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Gambogic acid suppresses cytochrome P450 3A4 by downregulating pregnane X receptor and up-regulating DEC1 in human hepatoma HepG2 cells

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Abbreviations:

GA, gambogic acid; CYP, cytochrome P450; PXR, pregnane X receptor; DEC1, differentiated embryonic chondrocyte gene1; RXR, retinoid X receptor; MDR1, multiple drug resistance 1; ACTD, actinomycin D

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Abstract

Gambogic acid (GA) was approved by the Chinese Food and Drug Administration for phase II clinical trials in patients with solid tumors. Recently, combinations of GA and chemotherapeutic agents represents a promising therapeutic approach for cancer. However, little is known about the effect of GA on drug metabolism enzymes. In this study, we report for the first time that GA is a potent repressor of the cytochrome P450 3A4 (CYP3A4) enzyme, which is responsible for the oxidative metabolism of more than 60% of all pharmaceuticals. After HepG2 cells were treated with GA, the expression of CYP3A4 decreased at both the mRNA and protein levels, and CYP3A4 enzymatic activity was also reduced. Moreover, GA-mediated repression of CYP3A4 occurred after the decrease of PXR. Knockdown of PXR decreased CYP3A4 at the mRNA and protein levels, whereas PXR overexpression increased the CYP3A4 mRNA and protein levels. GA markedly decreased CYP3A4 expression in both normal and PXR overexpressing cells. Meanwhile, differentiated embryonic chondrocyte gene 1 (DEC1) expression rapidly increased in response to GA treatment, and subsequently, CYP3A4 expression continuously decreased. Overexpression of DEC1 decreased CYP3A4 mRNA and protein levels, and knockdown of DEC1 partially abolished the GA-mediated decrease in CYP3A4 expression. Taken together, our data suggest that two transcriptional factors, PXR and DEC1, are involved in the GA-mediated suppression of CYP3A4 in HepG2 cells.

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1. Introduction

Cytochrome P450 3A4 (CYP3A4) is the most important human CYP450 enzyme because it contributes to the metabolism of the largest number of therapeutically important drugs [1,2]. CYP3A4 is responsible for the oxidative metabolism of more than 60% of all pharmaceuticals [3,4]. CYP3A4 is a highly polymorphic enzyme with pronounced, constitutive, inter-individual expression in the human population, and these characteristics form the basis for its clinically significant drug interactions and toxicities [5,6]. The existence of polymorphic CYP genes may also contribute to inter-individual variation [7]. However, other factors, such as age, diet, hormonal status, disease, and exposure to drugs or xenobiotics, may also contribute to the wide variability in the expression of certain CYPs (e.g., CYP3A4) [8,9], as polymorphisms are rare in these enzymes [10].

CYP3A4 is regulated by various liver-enriched transcription factors, such as hepatocyte nuclear factors (HNFs) and CCAAT/enhance-binding proteins (CEBP) [11]. *CYP3A4* is also regulated by the interaction of various transcription factors, including the nuclear receptor (NR), pregnane X receptor (PXR, NR1I2) [12], constitutive androstane receptor (CAR, NR1I3) [13], vitamin D receptor (VDR, NR1I1) [14] and glucocorticoid receptor (GR, NR3C1) [15]. PXR is a particularly important transcriptional regulator of CYP3A4 [12,16].

PXR is a xenobiotic-induced nuclear receptor, and it plays a central role in the regulation of CYP3A4 and other clinically important CYPs. Thus, PXR expression is crucial for the metabolism of many clinically relevant drugs [17]. PXR regulation has

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been studied extensively, but its precise role in *CYP3A4* regulation is not fully understood. PXR is activated in a ligand-dependent manner. Upon activation, PXR translocates from the cytosol to the nucleus, where it forms a heterodimer with RXR (retinoid X receptor) and binds to the promoter regions of its target genes [18]. PXR regulates *CYP3A4* by binding to response elements composed of various direct (DR) and everted (ER) repeats of the consensus motif AG(G/T)TCA [19]. The transcriptional activity of activated PXR also requires interaction with different co-activators, including HNF4α [20], peroxisome proliferator-activated receptor-γ co-activator (PGC-1) and steroid receptor co-activator-1 (SRC-1) [21].

Differentiated embryonic chondrocyte gene1 (DEC1) (BHLHE40/Stra13/Sharp2), DEC2 (BHLHE41/Sharp1), and basic helix-loop-helix (bHLH) transcription factors, have been implicated in apoptosis [22], cell differentiation [23,24], lymphocyte maturation [25], regulation of the molecular clock [26], and the maintenance of metabolic homeostasis [27]. DEC1 and DEC2 regulate the transcription of target genes in an E-box-dependent or -independent manner [28-30]. DEC1 expression is highly elevated in response to environmental stimuli, such as hypoxia, and cytokines, such as TNFα [31,32]. Based on our research, DEC1 is a transcriptional repressor of CYP3A4 [33].

Gambogic acid (GA) is a small molecule that is present in the traditional Chinese medicine gamboges, and it has been used for hundreds of years in China [34]. GA is cytotoxic toward a variety of tumors [35,36]. Unlike other chemotherapeutics, GA does not greatly effect the hematologic system [37,38]. Notably, the Chinese Food and Drug

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Administration approved GA for phase II clinical trials in solid cancer therapy. Several molecular targets of GA have been proposed [39,40]. Most recently, Li et al. reported that GA is a novel tissue-specific proteasome inhibitor, with potency comparable to Bortezomib, but with markedly less toxicity [41]. Moreover, GA only gains proteasome-inhibitory function after being metabolized by intracellular CYP2E1. Therefore, GA is a promising anticancer agent that is not toxic toward normal tissues. However, the effect of GA on drug-metabolizing enzymes remains unknown.

In the present study, we report for the first time that GA is a potent repressor of the cytochrome P450 3A4 enzyme, which plays an important role in oxidative biotransformation [42]. In HepG2 cells, GA treatment decreased CYP3A4 expression at both the mRNA and protein levels, and its enzymatic activity was significantly reduced. Two transcription factors, PXR and DEC1, are involved in the GA-mediated suppression of CYP3A4.

2. Materials and methods

2.1 Reagents

GA (99% purity) was supplied by Dr. Qidong You's laboratory (China Pharmaceutical University, China), and it was isolated from the resin of *Garcinia hanburyi* and purified according to the established methods [34]. GA was dissolved in DMSO as a stock solution at 1 mM and stored at -20 °C. To obtain the concentrations used in this study (0.25, 0.5, and 1.0 μM), the stock solution was diluted with cell culture medium. The working solution was prepared fresh in basal medium with a

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final DMSO concentration of 0.1%, and the controls were treated with an equivalent amount of DMSO.

PXR and MDR1 antibodies were obtained from Abcam (Cambridge, UK), and polyclonal antibodies (from rabbit) against DEC1 and CYP3A4 were kindly provided by Dr. Yan Lab (University of Rhode Island). The GAPDH antibody was purchased from Boster (Wuhan, China). The Histone-3 antibody was purchased from SAB (Baltimore, MD). IRDye™800-conjugated secondary antibodies were obtained from Rockland Inc. (Philadelphia, PA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

2.2 Cell culture

Human hepatocellular carcinoma HepG2 cells were obtained from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences). HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sijiqing, Zhejiang, China), 100 U/ml benzyl penicillin, and 100 U/ml streptomycin in a humidified environment with 5% CO_2 at 37 °C.

2.3 MTT assay

Cell toxicity was measured using MTT colorimetric assay, as described previously [43]. Experiments were performed in triplicate in a parallel manner for each concentration of GA used. Control cells were treated with culture medium containing 0.1% DMSO.

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After incubation for 24 h, absorbance (A) was measured at 570 nm. The survival ratio (%) was calculated using the following equation: survival ratio (%) = (A-treatment / A-control) × 100%. IC_{50} values (the concentration that caused 50% inhibition of cell proliferation) were calculated by the Logit method.

2.4 Enzyme activity assay for CYP3A4

HepG2 cells were plated in 48-well plates in DMEM supplemented with 10% fetal bovine serum overnight and were then treated with 0.25, 0.5, or 1 μM GA for 24 h. CYP3A4 activity was determined using a P450-Glo™ CYP3A4 assay kit (Promega, Madison, WI), according to the manufacturer's instructions. Briefly, the medium was replaced with a mixture of 150 μl DMEM and 150 μl Luciferin-IPA (the luminescent reaction), and the plates were incubated at 37 °C and 5% CO_2 for 60 min. Then, 50 μl of culture medium from each well (the luminescent reaction) was transferred to a 96-well opaque white luminometer plate, and 50 μl of Luciferin Detection Reagent was added to initiate the luminescent reaction. After another 10 min incubation at room temperature, the luminescent signal intensity was determined using a spectral scanning multimode reader (Thermo Scientific). Several controls were performed, including incubation without cells or the regeneration system.

2.5 Plasmid constructs

The expression construct encoding the human pregnane X receptor (hPXR) was described previously [44]. The DEC1 expression construct and DEC1 shRNA construct

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were kindly provided by Dr. Yan [33]. The PXR shRNA construct was purchased from GENECHEM (Shang Hai). CYP3A4 reporter was prepared by fusing the pGL3 basic vector to the proximal and distal elements of CYP3A4 (-362 to +53 and -7836 to -6093; CYP3A4-DP-Luc) [45,46].

2.6 Modulation of PXR and DEC1 expression by shRNA and overexpression

To define the roles of PXR and DEC1 in the GA-mediated down-regulation of CYP3A4, the expression levels of PXR and DEC1 were regulated by shRNA and overexpression. HepG2 cells were plated in 6-well plates in DMEM supplemented with 5% FBS at a density of 6×10^5 cells per well. Transfection was conducted using GeneJetTM (Ver II) (Gaithersburg, MD). For the shRNA experiment, cells were transfected with PXR and DEC1 shRNA constructs (800 ng/well). After a 12-h incubation, with the addition of fresh medium at 36 h, the transfected cells were treated with or without 1 μM GA for 24 h, and the expression levels of CYP3A4, PXR and DEC1 were evaluated by Western blotting. For overexpression, cells were transfected with human PXR and DEC1 constructs (800 ng/well) or the corresponding vector. After a 48-h incubation, the transfected cells were treated with or without 1 μM GA for 24 h, and the expression levels of CYP3A4, PXR and DEC1 were evaluated by Western blotting.

2.7 Transient co-transfection (luciferase assay)

Cells (HepG2) were plated in 48-well plates in DMEM supplemented with 10%

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delipidated fetal bovine serum at a density of 1x10^5 cells per well, and transfection was conducted using GeneJetTM (Ver II) (Gaithersburg, MD). Transfection mixtures contained 50 ng of a PXR plasmid, 50 ng of a reporter plasmid, and 5 ng of a pRL-TK luciferase plasmid. Cells were transfected for 6 h, after which the medium was replaced with fresh medium. After an additional 12 h, the medium was changed again with the same medium containing a chemical or solvent DMSO (final concentration of 0.1%). Transfected cells were incubated for an additional 24 h, washed once with PBS, and collected by scraping. The collected cells were subjected to two freeze/thaw cycles. Reporter enzyme activities were assayed using a Dual-Luciferase Reporter Assay System. This system contained two substrates, which were used to sequentially measure the activities of two luciferases. Firefly luciferase activity, which represented the reporter activity, was initiated by mixing an aliquot of lysates (10 μl) with Luciferase Assay Reagent II; then, firefly luminescence was quenched, and Stop & Glo Reagent was simultaneously added to activate Renilla luminescence. The firefly luminescence signal was normalized based on the Renilla luminescence signal, and the ratio of normalized luciferase activity from chemical over DMSO treatment was calculated as the fold induction.

2.8 Preparation of cytosolic and nuclear extracts

HepG2 cells were treated with or without 1 μM GA for 12 h, and then cells were collected and nuclear extracts were prepared with a Nuclear Extract kit (Keygen, Nanjing, China). Briefly, cells were lysed with buffer A (10 mM Hepes-KOH (pH

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7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and incubated on ice for 15 min. Then cells were centrifuged at 14,000 g for 15min at 4 °C. The supernatants were saved as the cytosolic fractions. The nuclear pellets were washed with buffer A and resuspended of the crude nuclei in high-salt buffer (20 mM Hepes, 0.5 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl, fluoride, pH 7.9) for 30 min on ice and then centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatants were saved as the nuclear fractions.

2.9 Real-time polymerase chain reaction

Total RNA was isolated using the Trizol reagent, according to the manufacturer's instruction. First-strand cDNA was synthesized using total RNA (1 μg) at 70 °C for 5 min, 42 °C for 60 min, and 95 °C for 10 min using an oligo (dT) 12-18 primer and submitted to real-time PCR. Amplification assays were performed on a 7500 Fast Real-Time PCR System using SDS software (Applied Biosystems) in 10 μl reactions containing 0.2 μM of each primer, 5 μl SYBR Green PCR master mix (2×) and 0.2 μl cDNA. After an initial denaturation at 95 °C for 30 s, amplifications were performed for 40 cycles at 95 °C for 5 s and 60 °C for 31 s. The signals from each target gene were normalized based on the signal from the corresponding GAPDH signal. The following PCR primers were used in this study: CYP3A4 F: GATTGACTCTCAGAATTCAAAAGAAACTGA, CYP3A4 R: GGTGAGTGGCCAGTTCATACATAATG; MDR1 F: GTCCCAGGAGCCCATCCT,

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MDR1 R: CCCGGCTGTTGTCTCCATA; PXR F: CGAGCTCCGCAGCATCA, PXR R: TGTATGTCCTGGATGCGCA; DEC1 F: GGCGGGGAATAAAACGGAGCGA, DEC1 R: CCTCACGGGCACAAGTCTGGAA; and GAPDH F: CAGTCCATGCCA TCACTGCCA, GAPDH R: CAGTGTAGCCCAGGATGCCCTT.

2.10 Western blot analysis

Cells were collected and lysed in lysis buffer (100 mM Tris-Cl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol, 200 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1 g/ml aprotinin), and the lysates were centrifuged at 12,000 *g* for 15 min at 4 °C. The supernatant was collected, and total protein concentrations were determined using the BCA assay using a Varioskan spectrofluorometer and spectrophotometer (Thermo, Waltham, MA). Then, the protein samples were separated by 10-15% SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA). Immune complexes were formed by incubating proteins with primary antibodies overnight at 4 °C, followed by incubation with an IRDyeTM 800-conjugated second antibody for 1 h at 37 °C. Immunoreactive protein bands were detected using an Odyssey Scanning System (LI-COR Inc., Superior St. Lincoln, NE).

2.11 Immunofluorescence analysis

Cells were rinsed with PBS and fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4) for 20 min at room temperature. After washing with PBS and blocking with 3% bovine serum albumin (BSA) in PBS for 1 h, the cells were incubated with

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anti-PXR and anti-DEC1 antibodies overnight at 4 °C. After washing with PBS, tetramethylrhodamine-labeled anti-rabbit IgG antibody (Rockland) was added to the cells, and the samples were incubated for 1 h at 37 °C. To stain nuclei, cells were rinsed with PBS and exposed to DAPI for 15 min. After washing with PBS, cells were examined under a laser scanning confocal microscope.

2.12 Inhibitor treatment

To determine the mechanism underlying the decrease in CYP3A4 mRNA, a transcriptional inhibition assay was performed using an RNA synthesis inhibitor, actinomycin D, to inhibit RNA synthesis. HepG2 cells were pretreated with actinomycin D for 1 h before the addition of 1 μM GA for another 24 h.

2.13 Statistics analysis

All results are presented as the mean ± SEM of triplicate experiments performed in parallel unless otherwise indicated. Statistical analyses were performed using an unpaired, two-tailed, Student's *t*-test or one-way ANOVA. All comparisons are made relative to untreated controls, and significant differences are indicated as $^{*}P<0.05$ and $^{**}P<0.01$.

3. Results

3.1 GA reduces CYP3A4 activity by decreasing the expression of CYP3A4 in human HepG2 cells

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After HepG2 cells were treated with different concentrations of GA for 24 h or 1 μM GA for 2, 4, 8, 12 and 24 h, CYP3A4 mRNA levels were monitored by qRT-PCR. CYP3A4 mRNA levels significantly decreased in a concentration-dependent manner in response to GA (Fig. 1A). The time course experiment revealed that CYP3A4 mRNA levels significantly decreased after four hours of GA treatment and continued to decrease in a time-dependent manner (Fig. 1B). To further investigate whether the decrease of CYP3A4 mRNA translated into a decrease at the protein level, Western blotting was used to monitor the expression of CYP3A4 protein after GA treatment. CYP3A4 protein levels decreased after GA treatment in a concentration- and time-dependent manner (Fig. 1C and 1D). The reduction of CYP3A4 protein levels corresponded to the changes observed at the mRNA level. Next, we detected the oxidative activity of CYP3A4 after GA treatment. As expected, CYP3A4 oxidative activity significantly decreased in GA-treated cells (Fig. 1E), corresponding with the decrease of CYP3A4 protein. Importantly, GA was not cytotoxic toward HepG2 cells at the concentrations tested (Fig. 1F).

3.2 Involvement of transcription in the GA-mediated repression of CYP3A4

To determine the mechanism underlying the GA-mediated decrease in the CYP3A4 mRNA expression, we performed a transcriptional inhibition assay using actinomycin D, a RNA synthesis inhibitor. HepG2 cells were pretreated with 0.1 μM actinomycin D for 1 h prior to the addition of 1 μM GA for another 24 h. Quantitative RT-PCR indicated that actinomycin D abolished the GA-mediated transcriptional repression of

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CYP3A4 in HepG2 cells (Fig. 2A). The fact that an RNA synthesis inhibitor completely blocked GA-mediated suppression of CYP3A4 suggests that GA decreases the expression of CYP3A4 by repressing its promoter. To test this hypothesis, a CYP3A4 promoter reporter and an hPXR construct were co-transfected in HepG2 cells. The CYP3A4 promoter reporter (CYP3A4-DP-luc) contains both the proximal and distal promoter regions that respond to many CYP3A4 inducers [45]. HepG2 cells were transfected with CYP3A4-DP-luc and Renilla plasmids in combination with hPXR and were then treated with 1 μM GA. After a 24-h incubation, the cells were lysed, and luciferase activity was measured. GA treatment significantly reduced the activity of the CYP3A4-DP promoter reporter (Fig. 2B). These data suggest that the GA-mediated repression of CYP3A4 occurs at the transcriptional level.

3.3 GA downregulates PXR expression

PXR is an important transcriptional regulator of CYP3A4, so we sought to determine whether PXR is involved in the GA-mediated repression of CYP3A4. HepG2 cells were treated with different concentrations of GA for 24 h or 1 μM GA for 2, 4, 8, 12 and 24 h, and PXR mRNA levels were evaluated using qRT-PCR; PXR protein levels were also analyzed by Western blotting. The results showed that the PXR mRNA levels significantly decreased in a concentration-dependent manner following GA treatment (Fig. 3A). PXR mRNA levels continuously decreased after 2 h of treatment with GA (Fig. 3B), whereas a significant decrease in CYP3A4 was not observed until

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4 h of treatment (Fig. 1B), suggesting that the decrease in the CYP3A4 expression occurred after the decrease of PXR in HepG2 cells. Western blotting confirmed that GA also reduced PXR protein levels (Fig. 3C and 3D). An immunofluorescence analysis revealed that PXR was mainly present in the cytosol of control cells, and its fluorescence intensity decreased in response to GA treatment for 12 h; this decrease was more prominent as GA concentrations increased (Fig. 3E). Moreover, after HepG2 cells were treated with 1 μM GA for 12 h, cytosolic and nuclear extracts were prepared, and western blot analysis showed that the expressions of PXR decreased both in nucleus and cytoplasm (Fig. 3F). These results demonstrate that GA down-regulates the expression of PXR and suggest that the inhibition of PXR might be responsible for the GA-mediated repression of CYP3A4. Our data also indicate that MDR1, another PXR target gene, decreased at both the mRNA and protein levels (Fig. 3G and 3H) after GA treatment.

3.4 PXR is required for GA-mediated suppression of CYP3A4 expression

To determine the role of PXR in the GA-mediated downregulation of CYP3A4, we performed knockdown and overexpression experiments to selectively modulate the expression of PXR. First, HepG2 cells were transfected with a PXR shRNA construct or the corresponding vector for 12 h. Then, the CYP3A4 and PXR expression levels were analyzed using qRT-PCR and Western blotting. PXR knockdown significantly decreased the expression of CYP3A4 at both the mRNA and protein levels (Fig. 4A and 4B). Next, a construct encoding hPXR was transfected into HepG2 cells. After

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treatment with 1 μM GA for 24 h, the expression of CYP3A4 and PXR were measured in cells transfected with hPXR. Contrary to the results of the PXR knockdown experiment, PXR overexpression significantly increased CPY3A4 (P<0.05, #), and GA markedly decreased CYP3A4 expression in PXR overexpressing and normal cells (Fig. 4C and 4D). Moreover, GA significantly decreased PXR expression in both the PXR overexpressing and normal cells. These results suggest that PXR is required for GA-mediated repression of CYP3A4 and that downregulation of PXR reduces CYP3A4 levels in HepG2 cells.

3.5 GA induces DEC1 expression and translocation

HepG2 cells were treated with different concentrations of GA for 24 h or 1 μM GA for 2, 4, 8, 12 and 24 h, and DEC1 mRNA and protein levels were assessed using qRT-PCR and Western blotting, respectively. DEC1 mRNA levels significantly increased after GA treatment in a concentration-dependent manner (Fig. 5A). DEC1 mRNA levels continuously increased after 2 h treatment with GA (Fig. 5B), whereas a significant decrease in CYP3A4 was not observed until 4 h after treatment (Fig. 1B), indicating that the decrease in the CYP3A4 expression occurred after the increase in DEC1. The GA-mediated increase in DEC1 protein was confirmed by Western blotting (Fig. 5C and 5D). Immunofluorescence revealed that DEC1 was present in the cytosol and nuclei of control cells, and it translocated to the nucleus in response to 12 h GA treatment; more prominent DEC1 fluorescence was observed as the concentration of GA increased (Fig. 5E). Moreover, after HepG2 cells were treated

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with 1 μM GA for 12 h, cytosolic and nuclear extracts were prepared, and western blot analysis showed that the expressions of DEC1 increased both in nucleus and cytoplasm, especially in nucleus (Fig. 5F). These results demonstrate that GA up-regulates DEC1 expression and translocation, indicating that it might be partially responsible for the GA-mediated repression of CYP3A4.

3.6 DEC1 is required for the GA-mediated suppression of CYP3A4 expression

To determine the role of DEC1 in the downregulation of CYP3A4 in response to GA, we performed knockdown and overexpression experiments to selectively modulate the expression of DEC1. First, HepG2 cells were transfected with a Flag-DEC1 construct or the corresponding vector for 48 h. Then, the expression levels of CYP3A4, DEC1 and PXR were analyzed in cells transfected with Flag-DEC1. DEC1 overexpression significantly decreased CPY3A4 expression (Fig. 6A and 6B), but it did not alter PXR expression (data not shown). Next, HepG2 cells were transfected with a DEC1 shRNA construct or the corresponding vector for 12 h. After treatment with 1 μM GA for 24 h, the expression levels of CYP3A4 and DEC1 were analyzed in cells transfected with DEC1 shRNA. DEC1 knockdown alone had a minor effect on CYP3A4 expression. Interestingly, GA markedly decreased CYP3A4 expression, and DEC1 knockdown partially abolished the GA-mediated suppression of CYP3A4 expression (Fig. 6C and 6D). These results suggest that DEC1 is required for GA-mediated repression of CYP3A4 and that overexpression of DEC1 reduced CYP3A4 in HepG2 cells.

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4. Discussion

The Chinese Food and Drug Administration approved GA, a promising anticancer agent that is not toxic toward normal tissues, for phase II clinical trials in solid cancer. Recently, GA was demonstrated to produce synergistic activities against gastrointestinal cancer when combined with docetaxel, oxaliplatin and 5-fluorouracil [47-49]. Moreover, GA increases the chemotherapeutic effect of CDDP [50], cisplatin [51] and Adriamycin [52], suggesting that the combination of GA and chemotherapeutic agents might be a promising therapeutic approach for cancer. However, the effect of GA on drug metabolism enzymes is unknown. In this study, we report for the first time that GA is a potent repressor of the cytochrome P450 3A4 enzyme, which is responsible for the oxidative metabolism of more than 60% of all pharmaceuticals [3,4].

CYP3A4 is a highly polymorphic enzyme with pronounced constitutive inter-individual expression in the human population, and these characteristics form the basis for its clinically significant drug interactions and toxicities [5,6]. In addition to polymorphic CYP genes, factors such as age, diet, hormonal status, disease, and exposure to drugs or xenobiotics, may contribute to the wide variability in the expression of certain CYPs [8,9]. An insulin-like growth factor 1 receptor inhibitor (BMS-665351) [53], baicalin [54] and 8-MOP [46] induce the expression of CYP3A4, whereas IL-6 [33] represses the expression of CYP3A4. Our results indicate that GA treatment decreased CYP3A4 at both the mRNA (Fig. 1A and 1B) and protein (Fig. 1C and 1D) levels in HepG2 cells, and similar results were confirmed by enzymatic

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assay (Fig. 1E). Thus, GA may decrease other drugs' biotransformations by suppressing CYP3A4 activity. The observed decrease in CYP3A4 mRNA and protein levels might be due to transcriptional repression and/or increased CYP3A4 mRNA degradation. In this study, the GA-mediated decrease in CYP3A4 was prevented by adding a RNA synthesis inhibitor (Fig. 2A), suggesting that GA-mediated CYP3A4 repression occurs at the transcriptional level, which was confirmed using CYP3A4 promoter experiments (Fig. 2B).

The hepatic expression of CYP3A4 is highly dependent on the activity of several transcription factors and nuclear receptors, among which PXR is a key regulator [16,55]. In the present study, the PXR mRNA and protein expression levels were both decreased after GA treatment (Fig. 3A-D). Immunofluorescence analysis confirmed that GA suppressed PXR and blocked its nuclear translocation (Fig. 3E and 3F). A continuous decrease in PXR mRNA was observed after 2 h treatment with GA (Fig. 3B), whereas a significant continuous decrease in CYP3A4 occurred after 4 h of treatment (Fig. 1B), suggesting that the decrease in CYP3A4 expression occurred after the decrease of PXR in HepG2 cells. In addition to decreased CYP3A4 expression, the expression of another PXR target, MDR1 [56], was also significantly decreased after GA treatment (Fig. 3F and 3G). The suppression of P-glycoprotein function by GA synergistically potentiates Adriamycin-induced apoptosis in lung cancer [52]. Our results suggest that PXR plays a key role in the GA-mediated suppression of MDR1.

The role of PXR in the GA-mediated regulation of CYP3A4 was confirmed by shRNA knockdown of PXR, which significantly reduced CYP3A4 expression at both the mRNA

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and protein levels in HepG2 cells (Fig. 4A and 4B). In contrast, overexpression of PXR dramatically increased CYP3A4 expression, and GA markedly decreased CYP3A4 expression in both PXR-overexpressing and normal cells (Fig. 4C and 4D), suggesting that GA represses the expression of CYP3A4 by downregulating PXR.

PXR is regulated by several cellular modulators and processes involving protein-protein interactions with different co-repressors [21,57] and coactivators [58,59]. In addition, PXR is post-transcriptionally regulated by miRNAs (miR-148a) [60]. Several post-translational modifications are involved in PXR regulation, including ubiquitination [61] and acetylation [62].

Recently, Cho et al. reported that DEC1 repressed the transactivation of RXR heterodimers, including the liver X receptor (LXR), farnesoid X receptor (FXR), vitamin D receptor (VDR), and retinoic acid receptor (RAR) [63]. One study showed that hepatic DEC1 mediates the ligand-dependent LXR signal to regulate the expression of genes that are involved in the hepatic clock system and metabolism [64]. We previously demonstrated that DEC1 transcriptionally downregulates the CYP3A4 expression in response to IL-6 [33], indicating for the first time that DEC1 is involved in metabolizing xenobiotics, including the vast majority of clinically used drugs, environmental pro-carcinogens and toxins. We demonstrated that IL-6 repressed CYP3A4 expression by increasing DEC1 expression in human hepatocytes and that DEC1 binds to the CCCTGC sequence in the CYP3A4 promoter to form a CCCTGC-DEC1 complex, which downregulates the activity of the CYP3A4 promoter.

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Here, we found that GA rapidly induces DEC1 expression, continuously reducing CYP3A4 expression in HepG2 cells. Meanwhile, the GA-mediated repression of CYP3A4 occurred after the increase in DEC1 in HepG2 cells (Fig. 2B and 5B). Therefore, the GA-mediated decrease in CYP3A4 expression in HepG2 cells is partially due to the rapid increase of DEC1 expression. To investigate the role of DEC1 in the downregulation of CYP3A4 in response to GA, we performed knockdown and overexpression experiments to selectively modulate the expression of DEC1. Overexpression of DEC1 dramatically decreased the expression of CYP3A4 (Fig. 6A and 6B), whereas DEC1 knockdown alone had little effect on CYP3A4 expression (Fig. 6C and 6D). Furthermore, GA significantly decreased CYP3A4 expression in cells transfected with the vector but not DEC1 shRNA.

This study produced several important conclusions. First, GA can repress CYP3A4 expression, which could reduce drug metabolism. Second, GA represses CYP3A4 by decreasing PXR expression in HepG2 cells; thus, GA may decrease the expression of all PXR target genes (such as MDR1). Therefore, GA could alter the function of PXR target genes and change the metabolism and transport of many drugs. Third, GA rapidly induces DEC1, and DEC1 binds to the CYP3A4 promoter to repress its activity, which partially contributes to the GA-mediated suppression of CYP3A4 (Fig. 7). Pharmacologically, the GA-mediated repression of PXR and induction of DEC1 suggests that these two important transcriptional factors influence a broad range of drug-drug interactions and that GA will alter the therapeutic effect and toxicity of many drugs.

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FIGURE LEGENDS

Figure 1. GA represses CYP3A4 expression and enzymatic activity in HepG2 cells. The effect of GA treatment on CYP3A4 mRNA levels in HepG2 cells. A, HepG2 cells were treated with the indicated concentrations of GA for 24 h, and CYP3A4 mRNA levels were evaluated by qRT-PCR. B, HepG2 cells were treated with 1 µM GA for 2, 4, 8, 12 and 24 h, and total RNA was isolated and subjected to qRT-PCR analysis to measure CYP3A4 mRNA levels. The effect of GA treatment on CYP3A4 protein expression in HepG2 cells. C, HepG2 cells were treated with the indicated concentrations of GA for 24 h, and CYP3A4 protein expression was evaluated by Western blotting. D, HepG2 cells were treated with 1 µM GA for 2, 4, 8, 12 and 24 h, and the expression level of CYP3A4 protein was determined by Western blotting. The effect of GA treatment on CYP3A4 enzymatic activity in HepG2 cells. E, HepG2 cells were treated with 0.25, 0.5 and 1.0 µM GA for 24 h, and the cells

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were prepared and assayed for CYP3A4 activity as described in the Materials and Methods. The effect of GA treatment on HepG2 cell viability. F, HepG2 cells were treated with 0.25, 0.5, 0.75, 1.0 and 1.25 μM GA for 24 h, and MTT assays were used to measure cell viability. All experiments were repeated at least three times, and the data are expressed as the mean ± SEM * $p<0.05$ and ** $p<0.01$ represent a statistically significant decrease in response to GA treatment.

Figure 2. GA suppresses CYP3A4 via a transcriptional mechanism. The effect of ACTD on the GA-mediated suppression of CYP3A4 mRNA in HepG2 cells. A, Cells were treated with 1 μM GA for 24 h in the absence or presence of 0.1 μM ACTD. Total RNA was prepared, and the levels of CYP3A4 and GAPDH were assessed by qRT-PCR. Repression of the CYP3A4-DP-Luc promoter reporter. B, HepG2 cells were transiently transfected with a mixture containing 50 ng of CYP3A4-DP-Luc and 5 ng of the Null-Renilla reniformis luciferase in the presence of PXR. The transfected cells were treated with 1 μM GA for 24 h. Luciferase activities were determined using a dual-luciferase reporter assay system, and the reporter activity was normalized to that of the Null-*Renilla reniformis* luminescence signal. All experiments were repeated at least three times, and the data are expressed as the mean ± SEM. * $p<0.05$ and ** $p<0.01$ represent a statistically significant decrease following GA treatment.

Figure 3. GA downregulates PXR expression. The effect of GA treatment on PXR mRNA levels in HepG2 cell. A, HepG2 cells were treated with the indicated

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concentrations of GA for 24 h, and PXR mRNA levels were evaluated by qRT-PCR. B, HepG2 cells were treated with 1 μM GA for 2, 4, 8, 12 and 24 h, and total RNA was isolated and subjected to qRT-PCR analysis to determine the expression level of PXR mRNA. The effect of GA treatment on PXR protein expression in HepG2 cells. C, HepG2 cells were treated with the indicated concentrations of GA for 24 h, and PXR protein expression was evaluated by Western blotting. D, HepG2 cells were treated with 1 μM GA for 2, 4, 8, 12 and 24 h, and PXR protein levels were determined by Western blotting. E, HepG2 cells were treated with the indicated concentrations of GA for 12 h, and PXR protein localization was detected by immunofluorescence analysis (400 ×), bar = 50 μm. F, Cells were treated with 1 μM GA for 12 h, and cytosolic and nuclear extracts were prepared, then PXR protein levels were determined by Western blotting. G and H, HepG2 cells were treated with the indicated concentrations of GA for 24 h, and the MDR1 mRNA level and protein expression levels were assessed by qRT-PCR and Western blotting, respectively. All experiments were repeated at least three times, and the data are expressed as the mean ± SEM. * $p<0.05$ and ** $p<0.01$ represent a statistically significant decrease following GA treatment.

Figure 4. The role of PXR in the GA-mediated suppression of CYP3A4 expression. The induction of CYP3A4 as a function of PXR. A and B, The effect of PXR shRNA on CYP3A4 suppression. HepG2 cells were transfected with vector or PXR shRNA constructs for 12 h, and total RNA and cell lysate were prepared to

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analyze the expression of CYP3A4, PXR and GAPDH by qRT-PCR and Western blotting, respectively. C and D, The effect of PXR overexpression on GA-mediated CYP3A4 suppression. Vector or human PXR was transfected into HepG2 cells for 48 h, and the cells were treated with GA (1 μM) for an additional 24 h. Total RNA and lysates were prepared to analyze the expression levels of CYP3A4, PXR and GAPDH by qRT-PCR and Western blotting, respectively. All experiments were repeated at least three times, and the data are expressed as the mean ± SEM. # $p<0.05$, ## $p<0.01$ represents a statistically significant increase by hPXR; ** $p<0.01$ represents a statistically significant decrease after GA treatment.

Figure 5. GA up-regulates DEC1 expression. The effect of GA treatment on the DEC1 mRNA level in HepG2 cells. A, HepG2 cells were treated with the indicated concentrations of GA for 24 h, and DEC1 mRNA levels were evaluated by qRT-PCR. B, HepG2 cells were treated with 1 μM GA for 2, 4, 8, 12 and 24 h, respectively; then, total RNA was isolated, and DEC1 mRNA expression was evaluated by qRT-PCR analysis. The effect of GA treatment on DEC1 protein expression in HepG2 cells. C, HepG2 cells were treated with the indicated concentrations of GA for 24 h, and DEC1 protein expression was evaluated by Western blotting. D, HepG2 cells were treated with 1 μM GA for 2, 4, 8, 12 and 24 h, DEC1 protein levels were determined by Western blotting. E, HepG2 cells were treated with the indicated concentrations of GA for 12 h, and DEC1 protein translocation was detected by immunofluorescence (400 ×), bar = 50 μm. F, Cells were treated with 1 μM GA for 12 h, and cytosolic and

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Figure 6. The role of DEC1 in the GA-mediated suppression of CYP3A4 expression.

Suppression of CYP3A4 as a function of DEC1. A and B, The effect of DEC1 overexpression on GA-mediated CYP3A4 suppression. HepG2 cells were transfected with vector or Flag-DEC1 for 48 h, and total RNA and lysates were prepared to analyze the expression of CYP3A4 and GAPDH by qRT-PCR and Western blotting, respectively. C and D, The effect of DEC1 shRNA on CYP3A4 expression. HepG2 cells were transfected with vector or DEC1 shRNA for 12 h, and total RNA and lysates were prepared to analyze the expression of CYP3A4 and GAPDH by qRT-PCR and Western blotting, respectively. All experiments were repeated at least three times, and the data are expressed as the mean ± SEM. # $p<0.05$ represents a statistically significant increase after DEC1 shRNA treatment; ** $p<0.01$ represents a statistically significant decrease after GA treatment.

Figure 7. Gambogic acid suppresses cytochrome P450 3A4 by downregulating pregnane X receptor and up-regulating DEC1 in human hepatoma HepG2 cells

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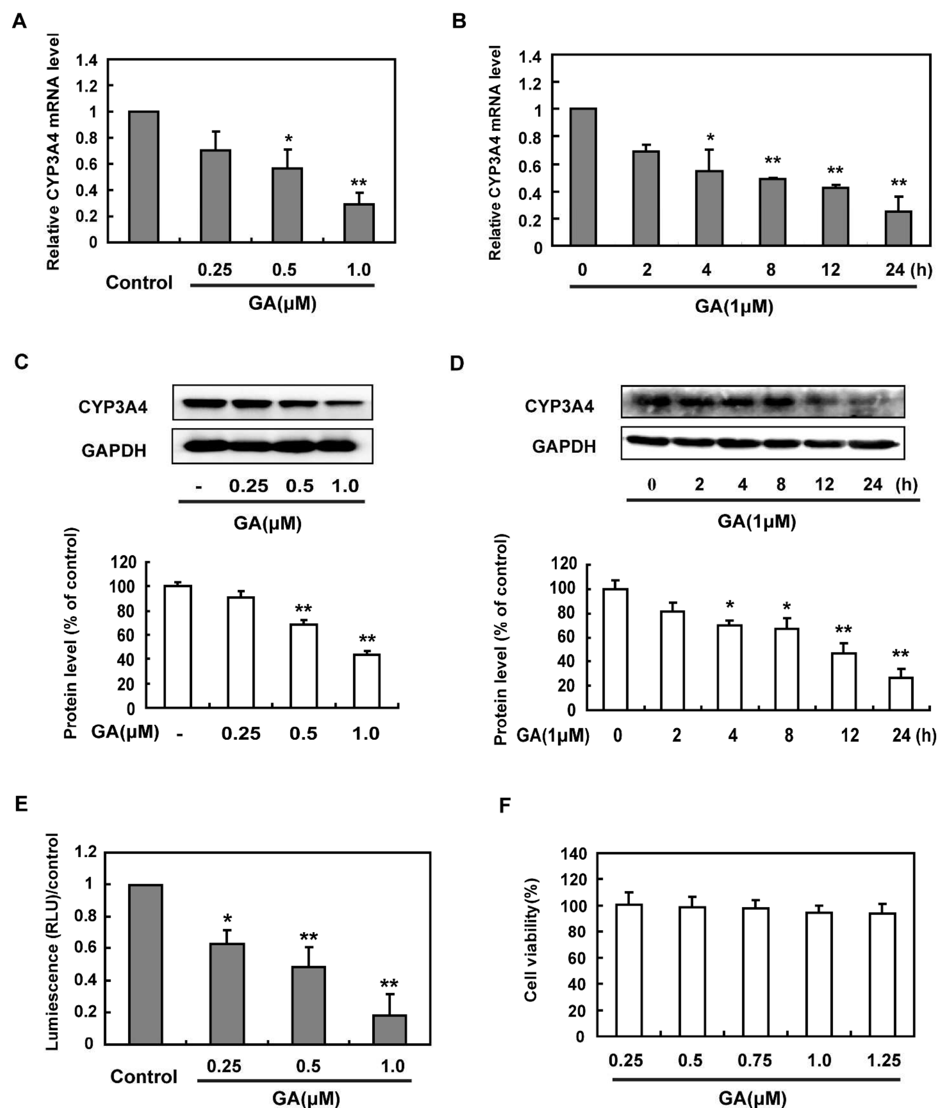

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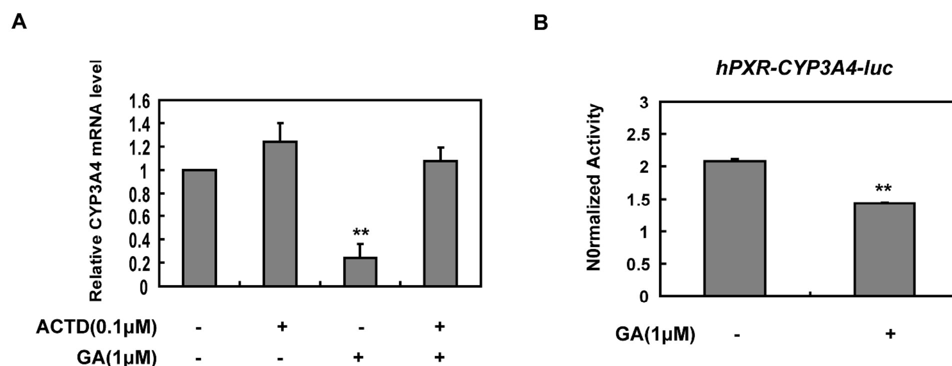

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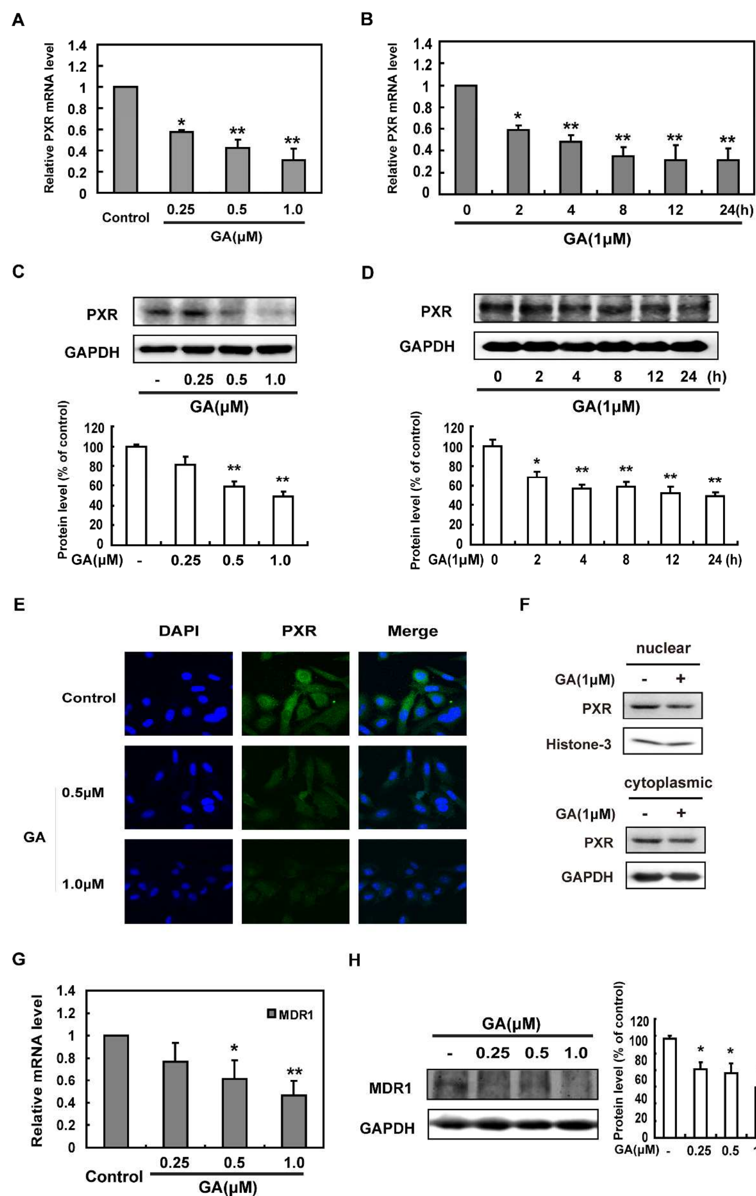

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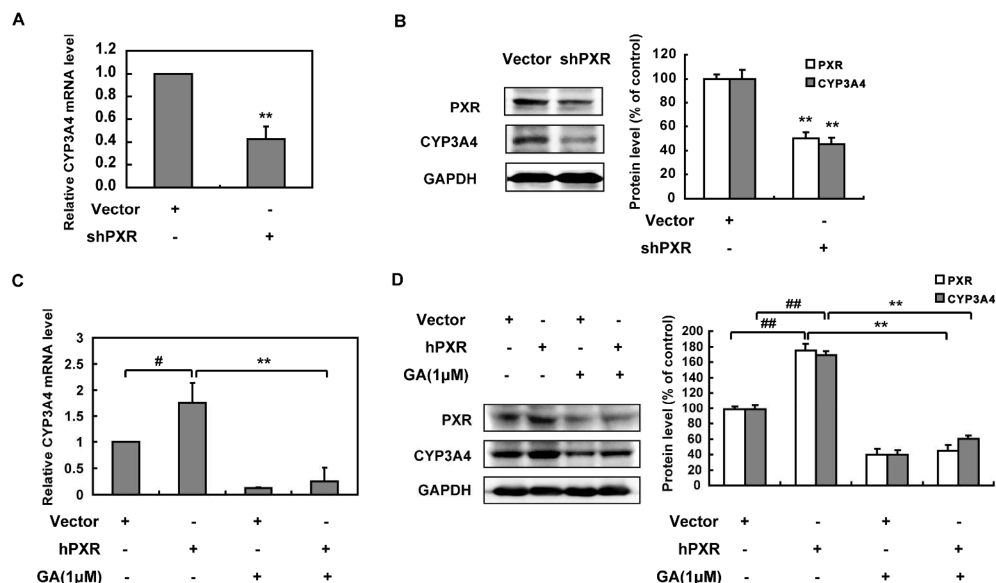

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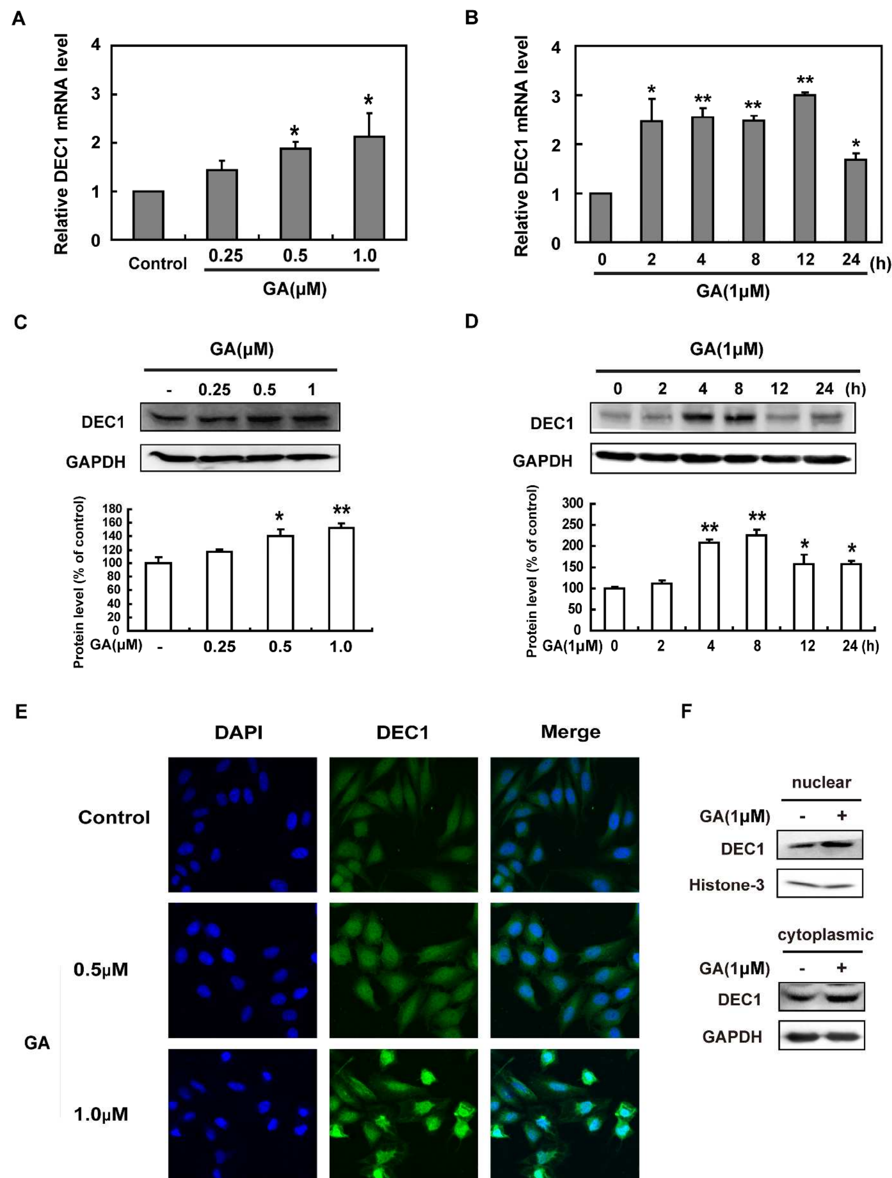

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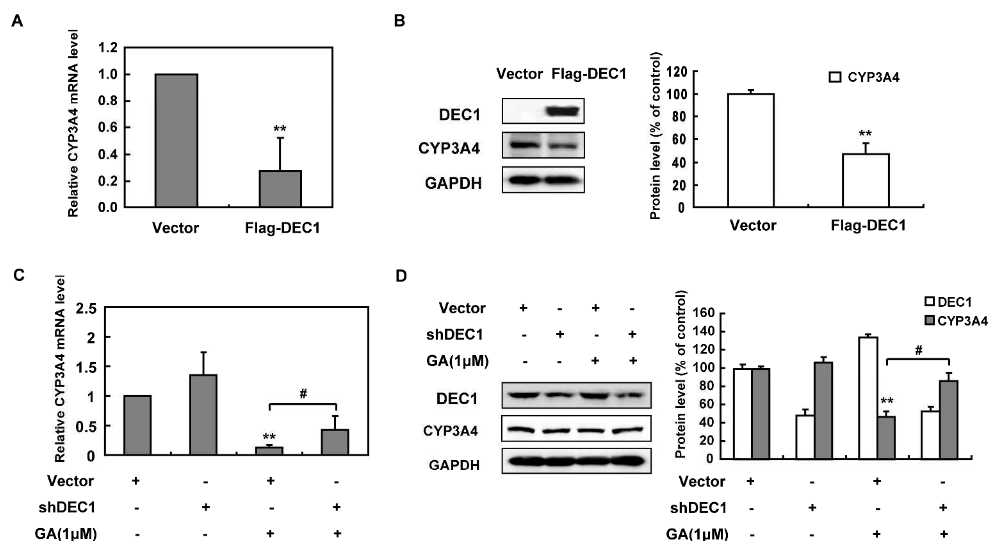

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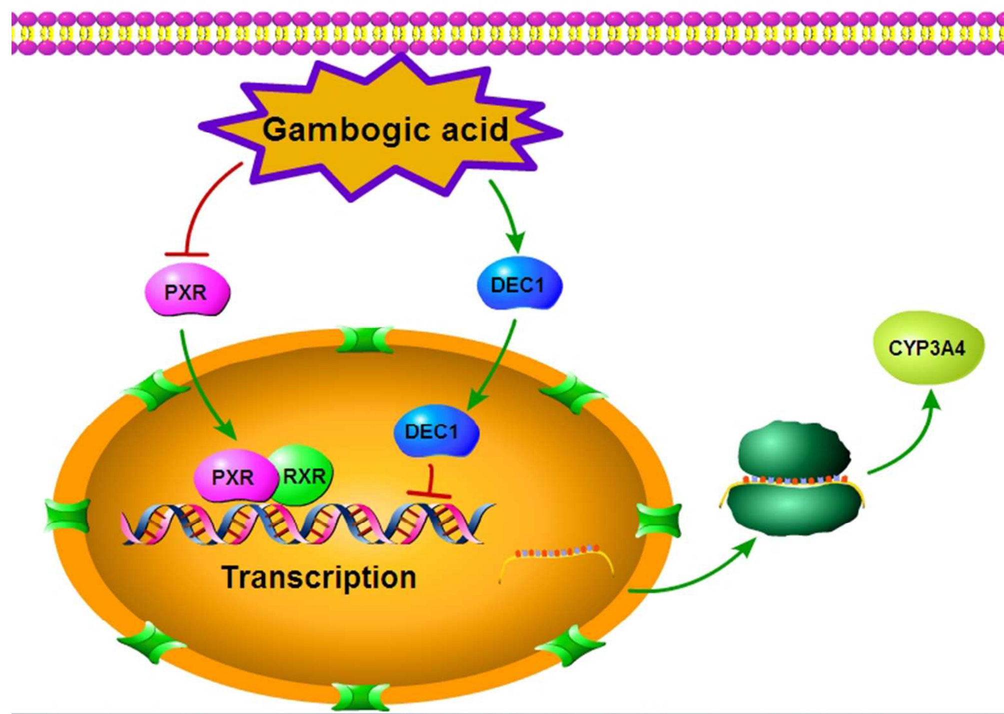

Figure 7. Gambogic acid suppresses cytochrome P450 3A4 by downregulating pregnane X receptor and up-regulating DEC1 in human hepatoma HepG2 cells
283x221mm (72 x 72 DPI)

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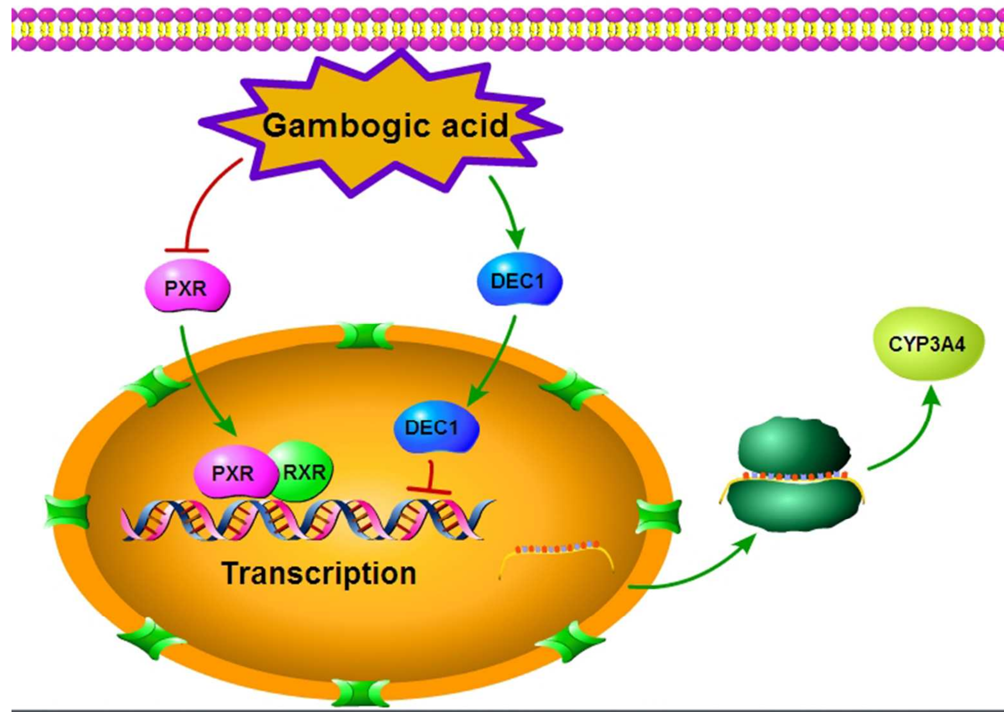

Figure 7. Gambogic acid suppresses cytochrome P450 3A4 by downregulating pregnane X receptor and up-regulating DEC1 in human hepatoma HepG2 cells
283x221mm (72 x 72 DPI)

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	GA reduces CYP3A4 activity by decreasing the expression of CYP3A4 in human HepG2 cells
	Involvement of transcription in the GA-mediated repression of CYP3A4
	GA downregulates PXR expression
	PXR is required for GA-mediated suppression of CYP3A4 expression
	GA induces DEC1 expression and translocation
	DEC1 is required for the GA-mediated suppression of CYP3A4 expression
	Gambogic acid suppresses cytochrome P450 3A4 by downregulating pregnane X receptor and up-regulating DEC1 in human hepatoma HepG2 cells

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