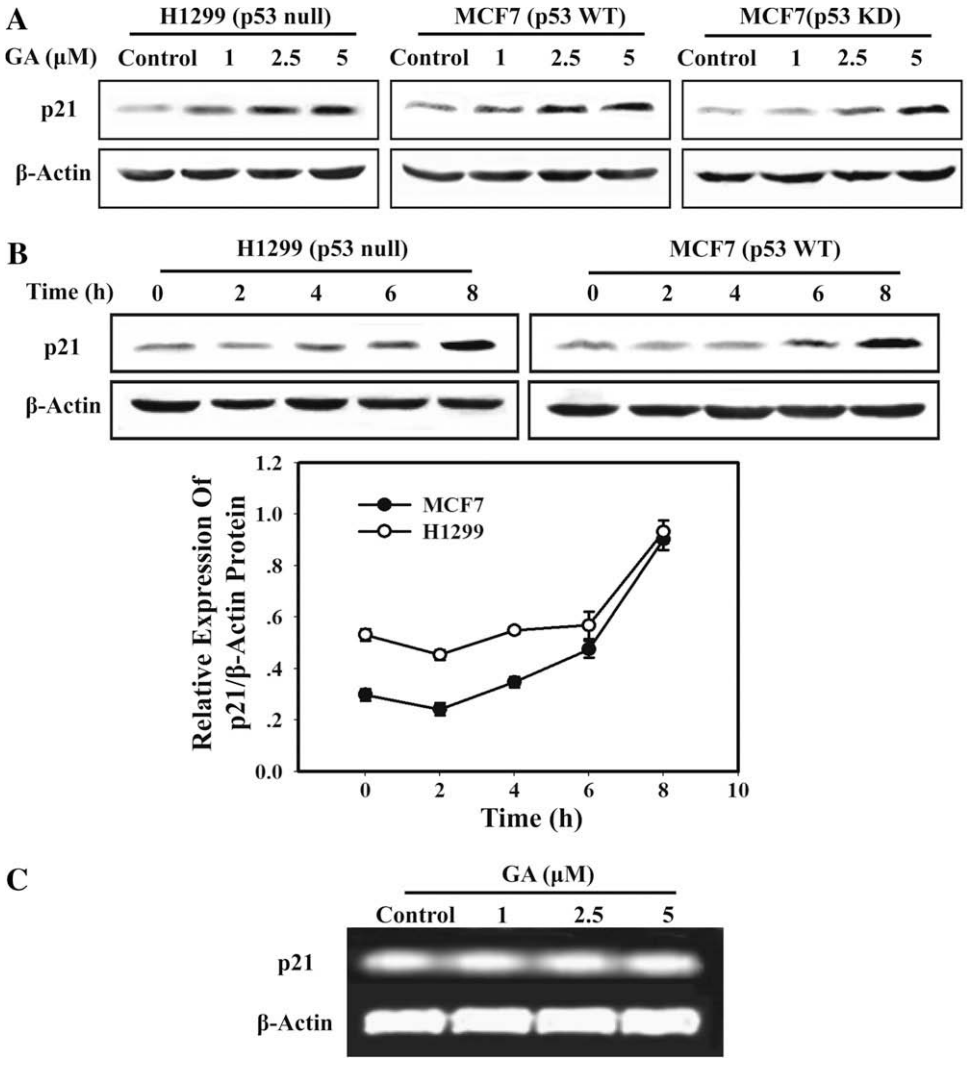

Fig. 5. GA elevates expressing of $p21^{Waf1/CIP1}$ independent of p53 but not mRNA level. (A) Protein level of $p21^{Waf1/CIP1}$ was increased by GA. H1299 (p53 null), MCF-7 (p53 WT), and MCF-7 cells (p53 KD) were treated with various concentrations (0, 1, 2.5, and 5 μM) of GA for 6 h, and protein $p21^{Waf1/CIP1}$ was detected by western blotting. (B) GA elevates $p21^{Waf1/CIP1}$ expressing time-dependently. H1299 and MCF-7 cells were treated with 5 μM GA for different time as indicated above, and then protein $p21^{Waf1/CIP1}$ levels were determined by western blotting. (C) GA has little effect on $p21^{Waf1/CIP1}$ expressing at RNA level. H1299 cells were treated with various concentrations of GA for 6 h followed by extraction of total RNA. cDNA fragments of $p21^{Waf1/CIP1}$ was amplified by RT-PCR.

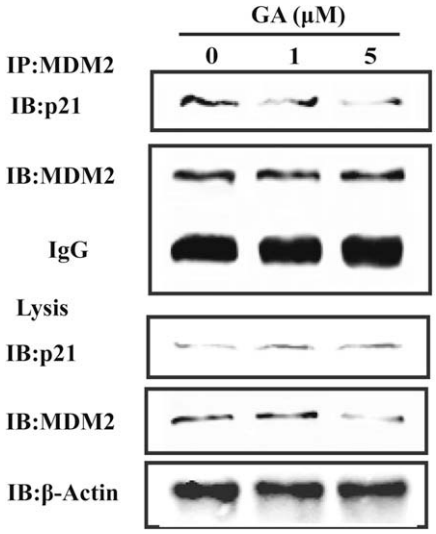

Fig. 6. GA attenuates the physical interaction between MDM2 and $p21^{Waf1/CIP1}$. H1299 cells were treated with various concentrations of GA for 6 h. Cells were lysed and the lysates were subjected to immunoprecipitation with anti-MDM2 antibody. Endogenous $p21^{Waf1/CIP1}$ binding with MDM2 was detected by anti-p21 antibody.

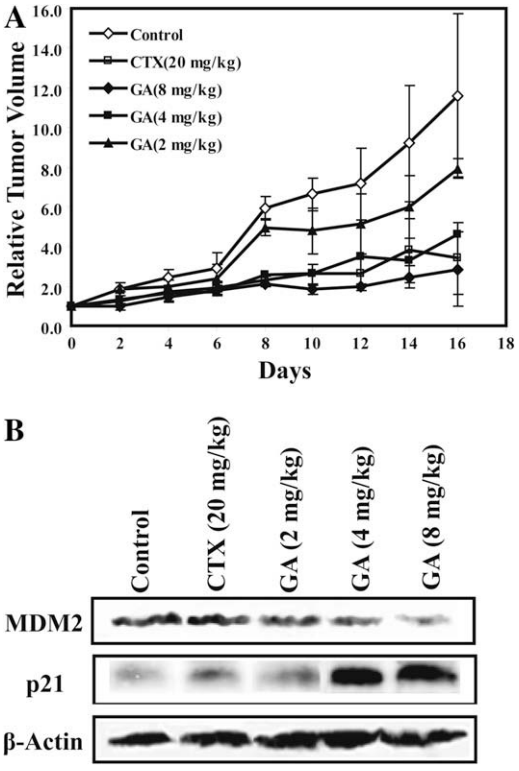

Fig. 7. GA inhibits tumor growth and MDM2 expression *in vivo* (A) anti-tumor activity of GA is confirmed in H1299 xenografts. H1299 cells were inoculated in the right flank region of BALB/cA nude mice. Each group was treated with 20 mg/kg CTX, 2, 4, and 8 mg/kg GA and 0.9% NaCl (vehicle), respectively, by i.v. once every 2 days for 3 weeks. Tumor volumes were measured as described in methods. (B) GA inhibits MDM2 expressing while elevates $p21^{Waf1/CIP1}$ expressing in H1299 xenografts. At the end of treatment, tumor xenografts were removed and proteins of tumor tissue homogenates were analyzed by western blotting with anti-MDM2 and anti-$p21^{Waf1/CIP1}$ antibodies.

ment group (Fig. 7B). These results showed *in vivo* anti-tumor activity of GA, potentially through modulation of MDM2 and $p21^{Waf1/CIP1}$.

4. Discussion

The purpose of this study is to determine whether GA affects the expression of the oncoprotein MDM2 p53-dependently and reveal the underlying mechanisms. There are three novel results presented here. Firstly, we proved that GA down-regulates MDM2 expression at both transcriptional and posttranslational levels. Both P1 and P2 promoters of MDM2 are responsive to GA-mediated down-regulation of MDM2 at transcriptional level. Further evidence of GA promoting autoubiquitination of MDM2 provides another possibility for regulatory mechanism at posttranslational level. Secondly, we found that $p21^{Waf1/CIP1}$ was up-regulated by GA independent of p53, probably due to alleviating the binding of MDM2 to $p21^{Waf1/CIP1}$ and stabilizing tumor suppressor $p21^{Waf1/CIP1}$. Lastly, GA exerts anti-tumor effects, including cell growth inhibition and inducing apoptosis as well cell cycle arrest, in cancer cells not expressing functional p53, as well as in inhibiting tumor growth in mice xenograft model, possibly through down-regulating MDM2.

It is well known that MDM2 expression is controlled by two different promoters, generating alternatively spliced transcripts [32]. The second promoter of MDM2 P2 was more extensively studied than P1 promoter, considering that p53 binds to the two p53-responsive elements in intron 1 and contributes to the major biological function of MDM2 [33,34]. Nevertheless, there are numbers of p53-independent response elements in MDM2 promoters, especially P1 promoter, regulated by different transcriptional factors, such as AP1, ETS, PTEN, and others factors [35,36]. Our results of RT-PCR and luciferase reporter assay supported that P1 promoter, accompanied by P2 promoter, was responsive to GA-mediated transcriptional inhibition of MDM2. It was reported that the ratio of the two MDM2 protein products, p90 to p76, was determined by the relative abundance of transcription initiated by P1 promoter which predominantly gives rise to p90 [37]. As in our observation, GA mainly affected the expression of MDM2-p90 whereas slightly regulated MDM2-p76 (data not shown). What still needs to be addressed, however, is which signaling effectors binding to the MDM2 promoter blocked by GA, leading to the suppression of MDM2 transcription. Moreover, MDM2 is the major regulator for p53 degradation mediated by 26S proteasome pathway, dependent on the E3 ubiquitin ligase activity of a RING finger domain located in the C terminus of MDM2 [7]. Meanwhile, it facilitates its own ubiquitination under certain conditions, consequently resulting in the destabilization of MDM2 itself [38]. The mechanism of controlling the switch between substrate ubiquitination and autoubiquitination of MDM2 is still not clear [17]. Here we showed that GA could promote the autoubiquitination of MDM2 at posttranslational level, which means GA may attenuate the stability of MDM2 to access its targets. We have proved that GA induced p53 stabilization in cytoplasm by means of suppressing the expression of MDM2 [27]. Consistently, the cytoplasmic MDM2 expression was observed to decline

in H1299 cells after the treatment of GA, even though MDM2 is known for its shuttle between cytoplasm and nucleus [39]. Still, whether GA affects cellular translocation of MDM2 needs to be investigated in future. More evidence has suggested that PI3K/AKT pathway plays an important role in MDM2 modification at posttranslational level [40]. And our previous work indeed identified the link between tyrosine kinase inhibition and GA's anti-tumor effects, which provided the possibility for elucidating GA's regulation of MDM2 in detail.

Beyond our initial expectations, the expression of $p21^{Waf1/CIP1}$, a typical transactivation target of p53, was enhanced at protein level by GA without p53 presence, which could be resulted from the inhibition of MDM2. This is a consensus that MDM2 contributes to the proteasome-mediated degradation of $p21^{Waf1/CIP1}$ [41,42]. We also observed that there was indeed a physical interaction between MDM2 and $p21^{Waf1/CIP1}$, and comparatively endogenous $p21^{Waf1/CIP1}$ was released from the complex followed by adding of GA. Thereby GA may stabilize $p21^{Waf1/CIP1}$ at posttranslational level, indicating an underlying connection between GA's anti-tumor effects and p53-independent activation of $p21^{Waf1/CIP1}$. Nevertheless, the regulation of $p21^{Waf1/CIP1}$ is more complicated than previous thoughts. There are many signaling molecules that regulate the transactivation and degradation of $p21^{Waf1/CIP1}$ via different pathways [43,44]. So whether there exist crosstalk between other factors and $p21^{Waf1/CIP1}$ pharmacologically induced by GA, such as Rb family, will be discussed in the future work.

In conclusion, our study provides further insight into the molecular mechanisms of GA: in the presence of p53, GA activates p53 via suppressing of MDM2; however, in the absence of p53, MDM2 inhibition still contributes to GA-mediated anti-tumor effects. Moreover, we found that GA could inhibit cell growth, induce apoptosis and cell cycle arrest in G2/M phase in cancer cells with or without functional p53. This novel finding provides implication for clinical application of GA, as p53 is genetically mutated in over 50% of human cancers. According to our previous studies, GA selectively inhibited tumor growth while showed lower toxic effects in normal cells, which raised the comparative advantages of GA [45]. It has been found that high MDM2 levels are associated with poor prognosis and resistance to chemotherapy [15]. Further research and trials are required to identify the role of MDM2 inhibitory action on the therapeutic uses of GA.

Conflicts of interest

None declared.

Acknowledgements

This project was supported by Natural Science Foundation of China (No. 30472044) and Natural Science Foundation of Jiangsu Province, China (No. BK2005096).

We thank Dr. Mosihe Oren for pCMV-Neo-Bam-*Mdm2* plasmid, Dr. Caicun Zhou for H1299 cancer cell line, and Dr. Feng Feng for gambogic acid purification.

References

[1] S.S. Fakharzadeh, S.P. Trusko, D.L. George, Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line, EMBO J. 10 (1991) 1565–1569.
[2] J. Momand, G.P. Zambetti, D.C. Olson, D. George, A.J. Levine, The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation, Cell 69 (1992) 1237–1245.
[3] J.D. Oliner, K.W. Kinzler, P.S. Meltzer, D.L. George, B. Vogelstein, Amplification of a gene encoding a p53-associated protein in human sarcomas, Nature 358 (1992) 80–83.
[4] Y. Haupt, R. Maya, A. Kazaz, M. Oren, Mdm2 promotes the rapid degradation of p53, Nature 387 (1997) 296–299.
[5] M.H. Kubbutat, S.N. Jones, K.H. Vousden, Regulation of p53 stability by Mdm2, Nature 387 (1997) 299–303.
[6] X. Wu, J.H. Bayle, D. Olson, A.J. Levine, The p53-mdm-2 autoregulatory feedback loop, Genes Dev. 7 (1993) 1126–1132.
[7] R. Honda, H. Yasuda, Activity of MDM2, a ubiquitin ligase, toward p53 or itself is dependent on the RING finger domain of the ligase, Oncogene 19 (2000) 1473–1476.
[8] Z. Zhang, R. Zhang, p53-independent activities of MDM2 and their relevance to cancer therapy, Curr. Cancer Drug Target. 5 (2005) 9–20.
[9] Z.X. Xiao, J. Chen, A.J. Levine, N. Modjtahedi, J. Xing, W.R. Sellers, et al., Interaction between the retinoblastoma protein and the oncoprotein MDM2, Nature 375 (1995) 694–698.
[10] K. Martin, D. Trouche, C. Hagemeier, T.S. Sørensen, N.B. La Thangue, T. Kouzarides, Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein, Nature 375 (1995) 691–694.
[11] A. Thomas, E. White, Suppression of the p300-dependent mdm2 negative-feedback loop induces the p53 apoptotic function, Genes Dev. 12 (1998) 1975–1985.
[12] V.S. Tompkins, J. Hagen, A.A. Frazier, T. Lushnikova, M.P. Fitzgerald, A. di Tommaso, et al., A novel nuclear interactor of ARF and MDM2 (NIAM) that maintains chromosomal stability, J. Biol. Chem. 282 (2007) 1322–1333.
[13] T. Juven-Gershon, O. Shifman, T. Unger, A. Elkeles, Y. Haupt, M. Oren, The Mdm2 oncoprotein interacts with the cell fate regulator Numb, Mol. Cell Biol. 18 (1998) 3974–3982.
[14] M.T. Boyd, N. Vlatkovic, D.S. Haines, A novel cellular protein (MTBP) binds to MDM2 and induces a G1 arrest that is suppressed by MDM2, J. Biol. Chem. 275 (2000) 31883–31890.
[15] K. Lundgren, R. Montes de Oca Luna, Y.B. McNeill, E.P. Emerick, B. Spencer, C.R. Barfield, et al., Targeted expression of MDM2 uncouples S phase from mitosis and inhibits mammary gland development independent of p53, Genes Dev. 11 (1997) 714–725.
[16] H. Wang, X. Zeng, P. Oliver, L.P. Le, J. Chen, L. Chen, et al., MDM2 oncogene as a target for cancer therapy: an antisense approach, Int. J. Oncol. 15 (1999) 653–660.
[17] Z. Lai, T. Yang, Y.B. Kin, T.M. Sielecki, M.A. Diamond, P. Strack, et al., Differentiation of hdm2-mediated p53 ubiquitination and hdm2 autoubiquitination activity by small molecular weight inhibitors, Proc. Natl. Acad. Sci. USA 99 (2002) 14734–14739.
[18] C.A. Midgley, J.M. Desterro, M.K. Saville, S. Howard, A. Sparks, R.T. Hay, et al., An N-terminal p14ARF peptide blocks mdm2-dependent ubiquitination in vitro and can activate p53 in vivo, Oncogene 19 (2000) 2312–2323.
[19] L. Zhao, Q.L. Guo, Q.D. You, Z.Q. Wu, H.Y. Gu, Gambogic acid induces apoptosis and regulates expressions of Bax and Bcl-2 protein in human gastric carcinoma MGC-803 cells, Biol. Pharm. Bull. 27 (2004) 998–1003.
[20] Z.Q. Wu, Q.L. Guo, Q.D. You, L. Zhao, H.Y. Gu, Gambogic acid inhibits proliferation of human lung carcinoma SPC-A1 cells in vivo and in vitro and represses telomerase activity and telomerase reverse transcriptase mRNA expression in the cells, Biol. Pharm. Bull. 27 (2004) 1769–1774.
[21] M.K. Pandey, B. Sung, K.S. Ahn, A.B. Kunnumakkara, M.M. Chaturvedi, B.B. Aggarwal, Gambogic acid, a novel ligand for transferrin receptor, potentiates TNF-induced apoptosis through modulation of the nuclear factor-kappaB signaling pathway, Blood 110 (2007) 3517–3525.
[22] J. Yu, Q.L. Guo, Q.D. You, L. Zhao, H.Y. Gu, Y. Yang, et al., Gambogic acid-induced G2/M phase cell-cycle arrest via disturbing CDK7-mediated phosphorylation of CDC2/p34 in human gastric carcinoma BGC-823 cells, Carcinogenesis 28 (2007) 632–638.
[23] Q. Zhao, Y. Yang, J. Yu, Q.D. You, S. Zeng, H.Y. Gu, et al., Posttranscriptional regulation of the telomerase hTERT by gambogic acid in human gastric carcinoma 823 cells, Cancer Lett. 262 (2008) 223–231.

[24] T. Yi, Z. Yi, S.G. Cho, J. Luo, M.K. Pandey, B.B. Aggarwal, et al., Gambogic acid inhibits angiogenesis and prostate tumor growth by suppressing vascular endothelial growth factor receptor 2 signaling, Cancer Res. 68 (2008) 1843–1850.
[25] Q. Qi, N. Lu, X.T. Wang, H.Y. Gu, Y. Yang, W. Liu, et al., Anti-invasive effect of gambogic acid in MDA-MB-231 human breast carcinoma cells, Biochem. Cell Biol. 86 (2008) 386–395.
[26] S. Kasibhatla, K.A. Jessen, S. Maliartchouk, J.Y. Wang, N.M. English, J. Drewe, et al., A role for transferrin receptor in triggering apoptosis when targeted with gambogic acid, Proc. Natl. Acad. Sci. USA 102 (2005) 12095–12100.
[27] H.Y. Gu, X.T. Wang, S. Rao, J. Wang, J. Zhao, F.L. Ren, et al., Gambogic acid mediates apoptosis as a p53 inducer through down-regulation of mdm2 in wild-type p53-expressing cancer cells, Mol. Cancer Ther. 7 (2008) 3298–3305.
[28] Q.D. You, Q.L. Guo, F. Feng, W.Y. Liu, Chinese Patent ZL01008049. 3, 2003.
[29] H.Z. Zhang, S. Kasibhatla, Y. Wang, J. Herich, J. Guastella, B. Tseng, et al., Discovery, characterization and SAR of gambogic acid as a potent apoptosis inducer by a HTS assay, Bioorg. Med. Chem. 12 (2004) 309–317.
[30] N.C. Arva, T.R. Gopen, K.E. Talbott, L.E. Campbell, A. Chicas, D.E. White, et al., A chromatin-associated and transcriptionally inactive p53-Mdm2 complex occurs in mdm2 SNP309 homozygous cells, J. Biol. Chem. 280 (2005) 26776–26787.
[31] Y. Hu, Y. Yang, Q.D. You, W. Liu, H.Y. Gu, L. Zhao, et al., Oroxylin A induced apoptosis of human hepatocellular carcinoma cell line HepG2 was involved in its antitumor activity, Biochem. Biophys. Res. Commun. 351 (2006) 521–527.
[32] C.Y. Brown, G.J. Mize, M. Pineda, D.L. George, D.R. Morris, Role of two upstream open reading frames in the translational control of oncogene mdm2, Oncogene 18 (1999) 5631–5637.
[33] T. Juven, Y. Barak, A. Zauberman, D.L. George, M. Oren, Wild-type p53 can mediate sequence-specific transactivation of an internal promoter within the mdm2 gene, Oncogene 8 (1993) 3411–3416.
[34] A. Zauberman, D. Flusberg, Y. Barak, M. Oren, A functional p53-responsive intronic promoter is contained within the human mdm2 gene, Nucleic Acid. Res. 23 (1995) 2584–2592.
[35] S. Ries, C. Biederer, D. Woods, O. Shifman, S. Shirasawa, T. Sasazuki, et al., Opposing effects of Ras on p53: transcriptional activation of mdm2 and induction of p19ARF, Cell (2000) 321–330.
[36] C.J. Chang, D.J. Freeman, H. Wu, PTEN regulates Mdm2 expression through the P1 promoter, J. Biol. Chem. 279 (2004) 29841–29848.
[37] L.J. Saucedo, C.D. Myers, M.E. Perry, Multiple murine double minute gene 2 (MDM2) proteins are induced by ultraviolet light, J. Biol. Chem. 274 (1999) 8161–8168.
[38] J.M. Stommel, G.M. Wahl, Accelerated MDM2 auto-degradation induced by DNA-damage kinases is required for p53 activation, EMBO J. 23 (2004) 1547–1556.
[39] J. Roth, M. Dobbelstein, D.A. Freedman, T. Shenk, A.J. Levine, Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein, EMBO J. 17 (1998) 554–564.
[40] M. Ashcroft, R.L. Ludwig, D.B. Woods, T.D. Copeland, H.O. Weber, E.J. MacRae, et al., Phosphorylation of HDM2 by Akt, Oncogene 21 (2002) 1955–1962.
[41] Y. Jin, H. Lee, S.X. Zeng, M.S. Dai, H. Lu, MDM2 promotes p21waf1/cip1 proteasomal turnover independently of ubiquitylation, EMBO J. 22 (2003) 6365–6377.
[42] Z. Zhang, H. Wang, M. Li, S. Agrawal, X. Chen, R. Zhang, MDM2 is a negative regulator of p21WAF1/CIP1, independent of p53, J. Biol. Chem. 279 (2004) 16000–16006.
[43] A. Wood, A. Shilatifard, Transcriptional blackjack with p21, Genes Dev. 20 (2006) 643–647.
[44] R.J. Sheaff, J.D. Singer, J. Swanger, M. Smitherman, J.M. Roberts, B.E. Clurman, Proteasomal turnover of p21Cip1 does not require p21Cip1 ubiquitination, Mol. Cell 5 (2000) 403–410.
[45] Y. Yang, L. Yang, Q.D. You, F.F. Nie, H.Y. Gu, L. Zhao, et al., Differential apoptotic induction of gambogic acid, a novel anticancer natural product, on hepatoma cells and normal hepatocytes, Cancer Lett. 256 (2007) 259–266.