BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.01069.x
www.brjpharmacol.org

RESEARCH PAPER

Byakangelicin induces cytochrome P450 3A4 expression via transactivation of pregnane X receptors in human hepatocytes

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Keywords
byakangelicin; cytochrome P450 3A4; CYP3A4; pregnane X receptor; transcriptional regulator; transactivation; hepatocyte

Received
3 April 2010
Revised
5 August 2010
Accepted
27 August 2010

BACKGROUND AND PURPOSE

Byakangelicin is found in extracts of the root of *Angelica dahurica*, used in Korea and China as a traditional medicine to treat colds, headache and toothache. As byakangelicin can inhibit the effects of sex hormones, it may increase the catabolism of endogenous hormones. Therefore, this study investigated the effects of byakangelicin on the cytochrome P450 isoform cytochrome (CY) P3A4 in human hepatocytes.

EXPERIMENTAL APPROACH

Cultures of human hepatocytes and a hepatoma cell line (Huh7 cells) were used. mRNA and protein levels were measured by quantitative reverse transcription-polymerase chain reaction and Western blot. Plasmid constructs and mutants were prepared by cloning and site-directed mutagenesis. Reporter (luciferase) activity was determined by transient co-transfection experiments.

KEY RESULTS

In human primary hepatocytes, byakangelicin markedly induced the expression of CYP3A4 both at the mRNA level (approximately fivefold) and the protein level (approximately threefold) but did not affect expression of human pregnane X receptor (hPXR). In reporter assays, byakangelicin activated CYP3A4 promoter in a concentration-dependent manner (EC_{50} = 5 μM), and this activation was enhanced by co-transfection with hPXR. Further reporter assays demonstrated that the eNR4 binding element in the CYP3A4 promoter was required for the transcriptional activation of CYP3A4 by byakangelicin.

CONCLUSIONS AND IMPLICATIONS

Byakangelicin induced expression and activity of CYP3A4 in human hepatocytes. This induction was achieved by the transactivation of PXR and not by increased expression of PXR. Therefore, byakangelicin is likely to increase the expression of all PXR target genes (such as MDR1) and induce a wide range of drug–drug interactions.

Abbreviations

CYP450, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hPXR, human pregnane X receptor; PCR, polymerase chain reaction; PXR, pregnane X receptor; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction

Introduction

Byakangelicin is one of the furanocoumarins extracted from the root of *Angelica dahurica* which has been used in Korea and China as a traditional medicine to treat colds, headache and toothache (Tang and Eisenbrand, 1992; Kimura *et al.*, 1996; The Pharmacopoeia Commission of PRC, 2005). Byakangelicin also protects against tacrine-induced cytotoxicity in HepG2 cells (Oh *et al.*, 2002). In addition to this hepatoprotective effect, byakangelicin exerts anti-inflammatory effects and protects against the effects of induced by lipopolysaccharide (Kim *et al.*, 1991; Ngwendson *et al.*, 2003; Song *et al.*, 2005a,b). The anti-inflammatory mechanisms include the inhibition of tumour necrosis factor-α (Kim *et al.*, 1991; Ok-Hwa *et al.*, 2007), of histamine release (Kimura and Okuda, 1997) and of PGE_2 through decreased cyclocoxygenase-2 (Ban *et al.*, 2003; Ok-Hwa *et al.*, 2007), besides its potent antioxidant effects (Piao *et al.*, 2004).

Early studies reported that byakangelicin could inhibit the effects of sex hormones (Pakrashi, 1967, 1968). Therefore, in this study, we investigated using human hepatocytes, the effects of byakangelicin on cytochrome P450 3A4 (CYP3A4; nomenclature follows Alexander *et al.*, 2009), an enzyme that plays an important role in the oxidative biotransformation and inactivation of many xenobiotic compounds. We show for the first time that byakangelicin is a potent inducer of this enzyme and, as a result, byakangelicin is likely to exert a wide range of effects on the actions of many endogenous and exogenous pharmacological agents (Mandlekar *et al.*, 2006; Murray, 2006; Rodeiro *et al.*, 2008).

Methods

The culture and treatment of human primary hepatocyte and Huh7 cells

Human primary cultured hepatocytes in 6-well plates were obtained from the Liver Tissues Procurement and Distribution System (University of Minnesota, Minneapolis, MN, USA or CellzDirect, Pittsboro, NC, USA). The eight hepatocyte donors (four white and four black) were five males (36–75 years old) and three females (35–77 years old). They are all non-smokers. Upon arrival, the media were replaced with Willians'E medium containing insulin-transferrin-selenium G supplement and penicillin/streptomycin (Yang and Yan, 2007). After incubation at 37°C with 5% CO_2 for 24 h, the hepatocytes were treated with the corresponding concentration of byakangelicin or rifampicin for 24 h (to determine the mRNA level) or 48 h (to determine the protein level). Hepatoma (Huh7) cells were purchased from American Type Culture Collection (Mannassas, VA, USA), and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% de-lipidated fetal bovine serum, penicillin/streptomycin and 1× non-essential amino acids. Huh7 cells were seeded at the density of 2.5×10^5 cells per well (12-well plates) in a regular medium for 12 h, and treated with the corresponding concentration of byakangelicin (10 μM), rifampicin (10 μM) or dimethyl sulphoxide (DMSO) (0.1%) for another 24 h (to determine the mRNA level). The treated cells were cultured in a 1% serum-reduced medium.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated by using a RNA-Bee (Tel-Test Inc., Friendswood, TX, USA) according to the manufacturer's instruction and checked by formaldehyde gel electrophoresis for quality control. The first-strand cDNA was synthesized using total RNA (1 μg) at 25°C for 10 min, 42°C for 50 min, and 70°C for 10 min by using random primers and Moloney murine leukaemia virus reverse transcriptase (Promega, Madison, WI, USA). The cDNAs were then diluted eight times and quantitative polymerase chain reaction (PCR) was conducted with TaqMan Gene Expression Assay kits (Applied Biosystems, Foster City, CA, USA). The TaqMan assay identification numbers are: CYP3A4, Hs00604506_m1; PXR (NR1I2), Hs00243666_m1; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 4352934E. A 20 μL PCR mix contained 10 μL of universal PCR master mixture, 1 μL of gene-specific TaqMan assay mixture (probe), 6 μL of diluted cDNA as template and 3 μL of water. Cycling profile was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, as recommended by the manufacturer. The PCR amplification and quantification were carried out in an Applied Biosystems 7900 real-time PCR system (Applied Biosystems).

Plasmid constructs

The expression construct encoding the human pregnane X receptor (hPXR) was described previously (Zhang *et al.*, 1999). Two CYP3A4 reporters were prepared with the pGL3 basic vector to contain the proximal element alone (–362 to +53, CYP3A4-P) or the proximal fused to the distal element (–362 to +53 and –7836 to –6093, CYP3A4-DP-Luc) (Song *et al.*, 2004; 2005b). Mutated constructs targeted on different distal element regions, which were presented diagrammatically in Figure 4A, were prepared by PCR using the targeting primers shown in Table 1. The fragments harbouring these elements were amplified by PCR with primers that were extended to include appropriate endonucleases (bglII/MulI) to facilitate the subsequent ligation. CYP3A4-DP-ER6M-Luc, CYP3A4-DP-DR3M-Luc, CYP3A4-DP-eNR4M1-Luc, CYP3A4-DP-eNR4M2-Luc were prepared from the CYP3A4-DP-Luc with site-directed mutagenesis and the primers were shown in Table 2. Complementary oligonucleotides were synthesized to introduce a substitution. The primers were annealed to human promoter constructs and subjected to a thermocycler for a total of 18 cycles. The resultant PCR-amplified constructs were then digested with DpnI to remove the non-mutated parent construct. The mutated PCR-amplified constructs were used to transform XL-1Blue bacteria. The sequences of all of the CYP3A4 reporter gene constructs were verified by direct DNA sequencing.

Transient co-transfection experiment

Huh7 cells were plated in 48-well plates in DMEM supplemented with 10% de-lipidated fetal bovine serum at a density of 8×10^4 cells·well^{-1}. Transfection was conducted by FUGENE HD (Roche Diagnostics, Indianapolis, IN, USA). The transfection mixtures contained 50 ng of PXR plasmid, 50 ng of a reporter plasmid and 5 ng of Null-*Renilla reniformis* luciferase plasmid. Huh7 cells were transfected for 12 h and the

Table 1

Primers used for preparation of mutated constructs of CYP3A4 reporter gene

Construct	Primer	5′–3′
CYP3A4(–7701/–6093)P-luc	sense	TTTACGCGTCCTCCAGCC TCTCGGTGCC CT
	anti-sense	TAGAGATCTTAGATCTTGAAACATGTTTCTTTCCTT
CYP3A4(–7658/–7200)P-luc	sense	TTTACGCGTGCCTCA TGAGGCATTA CAAAG
	anti-sense	TAGAGATCTTCGTCAACAGGTTAAAGGAGAATGG
CYP3A4(–7658/–7467)P-luc	sense	TTTACGCGTGCCTCA TGAGGCATTA CAAAG
	anti-sense	TAGAGATCTTGTTTACCATGTGCACATATTACC
CYP3A4(–7742/–7658)P-luc	sense	TTTACGCGTAGCTGAATGAACTTGCTGA
	anti-sense	TAGAGATCTCTTTGTAATGCCTCATGAGGC

The underlined letters indicate the restriction sites.

Table 2

Primers used for preparation of mutated constructs of CYP3A4 reporter gene

Construct	Primer	5′–3′
CYP3A4-DP-ER6M-luc	sense	TAGAATATGAATTcaaaggACGTCAGTGAGT
	anti-sense	ACTCACTGACGTcctttgAATTCATATTCTA
CYP3A4-DP-DR3M-luc	sense	TCTCAGCTGAAGCTTCTtgcTGACCCTCTG
	anti-sense	CAGAGGGTCAgcaAGAAGCTTCAGCTGAGA
CYP3A4-DP-eNR4M1-luc	sense	TATTAAACCTTATCGTgtgtTGACCC
	anti-sense	GGGTCAacacACGATAAGGTTTAATA
CYP3A4-DP-eNR4M2-luc	sense	TGTCCTGTGTTATCGCcaggTGAATC
	anti-sense	GATTCAcctgACGATAACACAGGACA

The underlined letters indicate the nucleotides substituted, and the highlight indicates half-site.

medium was replaced with fresh medium supplemented with 1% de-lipidated fetal bovine serum with the corresponding concentrations of byakangelicin (10 μM), rifampicin (10 μM) or DMSO (0.1%). After another 24 h of treatment, the cells were washed once with phosphate-buffered saline and then collected by scraping. The collected cells were subjected to two cycles of freeze/thaw. The reporter enzyme activities were assayed with Dual-Luciferase reporter assay system. This system contained two substrates, the firefly luminescence and *Renilla* luminescence, which were used to determine the activities of two luciferases sequentially. The firefly luciferase activity, which reflected the reporter activity, was initiated by mixing an aliquot of lysates (10 μL) with Luciferase Assay Reagent II (Promega). Then, the firefly luminescence was quenched, and the *Renilla* luminescence was simultaneously activated by adding Stop & Glo reagent (Promega) to the sample tubes. The firefly luminescence signal intensity was normalized based on the *Renilla* luminescence signal intensity, and the ratio of normalized luciferase activity from byakangelicin or rifampicin over DMSO treatment served as relative luciferase activity or fold of induction.

Western blot analysis

Human primary cultured hepatocytes lysates (8 μg) were resolved by 7.5% sodium dodecyl sulphate- polyacrylamide gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane. After non-specific binding sites were blocked with 5% non-fat milk, the blots were incubated with an antibody against CYP3A4 (1:2500), PXR (1:2500) and GAPDH (1:5000). The primary antibodies were raised against human PXR- and CYP3A4-specific peptides and the production and purification of antibodies were described previously (Lindley *et al.*, 2002; Sachdeva *et al.*, 2003). The primary antibodies were subsequently localized with goat anti-rabbit IgG conjugated with horseradish peroxidase, the activity of which was detected with a chemiluminescent kit (Pierce, Rockford, IL, USA). The chemiluminescent signal was captured by KODAK Image Station 2000 (Estman Kodak, Rochester, NY, USA), and the relative intensities were quantified by KODAK Image Analysis software (Estman Kodak).

Enzymic assays

Primary hepatocytes were treated with byakangelicin (10 μM), rifampicin (10 μM) or DMSO (0.1%) for 48 h, and

the cells were rinsed with phosphate-buffered saline and harvested in 200 μL of 100 mM potassium phosphate buffer (pH 7.4). The cell suspension was sonicated by a sonifier (Branson Ultrasonics Corporation, Danbury, CT, USA), and cell debris was removed by centrifugation at 12 000 g for 15 min at 4°C. The supernatant was assayed for CYP3A4 activity with a P450-Glo kit (CYP3A4) (Promega) (Yang and Yan, 2007) according to the manufacturer's manual. Briefly, cell lysates (16.5 μg in 12.5 μL) were mixed with 12.5 μL of CYP3A4 substrate Luciferin-BE (4×). After a 10 min pre-incubation at 37°C, the NADP regeneration mixture (25 μL containing 400 mM KPO4) was added to initiate the reaction. The reaction lasted for 30 min at 37°C and was terminated by adding 50 μL of Luciferin Detection Reagent (Promega). After additional 10 min incubation at room temperature, the luminescent signal was determined by EGΣG BERTHOLD Microplate Luminometer (PerkinElmer, Waltham, MA, USA). Several controls were performed including incubation without cell lysates or the regeneration system.

Protein concentrations were determined with BCA assay (Pierce) based on the albumin standard (Yang and Yan, 2007).

Data analysis

Data are presented as mean ± SEM from at least three independent experiments, except where the results of blots are shown, in which case a representative experiment is depicted in figures. Statistical analysis was performed using SAS software version 9.1 (SAS Institute, Cary, NC, USA). Significant differences between treatments were claimed at $P < 0.05$ based on one-way analysis of variance followed by Duncan's multiple comparison tests.

Materials

Byakangelicin was purchased from J & K Chemical Company and its purity is 99% (Beijing, China), Hank's balanced salt solution, actinomycin D and Williams'E medium were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM, high-fidelity platinum Taq DNA polymerase and insulin-transferrin-selenium G supplement were purchased from Invitrogen (Carlsbad, CA, USA). QuickChange Lightning Site-Directed Mutagenesis Kit was from Agilent Technologies (Santa Clare, CA, USA). Dual-luciferase reporter assay system was from Promega. De-lipidated and normal fetal bovine serum was from Hyclone Laboratories (Logan, UT, USA). The antibody against GAPDH was from Abcam (Cambridge, UK). The goat anti-rabbit IgG conjugated with horseradish peroxidase was from Pierce Chemical (Pierce). Nitrocellulose membrane was from Bio-Rad Laboratories (Hercules, CA, USA). All the other reagents were purchased from Fisher Scientific (Fair-Lawn, NJ, USA).

Results

Induction of CYP3A4 expression and its oxidative activity, by byakangelicin, in primary cultured human hepatocytes

Primary cultured human hepatocytes were treated with byakangelicin (5, 10 μM), rifampicin (10 μM) or DMSO (0.1%) for 24 h (to determine the mRNA level) or 48 h (to determine the protein level). Expression of CYP3A4 was monitored by qRT-PCR and Western blotting. As shown in Figure 1, although there was a variation in the basal CYP3A4 expression, byakangelicin induced CYP3A4 expression at both the mRNA level (~fivefold) and the protein level (~threefold). Similarly rifampicin, as a positive control, induced CYP3A4 expression at both the mRNA level (~sixfold) and the protein level (~4.5-fold) (Figure 1A,C). However, no increase was detected in the protein level of GAPDH, a housekeeping gene in the same experiment (Figure 1C).

To determine whether the increase of CYP3A4 mRNA can be translated into the increase in the oxidative activity, primary hepatocytes were treated with byakangelicin (10 μM), rifampicin (10 μM) or DMSO (0.1%) for 48 h, and the cell lysates were analysed for CYP3A4 oxidative activity. Our results showed that the oxidative activity of CYP3A4 was significantly increased in human hepatocytes treated with byakangelicin by about twofold, and after rifampicin by about threefold (Figure 1B), which agreed with the increase of CYP3A4 at both the mRNA level and the protein level(Figure 1A,C).

Expression of the PXR was not induced by byakangelicin in human hepatocytes

To determine whether the induction of the CYP3A4 expression by byakangelicin was due to the content of PXR in hepatocytes, the changes in the expression of PXR and CYP3A4 mediated by byakangelicin were observed in human hepatocytes and in Huh7 cells. Results indicated that the PXR expression in primary cultured hepatocytes treated with byakangelicin, rifampicin or DMSO did not change significantly (Figure 2C), although the CYP3A4 expression increased markedly (Figure 1A). Byakangelicin induced CYP3A4 expression in both a concentration-dependent manner (0–50 μM) and a time-dependent manner (0–24 h) but did not induce PXR expression in Huh7 cells (Figure 2A,B). These results suggest that the increased CYP3A4 expression induced by byakangelicin was not mediated by and increased expression of PXR.

Transcriptional activation involvement in CYP3A4 induction by byakangelicin

To determine the cause of the increase in the CYP3A4 mRNA level, a transcriptional inhibition assay was performed by using a RNA synthesis inhibitor, actinomycin D, to inhibit RNA synthesis (Yang and Yan, 2007). Huh7 cells were treated with byakangelicin (10 μM) and actinomycin D (5 μM) for 9 h separately and together. qRT-PCR results indicated that actinomycin D abolished the byakangelicin-mediated transcriptional activation of CYP3A4 in hepatoma Huh7 cells (Figure 3A).

The abolition of byakangelicin-mediated activation of CYP3A4 by a RNA synthesis inhibitor suggested that byakangelicin increased the expression of CYP3A4 through activating its promoter. To test this hypothesis, co-transfection was performed using a CYP3A4 promoter reporter and a human PXR (hPXR) construct in Huh7 cells. The CYP3A4-DP-Luc construct contained both proximal and distal promoter regions that respond to many CYP3A4 inducers (Song *et al.*,

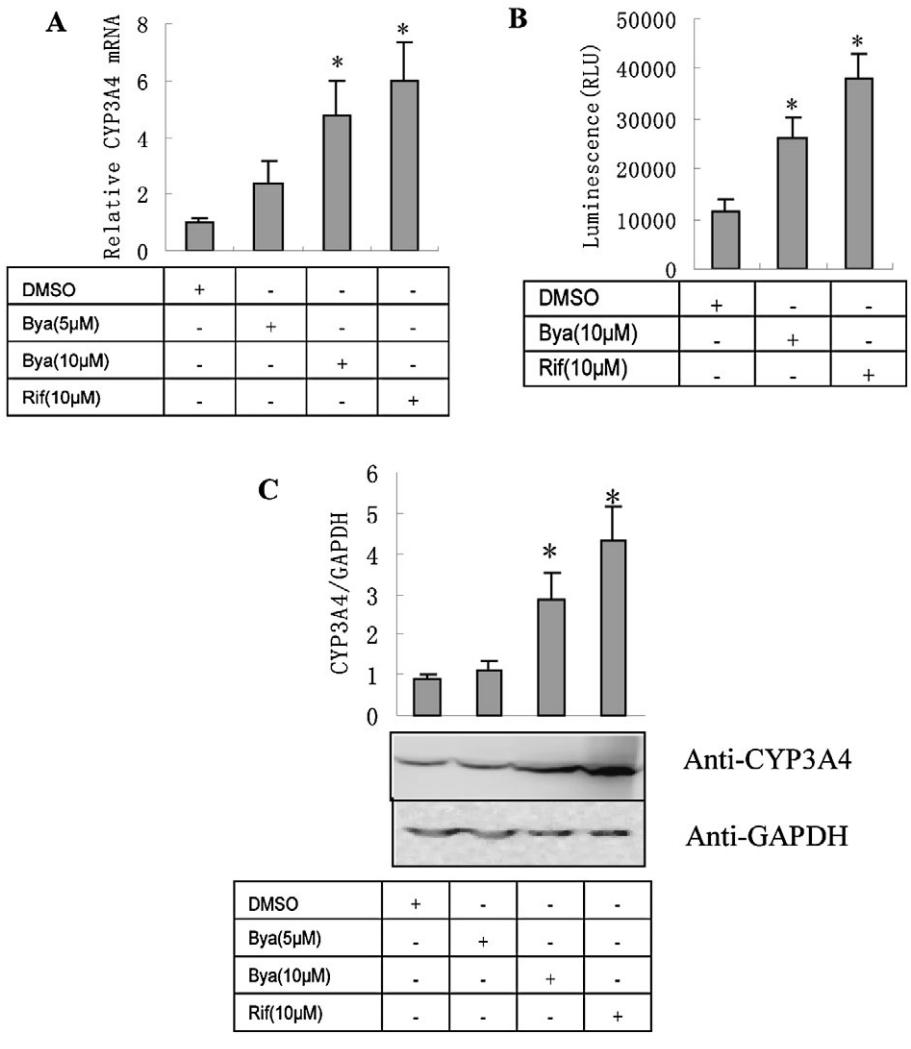

Figure 1

Effect of byakangelicin on cytochrome (CY)P3A4 expression and enzymatic activity in primary human hepatocytes. (A) Effect of byakangelicin (Bya) on the CYP3A4 mRNA level in primary human hepatocytes (4 individuals). Human primary hepatocytes were treated with 5, 10 μM byakangelicin, 10 μM rifampicin (Rif) or dimethyl sulphoxide (DMSO) (0.1%) for 24 h. Total RNA was isolated and subjected to quantitative reverse transcription-polymerase chain reaction analysis for the CYP3A4 mRNA level by TaqMan probes. The signals from each target were normalized based on the signal from glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the Ct of CYP3A4 in human hepatocytes was around 23 cycles ($n = 4$). (B) Effect of byakangelicin on enzymic activity of CYP3A4 in primary hepatocytes ($n = 3$). (C) Effect of byakangelicin on the CYP3A4 protein level in primary hepatocytes. Human hepatocytes were treated with byakangelicin (5, 10 μM), rifampicin (10 μM) or DMSO (0.1%) for 48 h (the media were changed every 24 h) and cell lysates were prepared and assayed for the expression and activities of CYP3A4 in human hepatocyte lysates (8 μg each sample) were subjected to Western blot analyses with antibody against CYP3A4, or GAPDH ($n = 3$). All experiments described in this figure were repeated at least three times, and data are expressed as mean ± SEM. *$P < 0.05$, statistically significant increase by byakangelicin or rifampicin treatment.

2005b). Hepatoma cells (Huh7) were transfected with CYP3A4-DP-Luc and Null-*Renilla* plasmid with or without hPXR, and then treated with byakangelicin (10 μM), rifampicin (10 μM) or DMSO (0.1%). After 24 h incubation, the cells were lysed and the luciferase activities were determined. The cells treated with byakangelicin significantly induced the activity of CYP3A4-DP promoter reporter with or without hPXR (Figure 3B left). Furthermore, the activation of CYP3A4 promoter reporter by byakangelicin (10 μM) in cells transfected with hPXR (5.06 ± 0.71-fold) was much higher than that without hPXR (1.35 ± 0.07-fold). The basal activity of CYP3A4-DP-Luc co-transfected with hPXR was much higher than that without hPXR (Figure 3B left). Similar results were obtained in Huh7 cells treated with rifampicin (as a positive control) (Figure 3B right). Byakangelicin activated CYP3A4-DP-Luc promoter reporter in a dosage-dependent manner and its EC_{50} was 5 μM, which was higher than that of rifampicin (EC_{50} = 3.7 μM) (Figure 3C). These data imply that the byakangelicin-mediated activation of CYP3A4 is likely to occur at the transcriptional level and the activation of PXR promotes the byakangelicin-mediated increase of the CYP3A4 expression in hepatocytes.

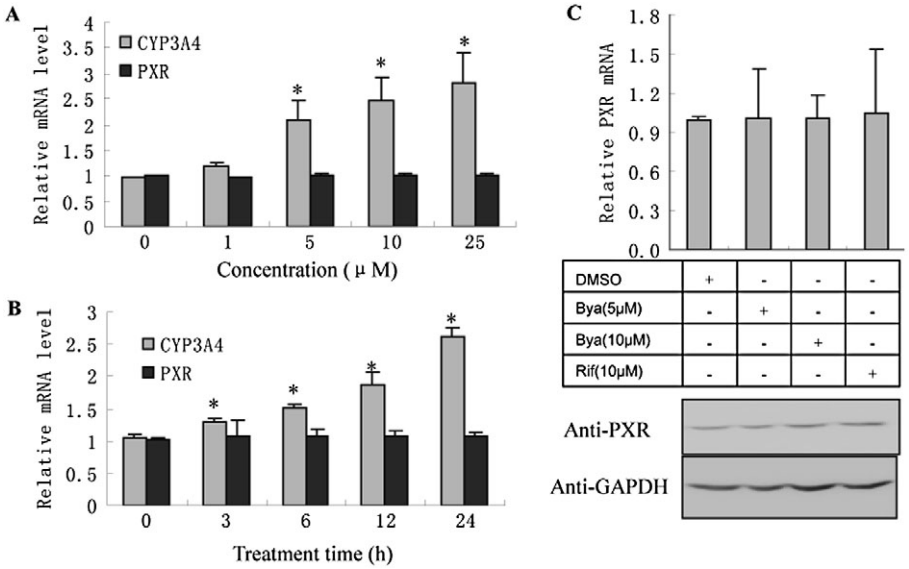

Figure 2

Byakangelicin increased cytochrome (CY)P3A4 expression but did not alter pregnane X receptor (PXR) expression in hepatocytes. (A) Concentration-dependent effect of byakangelicin (Bya) on mRNA for CYP3A4 and PXR. Huh7 cells were treated with various concentrations of byakangelicin (0–25 μM), and total RNA was prepared. The levels of CYP3A4 and PXR mRNA were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The mRNA levels were expressed relative to that in control cells (considered as 1) ($n = 4$). (B) Time course of CYP3A4 and PXR mRNA after byakangelicin. Huh7 cells were treated with byakangelicin for 0, 3, 6, 12 and 24 h, and total RNA was prepared at various time points after the initial treatment. The levels of CYP3A4 and PXR mRNA were determined by qRT-PCR. The mRNA levels were expressed compared to those in control cells (considered as 1) ($n = 4$). (C) Effect of byakangelicin on the PXR protein level in primary human hepatocytes. Human hepatocytes were treated with byakangelicin (5, 10 μM), rifampicin (Rif, 10 μM) or dimethyl sulphoxide (DMSO) (0.1%) for 48 h. Cell lysates were prepared and were subjected to Western blot analyses (8 μg each sample) with antibody against PXR, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ($n = 3$). All experiments described in this figure were repeated at least three times, and the data are expressed as mean ± SEM. *$P < 0.05$, statistically significant increase by byakangelicin or rifampicin treatment.

The mediation of the PXR distal response element region in CYP3A4 promoter to transcriptional activation by byakangelicin

The CYP3A4-DP-Luc had a DR3-containing fragment (distal region) fused to the proximal promoter (Figure 4A). Goodwin *et al*. (1999) reported that the proximal region and part of the distal region of the CYP3A4 promoter are required for its maximum activation. However, the response of the CYP3A4 promoter without the distal region to the PXR-mediated transactivation is much less than that of the promoter with the distal region (Song *et al*., 2005b). Next, we determined which part of the distal region was mainly responsible for the transactivation mediated by byakangelicin. Different CYP3A4 promoters including CYP3A4-DP-Luc, CYP3A4(–7701/–6093)P-Luc, CYP3A4-(–7658/–7200)P-Luc, CYP3A4(–7658/–7467)P-Luc, CYP3A4(–7742/–7658)P-Luc and CYP3A4-P-Luc were tested for the differential transactivation mediated by byakangelicin. Based on the fold of induction, all the CYP3A4 promoter reporters we used exhibited significant activation in response to byakangelicin except CYP3A4(–7742/–7658)P-Luc and CYP3A4-P-Luc. The maximum activation (5.06 ± 0.76-fold) mediated by byakangelicin was detected in the CYP3A4-DP-Luc reporter, and the minimum (1.39 ± 0.31-fold) was detected in the CYP3A4-P-Luc reporter (Figure 4B). Similar pattern results were obtained in the cells treated with rifampicin, although the transactivation in response to rifampicin was higher than that in response to byakangelicin (Figure 4B,C). These results suggest that one of the critical transactivation regions of byakangelicin is located in the distal region (–7658 to –7467) of CYP3A4 promoter, which is contained the PXR binding element (eNR3A4)(Toriyabe *et al*., 2009).

The PXR binding element eNR3A4 is required for the transcriptional activation of CYP3A4 by byakangelicin

To further elucidate the role of three PXR binding elements in the byakangelicin-induced transactivation of the CYP3A4 gene, CYP3A4-DP-ER6M-Luc, CYP3A4-DP-DR3M-Luc, CYP3A4-DP-eNR4M1-Luc and CYP3A4-DP-eNR4M2-Luc(Figure 5, left) were used. As shown in Figure 5, byakangelicin significantly transactivated the CYP3A4-DP-ER6M-Luc, CYP3A4-DP-DR3M-Luc but did not significantly transactivate CYP3A4-DP-eNR4M1-Luc or CYP3A4-DP-eNR4M2-Luc, although the transactivation of CYP3A4-DP-ER6M-Luc and CYP3A4-DP-DR3M-Luc is much lower than that of wild-type CYP3A4-DP-Luc. These results were consistent with the results from the CYP3A4-DP-Luc, CYP3A4(–7701/–6093)P-Luc(DR3 deleted), CYP3A4-(–7658/–7200)P-Luc, CYP3A4(–7658/–7467)P-Luc(eNR4 deleted). The data suggest that the binding element eNR4 is

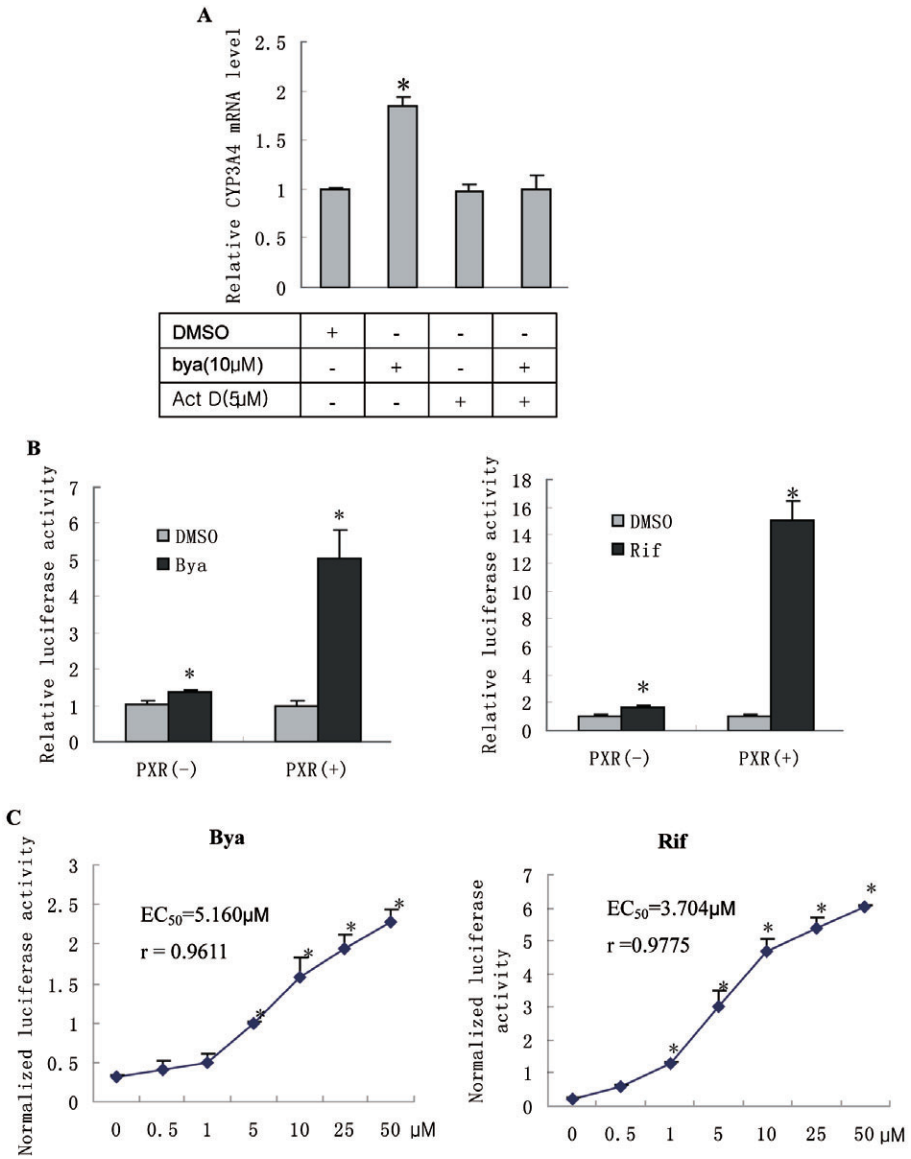

Figure 3

Pregnane X receptor (PXR) and transcriptional involvement in transactivation of cytochrome (CY)P3A4 by byakangelicin. (A) Effect of actinomycin D on the transactivation of CYP3A4 mRNA hepatocytes. Huh7 cells were treated with byakangelicin (Bya; 10 μM) or dimethyl sulphoxide (DMSO) (0.1%) in the absence or presence of 5 μM actinomycin D (Act D) for 9 h. Total RNA was prepared and analysed for CYP3A4 mRNA level by quantitative reverse transcription-polymerase chain reaction (n = 3), and data are expressed as mean ± SEM. *P < 0.05, statistically significant increase by byakangelicin treatment. (B) PXR involvement in the transactivation of CYP3A4 promoter reporters by byakangelicin. Huh7 cells in 48-well plates (8×10^4 cells per well) were transiently co-transfected by FUGENE HD with a mixture containing 50 ng of CYP3A4-DP-Luc with or without 50 ng human PXR (hPXR), and along with 5 ng of Null-*Renilla reniformis* plasmid (reference activity). After 12 h incubation, the transfected cells were treated with byakangelicin (10 μM), rifampicin (Rif, 10 μM) or DMSO (0.1%) for another 24 h, then the luciferase activities were determined with Dual-Luciferase reporter assay system and reporter activity was normalized based on Null-*Renilla reniformis* luminescence signal. The data are expressed as relative luciferase activity (the ratio of normalized luciferase activity from byakangelicin or rifampicin over DMSO treatment) (n = 4). (C) Activation of PXR by byakangelicin. Huh7 cells were co-transfected with human PXR and CYP3A4-DP-Luc and the transfected cells were treated with various concentrations of byakangelicin or rifampicin (0–50 μM). The reporter activities were determined with Dual-Luciferase reporter assay system and are expressed as normalized luciferase activity (based on Null-*Renilla reniformis* luminescence signal) (n = 3). All experiments described in this figure were repeated at least three times, and data are expressed as mean ± SEM. *P < 0.05, statistically significant increase by byakangelicin or rifampicin treatment.

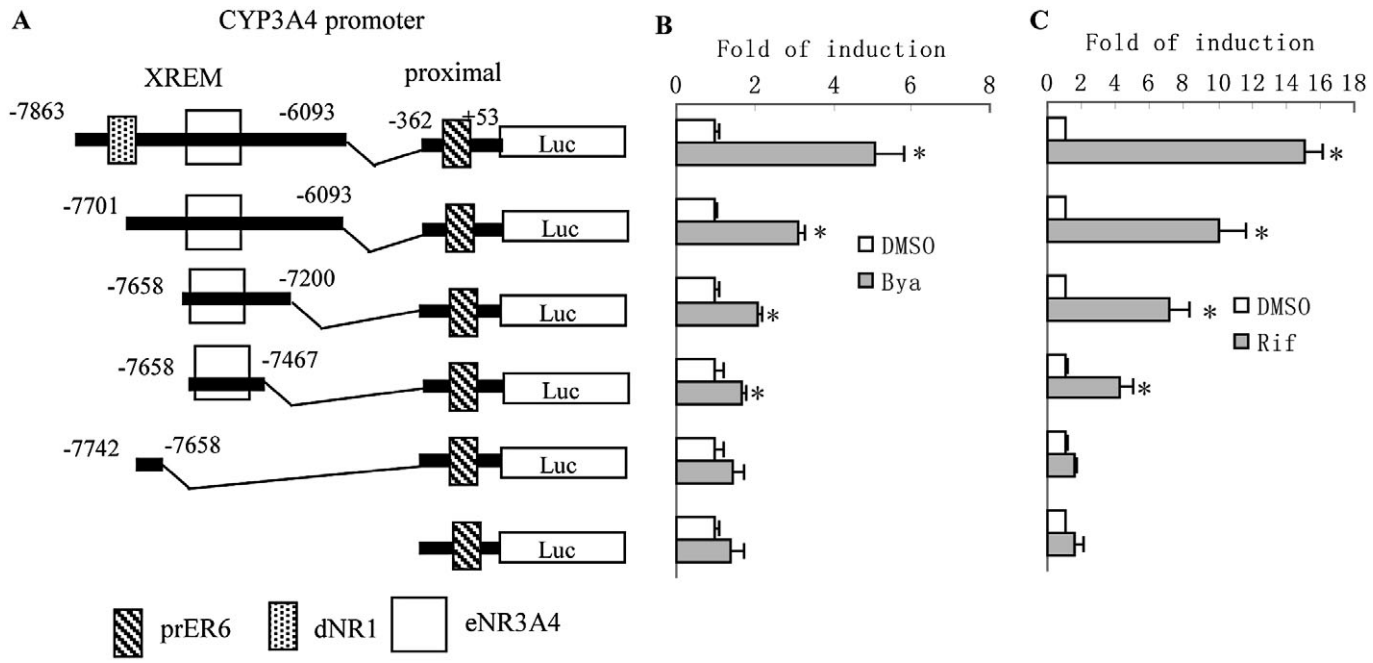

Figure 4

Differential activation of different cytochrome (CY)P3A4-Luc promoter reporters. (A) Diagrammatic presentation of a series of CYP3A4 luciferase reporter gene constructs. A series of CYP3A4 reporter constructs were prepared. (B) Differential activation of different CYP3A4 reporters by byakangelicin (Bya). (C) Differential activation of different CYP3A4 reporters by rifampicin (Rif). Huh7 cells in 48-well plates (8×10^4 cells per well) were transiently co-transfected different CYP3A4-DP-Luc which contained different distal region or CYP3A4-P-Luc and human pregnane X receptor (hPXR). After 12 h incubation, the transfected cells were treated with byakangelicin (10 μM), rifampicin (10 μM) or dimethyl sulphoxide (DMSO) (0.1%) as described above, then, the luciferase activities were determined with Dual-Luciferase reporter assay system, and reporter activity was normalized based on Null-*Renilla reniformis* luminescence signal (n = 3). The data are expressed as fold of induction (the ratio of normalized luciferase activity from byakangelicin over DMSO treatment). Three independent experiments were performed and data are expressed as mean ± SEM. *P < 0.05, statistically significant increase by byakangelicin or rifampicin treatment.

essential for the transcriptional activation of CYP3A4 by byakangelicin.

Discussion

Byakangelicin is a furanocoumarin extracted from the roots of *Angelica dahurica*. As these extracts have anti-inflammatory and analgesic effects (Tang and Eisenbrand, 1992; Ngwendson *et al*., 2003; Song *et al*., 2005a; Ok-Hwa *et al*., 2007), they are used for treating colds, flu, acne, sinusitis, headache, toothache, menstrual disorder and neuralgia (Tang and Eisenbrand, 1992; Lechner *et al*., 2004; The Pharmacopoeia Commission of PRC, 2005; Shinsuke and Mitsuo, 2010). Early studies reported that byakangelicin could inhibit the effects of sex hormones, which implied that byakangelicin was likely to increase the metabolism of the endogenous hormones (Pakrashi, 1967; 1968). In this study, we report for first time that byakangelicin is an effective inducer of CYP3A4, a major enzyme involved in the metabolism of a wide range of drugs and other xenobiotics. In human primary hepatocytes and hepatoma cells (Huh7), byakangelicin markedly increased the expression of CYP3A4 (Figures 1A,2A,B). The increase of CYP3A4 mRNA suggests two possibilities: (i) byakangelicin stimulates the transcription and/or (ii) decreases the degradation of mRNA. We have presented evidence to support the first possibility. First, the byakangelicin-mediated activation was abolished by actinomycin D, a RNA synthesis inhibitor (Figure 3A), suggesting that there is an involvement of transcriptional activation mechanism. Second, CYP3A4-DP-Luc was markedly activated in Huh7 cells treated with byakangelicin (Figure 3B,C). More importantly, the activation of the promoter mediated by byakangelicin was comparable with the extent of the increased mRNA and protein (Figures 1,2A,B, 3B,C), which provides direct evidence to confirm that the transcriptional activation is mainly responsible for the increase of the CYP3A4 expression. Finally, we have located a genomic sequence in the CYP3A4 gene which determines response to byakangelicin. Studies on the CYP3A4 gene promoter transactivation via ligand-activated nuclear receptors have uncovered several functional acting response elements that are common for PXR and other nuclear receptors. Barwick *et al*. identified two AG(G/T)TCA hexamers composing an everted repeat separated by six nucleotides (ER6) in the proximal promoter of CYP3A4 (prER6; –169/–152)(Barwick *et al*., 1996), and this region was later identified as a PXR binding response element(Blumberg *et al*., 1998). Goodwin *et al*. discovered an xenobiotic-responsive enhancer module located between –7.8 and –7.2 kb upstream of the CYP3A4 transcription start site with a functional DR3

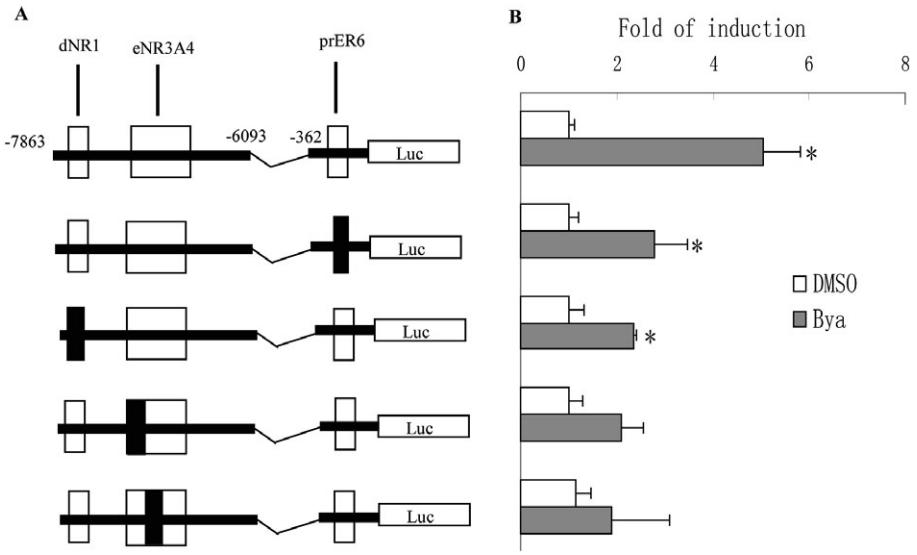

Figure 5

Evaluation of the role of three pregnane X receptor (PXR) binding elements in the byakangelicin-induced transactivation of cytochrome (CY)P3A4 gene in Huh7 cells. A. Diagrammatic presentation of CYP3A4 promoter mutants. PXR binding elements in the CYP3A4-DP-luc were mutated. Open and closed boxes represent wild-type and mutated PXR binding elements, respectively. B. Differential activation of CYP3A4 promoter mutants by byakangelicin (Bya). Huh7 cells were co-transfected with CYP3A4-DP-Luc or each mutant of CYP3A4-DP-Luc and human PXR, and the transfected cells were treated with byakangelicin (10 μM) or dimethyl sulphoxide (DMSO) (0.1%) for 24 h. the luciferase activities were determined with Dual-Luciferase reporter assay system. The data are expressed as fold of induction (the ratio of normalized luciferase activity from byakangelicin over DMSO treatment) ($n = 3$). Three independent experiments were performed and data are expressed as mean ± SEM. *$P < 0.05$, statistically significant increase by byakangelicin treatment.

nuclear receptor-binding element 1 (dNR1 or DR3, –7733/–7719)(Goodwin *et al.*, 1999). Recently, another DR4-type PXR binding *cis*-element (eNR3A4 or eNR4) in the CYP3A4 promoter region (–7618/–7558) was discovered (Toriyabe *et al.*, 2009). Using transient transfection assays with a set of deleted CYP3A4 reporter constructs containing different PXR response element, we found that byakangelicin significantly activated the promoter reporters of CYP3A4(–7836/–6093 362/+53)P-Luc(wild-type CYP3A4-DP-Luc), CYP3A4(–7701/–6093)P-Luc, CYP3A4 (–7658/–7200) P-Luc and CYP3A4 (–7658/–7467)P-Luc ($P < 0.05$, without activating the reporters of CYP3A4 (–7742/–7658)P-Luc and CYP3A4(–362/+53)-Luc(CYP3A4-P-Luc)($P > 0.05$) (Figure 4B). The results suggest that the sequence (–7658 to –7467) of the CYP3A4 promoter is a critical region, containing DR4-type PXR binding *cis*-element (eNR3A4) (Toriyabe *et al.*, 2009; Pavek *et al.*, 2010) for the response to byakangelicin. To further elucidate the role of three PXR binding elements in the byakangelicin-induced transactivation of CYP3A4 gene, the mutants of CYP3A4-DP-ER6M-Luc, CYP3A-DP-DR3M-Luc, CYP3A4-DP-eNR4M1-Luc and CYP3A4-DP-eNR4M2-Luc (Figure 5A) were prepared and used. The results showed that byakangelicin significantly transactivated the CYP3A4-DP-ER6M-Luc, CYP3A4-DP-DR3M-Luc but did not transactivate CYP3A4-DP-eNR4M1-Luc and CYP3A4-DP-eNR4M2-Luc, although the transactivation of CYP3A4-DP-ER6M-Luc and CYP3A4-DP-DR3M-Luc is much lower than that of wild-type CYP3A4-DP-Luc(Figure 5). Taken together, the results from the deleted and the base pair(s) mutated ER6 or eNR4 in CYP3A4-DP-Luc suggest that eNR4 is required for the transcriptional activation of CYP3A4 by byakangelicin.

Additionally, in the reporter assay, byakangelicin stimulated the activity of CYP3A4-DP-Luc reporter with PXR to a greater extent than that without PXR, although the basal activity of CYP3A4-DP-Luc promoter with PXR was higher than that without PXR (Figure 3B). Our data suggest that the induction of the CYP3A4 expression occurred at the transcriptional level and PXR was involved in this process.

Based on the fact that the PXR has been recognized as a key regulator that mediates the induction of many genes involved in catabolism of endogenous and exogenous substrates, including that for CYP3A (Watkins *et al.*, 2001; Xie *et al.*, 2001; Kliewer and Willson, 2002), the induction of the CYP3A4 expression mediated by byakangelicin might result from one or the other, or even both, of the following pathways: (i) byakangelicin could activate PXR as an agonist (like rifampicin) to induce CYP3A4 expression; (ii) byakangelicin could induce PXR expression and the increased PXR would promote CPY3A4 expression. In order to test these possibilities, we examined the PXR and CYP3A4 expression simultaneously in human primary hepatocytes and hepatoma Huh7 cells treated with byakangelicin. The result was that CYP3A4 expression mediated by byakangelicin increased markedly (Figure 1A), whereas PXR expression did not change (Figure 2C) in human primary hepatocytes. Moreover, byakangelicin induced CYP3A4 expression both in a concentration-dependent (0–50 μM) manner and a time-dependent (0–24 h) manner but did not induce PXR expres-

sion (Figure 2A,B) in hepatoma Huh7 cells. These data imply that byakangelicin induce CYP3A4 expression not through inducing the PXR expression but through promoting PXR transactivaction.

Clearly, such effective activation of PXR by byakangelicin suggests that this agent may induce many drug-metabolizing enzymes and transporters. Clearly, clinical studies are needed to determine the extent of pharmacokinetic interaction(s) resulting from PXR activation by byakangelicin. The concentration of byakangelicin used in this study was 10 μM (a typical concentration for *in vitro* induction studies), which caused a fivefold induction of CYP3A4 (Figure 1A) in primary human hepatocytes. Although the actual concentration in patients who take byakangelicin or the herbal medicine (*Angelica dahurica*) has not been firmly established, in dogs, a single oral administration of *Angelica dahurica* extract (30 $mg \cdot kg^{-1}$), which is comparable to 3.66 $mg \cdot kg^{-1}$ byakangelicin, gives a plasma Cmax of 1475 ± 400 $ng \cdot mL^{-1}$ byakangelicin (Xie *et al.*, 2007). These plasma levels are equivalent to concentrations of 3.3–5.6 μM. Based on current knowledge of byakangelicin plasma concentrations, it is unlikely that an interaction would occur in humans but that confirmation of the clinical relevance of the observation will require further study.

In summary, this study leads to several important conclusions. First, byakangelicin can activate CYP3A4 expression and its function. Therefore, the increased CYP3A4 is likely to increase the metabolism of many other drugs which may be given with byakangelicin. Second, the increase of CYP3A4 expression by byakangelicin is achieved not through increasing PXR expression but through activating PXR function in human hepatocytes. Thus byakangelicin is likely to increase the expression of all PXR target genes (such as MDR1). Third, byakangelicin is likely to be a ligand (an agonist) of human PXR (as is rifampicin), and the eNR4 binding element in the CYP3A4 promoter is required for the activation of CYP3A4 mediated by byakangelicin in human hepatocytes. Pharmacologically, the increase of CYP3A4 mediated by byakangelicin suggests that this agent could cause a wide range of drug–drug interactions, altered therapeutic action and even toxicity of many drugs, if co-administered with byakangelicin.

Acknowledgements

This work is supported by the Natural Science Foundation of China (No. 30772616), the National Key Basic Research Program of China (No.2009CB521906), and grants from the National Institutes of Health, USA (R01GM61988; F05AT003019).

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC). 4th edn. Br J Pharmacol 158 (Suppl. 1): S1–S254.

Ban HS, Lim SS, Suzuki K, Jung SH, Lee S, Lee YS *et al.* (2003). Inhibitory effects of furanocoumarins isolated from the roots of *Angelica dahurica* on prostaglandin E_2 production. Planta Med 69: 408–412.

Barwick JL, Quattrochi LC, Mills AS, Potenza C, Tukey RH, Guzelian PS (1996). Trans-species gene transfer for analysis of glucocorticoid-inducible transcriptional activation of transiently expressed human CYP3A4 and rabbit CYP3A6 in primary cultures of adult rat and rabbit hepatocytes. Mol Pharmacol 50: 10–16.

Blumberg B, Sabbagh WJ, Juguilon H, Bolado JJ, van Meter CM, Ong ES (1998). SXR, a novel steroid and xenobiotic-sensing nuclear receptor. Genes Dev 12: 3195–3205.

Goodwin B, Hodgson E, Liddle C (1999). The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. Mol Pharmacol 56: 1329–1339.

Kim CM, Heo MY, Kim HP, Sin KS, Pachaly P (1991). Pharmacological activities of water extracts of Umbelliferae plants. Arch Pharm Res 14: 87–92.

Kimura T, But PPH, Guo JX, Sung CK (1996). International Collection of Traditional and Folk Medicine: Part 1. World Scientific: Singapore.

Kimura Y, Okuda H (1997). Histamine-release effectors from Angelica dahurica var. dahurica root. J Nat Prod 60: 249–251.

Kliewer SA, Willson TM (2002). Regulation of xenobiotic and bile acid metabolism by the nuclear pregnane X receptor. J Lipid Res 43: 359–364.

Lechner D, Stavri M, Oluwatuyi M, Pereda-Miranda R, Gibbons S (2004). The anti-staphylococcal activity of Angelica dahurica (Bai Zhi). Phytochemistry 65: 331–335.

Lindley C, Hamilton G, McCune JS, Faucette S, Shord SS, Hawke RL *et al.* (2002). The effect of cyclophosphamide with and without dexamethasone on cytochrome P450 3A4 and 2B6 in human hepatocytes. Drug Metab Dispos 30: 814–822.

Mandlekar JL, Hong JL, Kong AN (2006). Modulation of metabolic enzymes by dietary phytochemicals: a review of mechanisms underlying beneficial versus unfavorable effects. Curr Drug Metab 7: 661–675.

Murray M (2006). Altered CYP expression and function in response to dietary factors: potential roles in disease pathogenesis. Curr Drug Metab 7: 67–81.

Ngwendson JN, Bedir E, Efange SM, Okunji CO, Iwu MM, Schuster BG *et al.* (2003). Constituents of Peucedanum zenkeri seeds and their antimicrobial effects. Pharmazie 58: 587–589.

Oh H, Lee HS, Kim T, Chai KY, Chung HT, Kwon TO (2002). Furocoumarins from Angelica dahurica with hepatoprotective activity on tacrine-induced cytotoxicity in Hep G2 cells. Planta Med 68: 463–464.

Ok-Hwa K, Go-Hoon L, Hyuk JC, Pil SP, Hee-Sung C, Seung-Il J *et al.* (2007). Ethyl acetate extract from Angelica Dahuricae Radix inhibits lipopolysaccharide-induced production of nitric oxide, prostaglandin E2 and tumor necrosis factor-alpha via

mitogen-activated protein kinases and nuclear factor-kappaB in macrophages. Pharmacol Res 55: 263–270.

Pakrashi A (1967). Endocrinological studies on plant products: V. Effect of byakangelicin on female sex hormones & on fertility of rats. Indian J Exp Biol 5: 75–79.

Pakrashi A (1968). Endocrinological studies on plant products. 8. Inhibitory effect of byakangelicin on and presence of sulphydryl group in pregnant mare's serum gonadotropin. Indian J Exp Biol 6: 212–215.

Pavek P, Pospechova K, Svecova L, Syrova Z, Stejskalova L, Blazkova J (2010). Intestinal cell-specific vitamin D receptor (VDR)-mediated transcriptional regulation of CYP3A4 gene. Biochem Pharmacol 79: 277–287.

Piao XL, Park IH, Baek SH, Kim HY, Park MK, Park JH (2004). Antioxidative activity of furanocoumarins isolated from Angelicae dahuricae. J Ethnopharmacol 93: 243–246.

Rodeiro I, Donato MT, Lahoz A, Garrido G, Delgado R, Gomez-Lechon MJ (2008). Interactions of polyphenols with the P450 system: possible implications on human therapeutics. Mini-Rev. Med Chem 8: 97–106.

Sachdeva K, Yan B, Chichester C (2003). Lipopolysaccharide and cecal ligation/puncture differentially affect the subcellular distribution of the pregnane X receptor but consistently cause suppression of its target genes CYP3A. Shock 19: 469–474.

Shinsuke M, Mitsuo M (2010). Beta-secretase inhibitory effects of furanocoumarins from the root of Angelica dahurica. Phytother Res 24: 510–513.

Song DK, Kim JY, Li G, Lee KS, Seo CS, Yan JJ (2005a). Agents protecting against sepsis from the roots of Angelica dahurica. Biol Pharm Bull 28: 380–382.

Song X, Xie M, Zhang H, Li Y, Sachdeva K, Yan B (2004). The pregnane X receptor binds to response elements in a genomic context-dependent manner, and PXR activator rifampicin selectively alters the binding among target genes. Drug Metab Dispos 32: 35–42.

Song X, Li Y, Liu J, Mukundan M, Yan B (2005b). Simultaneous substitution of phenylatine-305 and aspartate-318 of rat pregnane X receptor with the corresponding human residues abolishes the ability to transactivate the CYP3A23 promoter. J Pharmacol Exp Ther 312: 517–528.

Tang W, Eisenbrand G (1992). *Chinese Drugs of Plant Origin, Chemistry Pharmacology, and Use in Traditional and Modern Medicine*. Springer: Berlin.

The Pharmacopoeia Commission of PRC (2005). *The Pharmacopoeia of People's Republic of China, Part I*. Chemical Industry Press: Beijing, China, p. 69.

Toriyabe T, Nagata K, Takada T, Aratsu Y, Matsubara T, Yoshinari K *et al*. (2009). Unveiling a new essential cis-element for the transactivation of the CYP3A4 gene by xenobiotics. Mol Pharmacol 75: 677–684.

Watkins RE, Wisely GB, Moore LB, Collins JL, Lambert MH, Williams SP *et al*. (2001). The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. Science 292: 2329–2333.

Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES *et al*. (2001). An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. Proc Natl Acad Sci U S A 98: 3375–3380.

Xie Y, Chen Y, Lin M, Wen J, Fan G, Wu Y (2007). High-performance liquid chromatographic method for the determination and pharmacokinetic study of oxypeucedanin hydrate and byakangelicin after oral administration of Angelica dahurica extracts in mongrel dog plasma. J Pharm Biomed Anal 44: 166–172.

Yang J, Yan B (2007). Photochemotherapeutic agent 8-methoxypsoralen induces Cytochrome P450 3A4 and Carboxylesterase HCE2: Evidence on an Involvement of the Pregnane X Receptor. Toxicol Sci 95: 13–22.

Yang Y, Zhou F, Fang Z, Wang L, Li Z, Sun L *et al*. (2009). Post-transcriptional and post-translational regulation of PTEN by transforming growth factor-beta1. J Cell Biochem 106: 1102–1112.

Zhang H, LeCulyse E, Liu L, Hu M, Matoney L, Zhu W *et al*. (1999). Rat pregnane X receptor: molecular cloning, tissue distribution and xenobiotic regulation. Arch Biochem Biophys 368: 14–22.