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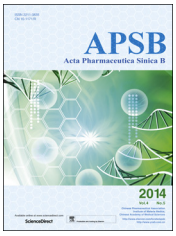

ORIGINAL ARTICLE

Three new shRNA expression vectors targeting the CYP3A4 coding sequence to inhibit its expression

Siyun Xu[a], Yongsheng Xiao[a], Li Li[a], Lushan Yu[a], Huidi Jiang[a], Aiming Yu[b], Su Zeng[a,*]

[a]*Department of Pharmaceutical Analysis and Drug Metabolism, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China*
[b]*Department of Biochemistry & Molecular Medicine, UC-Davis Medical Center, Sacramento, CA 95817, USA*

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Abstract RNA interference (RNAi) is useful for selective gene silencing. Cytochrome P450 3A4 (CYP3A4), which metabolizes approximately 50% of drugs in clinical use, plays an important role in drug metabolism. In this study, we aimed to develop a short hairpin RNA (shRNA) to modulate CYP3A4 expression. Three new shRNAs (S1, S2 and S3) were designed to target the coding sequence (CDS) of CYP3A4, cloned into a shRNA expression vector, and tested in different cells. The mixture of three shRNAs produced optimal reduction (55%) in CYP3A4 CDS-luciferase activity in both CHL and HEK293 cells. Endogenous CYP3A4 expression in HepG2 cells was decreased about 50% at both mRNA and protein level after transfection of the mixture of three shRNAs. In contrast, CYP3A5 gene expression was not altered by the shRNAs, supporting the selectivity of CYP3A4 shRNAs. In addition, HepG2 cells transfected with CYP3A4 shRNAs were less sensitive to Ginkgolic acids, whose toxic metabolites are produced by CYP3A4. These results demonstrate that vector-based shRNAs could modulate CYP3A4 expression in cells through their actions on CYP3A4 CDS, and CYP3A4 shRNAs may be utilized to define the role of CYP3A4 in drug metabolism and toxicity.

*Corresponding author. Tel./fax: +86 571 88208407.
E-mail address: zengsu@zju.edu.cn (Su Zeng).

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1. Introduction

RNA interference (RNAi) is an evolutionary conserved, cellular defensive mechanism that down-regulates gene expression at the post-transcriptional level[1,2]. RNAi mediated by small interfering RNA (siRNA) causes sequence-specific mRNA degradation of the targeted genes. This process involves multiple steps including the assembly of siRNA into the RNA-induced silencing complex (RISC), activation of the RISC, target recognition and binding, and thus target cleavage[3,4]. There are different ways to generate siRNAs for gene knockdown. The chemically synthesized siRNA oligos are suitable for target verification. The double strand RNAs (dsRNAs) that are transcribed and digested *in vitro* are usually used for the screening of siRNAs. The small hairpin RNAs (shRNAs) expressed from plasmid can be used for stable gene silencing and long-term studies. Therefore, vector-based expression of shRNA is increasingly used for delivery of siRNAs into mammalian cells[5,6].

The cytochrome P450 (CYP or P450) enzymes are encoded by 57 functional genes in humans; these enzymes catalyze the oxidative metabolism of a vast array of chemicals including drugs, carcinogens and toxins[7]. P450s are also involved in the biosynthesis or biodegradation of many endogenous compounds such as steroids, fatty acids and prostaglandins. Among them, CYP3A4 is the most abundant isoform expressed in human adult liver (60% of total P450) and intestine (70% of total P450), and plays an important role in metabolizing approximately 50% of drugs in clinical use[8,9]. The transcription of CYP3A4 is regulated by a number of nuclear receptors such as pregnane X receptor (PXR)[10,11] and constitutively activated receptor (CAR)[12]. In contrast to the understanding of nuclear receptor controlled transcriptional regulation of CYP3A4, there are only limited studies on the post-transcriptional regulation of CYP3A4 using RNAi at the 3′-untranslated region[13,14]. Therefore, we aimed to investigate the inhibitory effects of three new shRNA expression vectors that target the coding region sequence (CDS) of CYP3A4 in different cell models, and the consequent influence on the sensitivity of cells to CYP3A4 substrates.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco′s modified Eagle's medium, fetal bovine serum, Trypsin, Lipofectamine 2000 and TRIzol reagent were all purchased from Invitrogen (Carlsbad, CA). RevertAid™ Reverse Transcriptase was obtained from MBI Fermentas (Hanover, MD). Dual-Luciferase® Reporter Assay Kit was ordered from Promega (Madison, WI). Plasmid miniprep kit was purchased from Qiagen (Hilden, Germany). Oligonucleotide primers were synthesized by Sangon Co. (Shanghai, China). Antibodies of rabbit anti-human CYP3A4 were provided by AVIVA Systems Biology (California, U.S.A.). Alexa Fluor 488 F[ab']2 of goat anti-rabbit IgG [H+L] was from MultiSciences Biotech Co., Ltd. (Hangzhou, China), and horseradish peroxidase-labeled goat anti-rabbit IgG was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rifampin was purchased from MP Biomedicals Inc. (Eschwege, Germany).

2.2. Cell culture

Chinese hamster lung cells (CHL), Human Embryonic Kidney 293 cells (HEK293) and Human Hepatocellular Carcinoma cells (HepG2), all purchased from ATCC, were cultured in DMEM medium, supplemented with 10% FBS, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfates. Cell cultures were grown at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.3. Design of hairpin siRNA template oligonucleotides and construction of plasmids

Three hairpin siRNA (shRNA) template oligonucleotides (S1, S2 and S3), based on three different segments of the human CYP3A4 gene (GenBank accession no. NM_017460), were designed using the siRNA Target Finder and Design Tool available at http://www.ambion.com. The shRNA target sequences and the oligonucleotides designed to produce shRNAs are shown in Table 1. Two complementary oligonucleotides that encoded a hairpin structure with a 19-mer stem based on the mRNA target site were cloned

Table 2 IC_{50} of GA (15:1 and 17:1) in transfected HepG2 cells.

Group	IC_{50} (μmol/L)	
	GA 15:1	GA 17:1
Induced HepG2+shRNA plasmid	109±78	101±44
Induced HepG2+Negative plasmid	64±28	52±14

Table 1 Sequences and target of shRNA template oligonucleotides used in this study.

Sequence and target of shRNA template oligonucleotide	
Target sequence 1	AAAGTCGCCTCGAAGATACAC
Sense strand	5′–GATCCAGTCGCCTCGAAGATACACTTCAAGAGA GTGTATCTTCGAGGCGACTTTA-3'
Antisense strand	5′–AGCTTAAAGTCGCCTCGAAGATACACTCTCTTGAAGTGTAT CTTCGAGGCGACTG-3'
Position	773
Target sequence 2	AAACCACGAGCAGTGTTCTCT
Sense strand	5′–GATCCACCACGAGCAGTGTTCTCTTTCAAGAGAAGAGAAC ACTGCTCGTGGTTTA-3'
Antisense strand	5′–AGCTTAAACCACGAGCAGTGTTCTCTTCTCTTGAAAGAGA ACACTGCTCGTGGTG-3'
Position	923
Target sequence 3	AAGCTATGCTCTTCACCGTGA
Sense strand	5′–GATCCGCTATGCTCTTCACCGTGATTCAAGAGATCACGGTGAAGAGCATAGCTTA-3'
Antisense strand	5′–AGCTTAAGCTATGCTCTTCACCGTGATCTCTTGAATCACGGTGAAGAGCATAGCG-3'
Position	1191

into the pSilencer 4.1 CMV vector for siRNA expression. The pSilencer 4.1 CMV vector contained the restriction sites of BamHI and HindIII to ensure correct cloning into the linearized vector. The oligonucleotides were annealed and cloned into the pSilencer 4.1 CMV vector. Then pSilencer vectors were sequenced with universal primer to confirm that the oligonucleotides were connected to the desired site. A negative control vector was also designed by scrambling the nucleotide sequence of the gene-specific shRNA and conducting a blast search to ensure it lacks homology to any other gene. CYP3A4 CDS (1512bp), which was amplified from PGEMT-CYP3A4 CDS vector with primers with the forward oligonucleotides 5′–GCTCTAGAATGGC-TCTCATCCCAGACTT-3′ and reverse oligonucleotides 5′–GCGGCCGGCCTCAGGCTCCACTTACGGTGC-3′, was cloned into XbaI/FseI-digested pGL3 promoter vector (Promega). CYP3A4 CDS was inserted downstream of the Firefly luciferase gene.

2.4. *Luciferase assay*

All the transfection experiments were conducted with Lipofectamine 2000, following the manufacturer's instructions. Briefly, CHL cells were transiently cotransfected with pGL3/CYP3A4 CDS-luciferase reporter plasmid (0.1 μg) and shRNA plasmid or pS-NC plasmid (0.4 μg), together with pRL-TK plasmid (0.01 μg) which expresses Renilla luciferase. Luciferase activities were assayed 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Likewise, HEK293 cells were cotransfected with pGL3/CYP3A4 CDS-luciferase reporter plasmid (0.1 μg) and shRNA plasmid mixture or pS-NC plasmid (0.4 μg). Firefly luciferase activity was determined and normalized against Renilla luciferase activity and compared among different treatments[15].

2.5. *RT-PCR*

Specific PCR primer pairs for human *CYP3A4* and *CYP3A5* gene were derived from publication[14]. HepG2 cells treated with rifampicin were seeded in 24-well plates at a density of 2×10^5 cells per well and incubated overnight. The cells were transfected with shRNA plasmid or pS-NC plasmid. Total RNA was isolated from cultured cells using the TRIzol reagent following the instructions from the manufacturer 48 h later. The purity and integrity of the RNA was confirmed using an ultraviolet spectrometer before use. The RT reaction was carried out in a total volume of 20 μL, containing 2 μg of RNA, 50 μg/L $Oligo(dT)_{18}$ (Sangon Co.), 10 mmol/L dNTP mix, M-MLV 5 × reaction buffer, 40 U of RNase inhibitor, and 200 U of M-MLV at 42 °C for 1 h. PCR was performed by using the *CYP3A4* primers 5′–AAATCTGAGGCGGGAAGC-3′ (forward; F) and 5′–TTGGGATGAGGAATGGAAAG-3′ (reverse; R)[14]; *CYP3A5* primers 5′–ATGGAAAAATGTGGGGAACG-3′ (forward; F) and 5′–CGCTGGTGAAGGTTGGAGAC-3′ (reverse; R)[14,16]. The PCR reaction was conducted in a volume of 20 μL containing 0.3 μL of Taq enzyme (2.5 U/μL), 2 μL of dNTP at 2 mmol/L, 4 μL of RT product, 1 μL of each primer at 10 pmol, 2 μL of 10 × PCR reaction buffer. The PCR conditions were as follows: 95 °C for 2 min; 30 cycles of 95 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s; followed by incubation at 72 °C for 10 min. RT-PCR was also performed using primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene messages as a control. Five microliters of the amplified products were resolved by electrophoresis in a 1.0% agarose gel, and band intensities were determined by QuantityOne analysis software (Bio-Rad Laboratories, Segrate, Italy).

2.6. *Immunofluorescence chemistry*

HEK293 cells were seeded in 24-well plates at a density of 5×10^4 cells per well and incubated overnight. The cells were transiently cotransfected with pGL3/CYP3A4 CDS-luciferase reporter plasmid (0.1 μg) and shRNA plasmid or pS-NC plasmid (0.4 μg). 48 h later, the cells were fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). Then the cells were permeabilized with in 0.1% Triton X-100 plus 1% BSA in PBS/pH 7.3 for 10 min on ice, and blocked for 30 min in 1% BSA-PBS. The cells were incubated with primary antibody against CYP3A4 (AVIVA Systems Biology, California, USA) diluted in 1% BSA-PBS at 4 °C overnight and washed three times with PBS. Then the cells were incubated with secondary antibody (DyLight 488 conjugated of Goat Anti-rabbit IgG, MultiSciences Biotech Co., Ltd., Hangzhou, China) diluted in 1% BSA-PBS for 2 h at room temperature, and washed three times with PBS[17]. The antibody distribution was visualized with a DMI 3000B microscope (Leica), and images were acquired using Leica FW4000 version 1.2.1 software.

2.7. *Western blot analysis*

HepG2 cells treated with rifampicin or DMSO were seeded in 6-well plates at a density of 5×10^5 cells per well and incubated overnight. Cells were harvested at 48 h after transfection with 10 μL Lipofectamine 2000 and shRNA plasmid or pS-NC plasmid. The cell lysates were prepared with a buffer containing 50 mmol/L Tris- HCl, pH 7.5, 100 mmol/L NaCl, 5 mmol/L EDTA, 1% (*v*/*v*) Triton X-100, 1 mmol/L NaF, 1 mmol/L Na_3VO_4, 0.2 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. Lysates were centrifuged at $12{,}000 \times g$ at 4 °C for 10 min[14]. Protein concentrations were determined using the BCA Protein Assay Kit. Twenty-five μg of protein were separated on 7.5% SDS-polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes. The membranes were blotted with antibodies against antibodies of rabbit anti-human CYP3A4 (AVIVA Systems Biology, California, USA). To assure equivalent protein loading, the membranes were also incubated with mouse anti-human GAPDH monoclonal antibodies and subsequently with a corresponding horseradish peroxidase-conjugated second antibody IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The proteins were visualized with chemiluminescence detection kit for HRP (Biological Industries, Kibbutz Beit-Haemek, Israel). Images were acquired and densitometric analyses were conducted using QuantityOne analysis software (Bio-Rad Laboratories, Segrate, Italy).

2.8. *Cytotoxicity assay*

Cell viability was determined by MTT method. Five thousand cells/well were seeded in 96-well plates (Costar, Ann Arbor, MI). The next day, the HepG2 cells treated with rifampicin or DMSO were transfected with shRNA plasmid (0.2 μg) or pS-NC plasmid (0.2 μg). Drugs were added at increasing concentrations 48 h after transfection. Control cells were treated with an equal concentration of vehicle alone. 24 h later, the drug-containing medium was

replaced by fresh medium and an aliquot of 20 μL of MTT (5 mg/mL) were added to each well. After incubation for 4 h at 37 °C, the supernatant was removed, and 150 μL of DMSO was added. The plates were vigorously shaken for 10 min to solubilize the MTT-formazan product. The absorbance was read using SpectraMax M2 reader (Molecular Devices, California, U.S.A) at a wavelength of 570 nm. Vehicle-treated cells were assigned a value of 100%[13,15]. Each experiment was performed in triplicate wells for each drug concentration and carried out independently three times in different days. The cytotoxicity was evaluated with reference to the IC_{50} value. IC_{50} values were calculated from dose-response curves (*i.e.*, cell survival *versus* drug concentration) obtained in multireplicated experiments using GraphPad Prism 5.0.

2.9. *Statistical analysis*

All values were expressed as mean ± SD. Statistical analyses were conducted using one-way ANOVA with Dunnett's posttest, and multiple variances were analyzed by two-way ANOVA (GraphPad Prism). *P* values less than 0.05 were considered as statistically significant.

3. Results

3.1. *CYP3A4 CDS can be targeted by individual or combined shRNAs*

To test if CYP3A4 CDS can be targeted by siRNA, three shRNA constructs (S1, S2 and S3) were designed to act on different regions of the CYP3A4 mRNA sequence (Table 1). CHL cells were transiently cotransfected with CYP3A4 CDS-luciferase reporter plasmid and shRNA or pS-NC plasmid. Results found slight (< 30%) to no decrease in CYP3A4 CDS-luciferase activity by individual shRNAs (Fig. 1A). Interestingly, use of combined shRNAs led to significantly increased suppression (up to 55%) of CYP3A4 CDS-luciferase activity (Fig. 1B). Therefore, the mixture of three shRNAs (named S1+S2+S3; at a ratio of 1:1:1) was utilized to assess the capacity of RNAi on CYP3A4 gene expression in the following studies.

HEK293 cells were further used to evaluate the efficiency of CYP3A4 shRNAs, following a transient cotransfection with pGL3/CYP3A4 CDS-luciferase reporter plasmid and shRNA or pS-NC plasmid. Luciferase reporter assay showed that the luciferase activity decreased by 53 ± 2.2% in the HEK293 cells by the shRNAs mixture (Fig. 2A). In addition, our immunofluorescence study demonstrated that CYP3A4 protein level was decreased dramatically in HEK293 cells transfected with the shRNA mixture (Fig. 2B) These results indicate that designated shRNAs are able to target CYP3A4 CDS sequences to induce gene suppression.

3.2. *Expression of endogenous CYP3A4 mRNA is selectively reduced by combined shRNAs in HepG2 cells*

To investigate the impact of shRNAs on endogenous CYP3A4 expression, we utilized the human hepatocellular carcinoma cell line HepG2 which is an *in vitro* model system for the study on human CYP gene regulation. Since the basal level of CYP3A4 is low in HepG2 cells, we first employed rifampicin to induce CYP3A4 expression. The treatment of rifampicin (50 μmol/L) increased CYP3A4 mRNA level by 2-fold in HepG2 cells

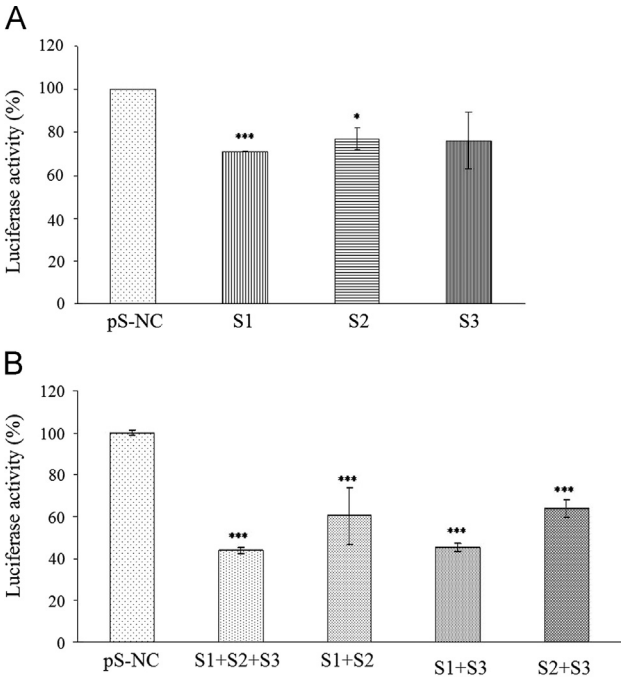

Figure 1 CYP3A4 CDS-luciferase activities are reduced by individual or combined shRNAs in CHL cells. (A) CHL cells co-transfected with CYP3A4 CDS-luciferase reporter, pRL-TK and different shRNA expression plasmids showed 20%–30% lower luciferase activities, compared to cells transfected with the pS-NC plasmid. (B) CHL cells transfected with CYP3A4 CDS-luciferase reporter, pRL-TK and different combinations of shRNA expression plasmids showed 40%–50% lower luciferase activities, compared to cells transfected with the pS-NC plasmid. Firefly luciferase activity was normalized to corresponding Renilla luciferase activity, and the control group was set as 100%. $^{*}P<0.05$; $^{***}P<0.001$ compared to the corresponding control ($n=3$ in each group, which refers to the number of independent transfection samples in a representative experiment).

(Fig. 3A). Following the induction of CYP3A4 by rifampicin, the shRNA plasmid mixture was revealed to reduce CYP3A4 mRNA expression by 35 ± 15.9% and 47.5 ± 13.8% in HepG2 cells after a 24 h and 48 h treatment, respectively, as demonstrated by the RT-PCR (Figs. 3B and 3C) analyses.

Because CYP3A4 and CYP3A5 genes exhibit 84% similarity in sequence, we further evaluated the selectivity of CYP3A4 shRNAs. As expected, treatment of HepG2 cells with shRNA plasmid mixture did not alter the expression of CYP3A5 mRNA expression (Fig. 3D). Together, these results indicate that the shRNA mixture selectively suppresses endogenous CYP3A4 mRNA expression in HepG2 cells.

3.3. *Expression of endogenous CYP3A4 protein is suppressed by combined shRNAs in HepG2 cells*

Immunoblot analyses were conducted with CYP3A4-selective antibody to examine whether shRNAs reduce CYP3A4 protein expression. The data (Fig. 4) showed that CYP3A4 protein level was reduced about 50% in HepG2 cells after transfection with the mixture of three shRNAs. This was associated with a reduction of CYP3A4 mRNA expression (Fig. 3B). These results suggest that shRNAs effectively repress CYP3A4 protein expression in HepG2 cells through the down-regulation of CYP3A4 mRNA expression.

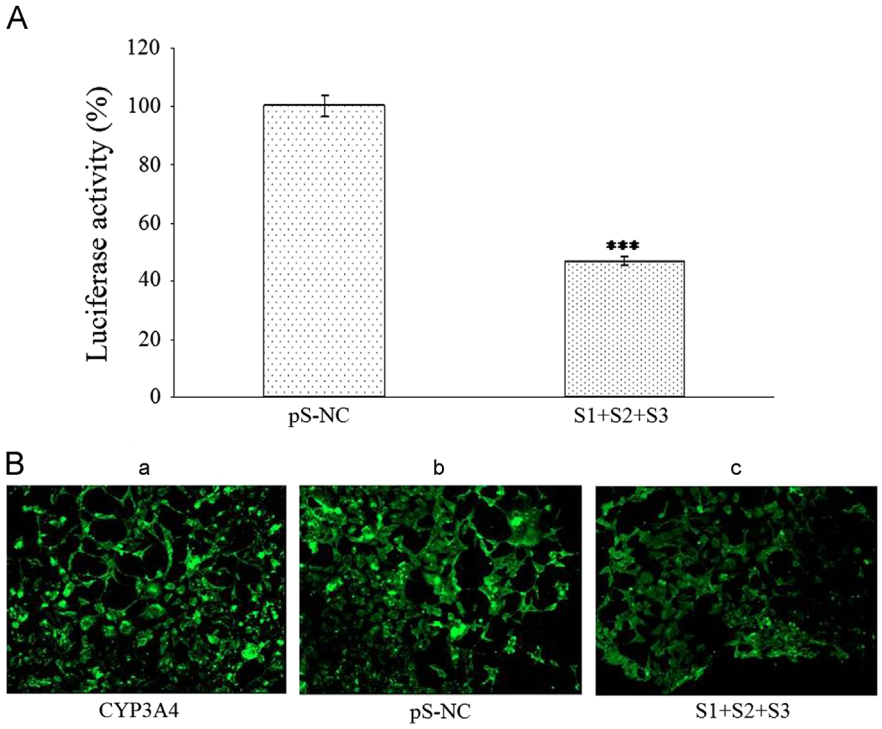

Figure 2 Combined shRNAs are effective in targeting CYP3A4 CDS in HEK293 cells. (A) HEK293 cells transfected with CYP3A4 CDS-luciferase reporter and three shRNA expression plasmids showed about 50% lower luciferase activities, compared to cells transfected with the pS-NC plasmid. ***$P<0.001$ compared to the control ($n=3$ in each group, which refers to the number of independent transfection samples in a representative experiment). (B) Fluorescent microscopic pictures of CYP3A4 expression in HEK293 cells at 48 h after transient transfection with CYP3A4 CDS-luciferase reporter plasmid alone (CYP3A4), along with control plasmid (pS-NC) or three shRNA expression plasmids (S1+S2+S3).

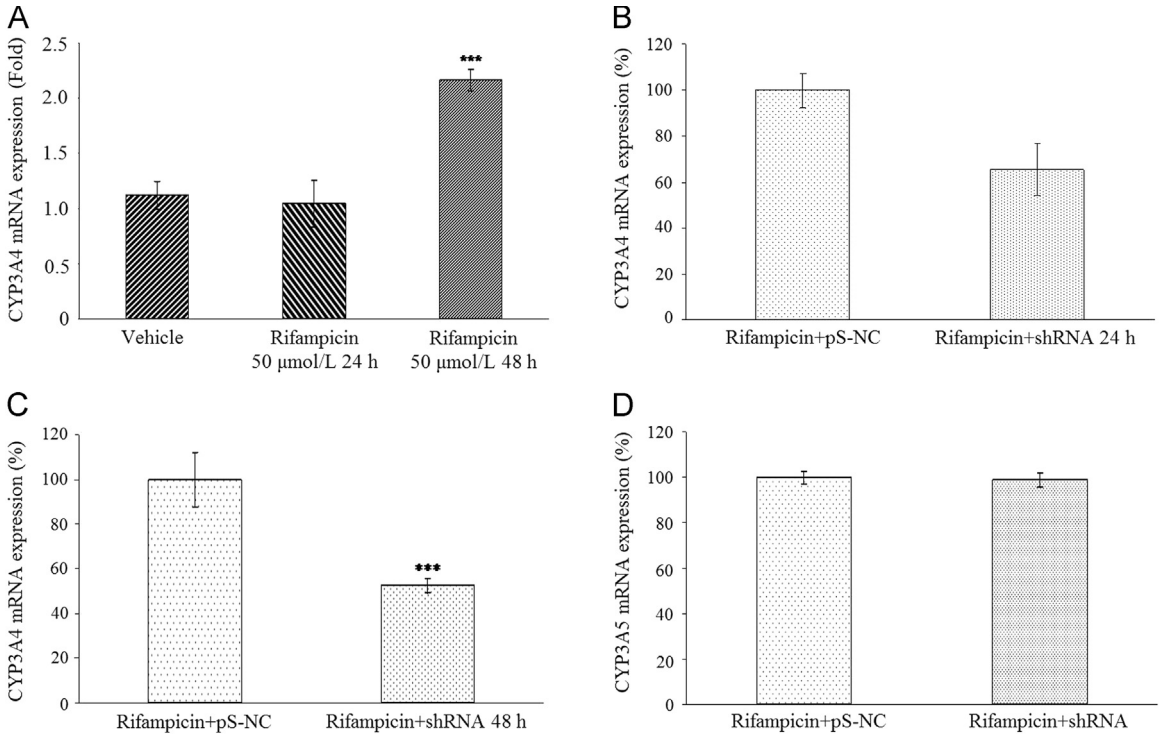

Figure 3 The mixture of three shRNA plasmids inhibits endogenous CYP3A4 mRNA expression in HepG2 cells. (A) CYP3A4 mRNA expression was elevated 2-fold in HepG2 cells by rifampicin (50 μmol/L for 48 h), as determined by RT-PCR analysis. (B) and (C) The shRNA expression plasmid mixture suppressed CYP3A4 mRNA levels in HepG2 cells. (D) The shRNA expression plasmid mixture did not change CYP3A5 mRNA expression in the HepG2 cells. Relative CYP3A4/5 mRNA expression level was normalized to corresponding GAPDH mRNA level, and the control group was set as 100%. ***$P<0.001$, compared to the vehicle control. $n=3$ in each group, which refers to the number of independent transfection samples in a representative experiment.

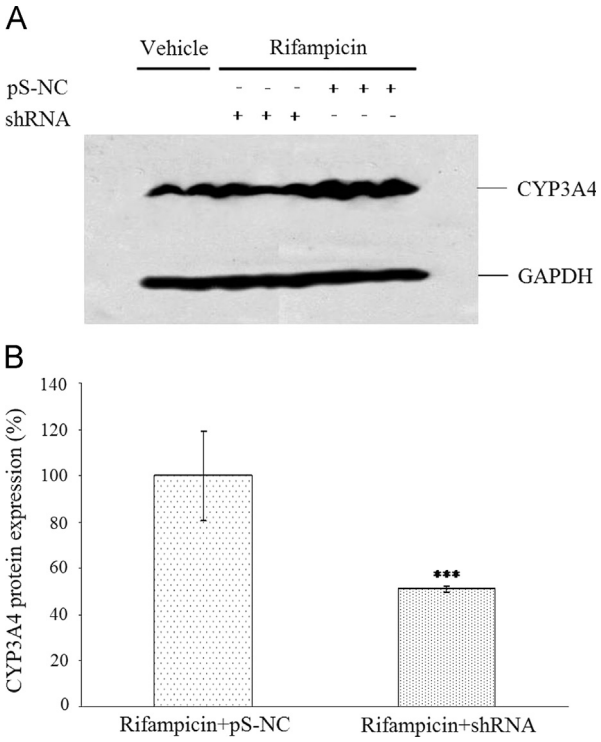

Figure 4 Expression of endogenous CYP3A4 protein is suppressed by combined shRNAs in HepG2 cells. CYP3A4 protein expression (A) in HepG2 cells (treated with rifampicin) was suppressed by about 50% (B) after the transfection with the shRNA expression plasmid mixture, compared to pS-NC control plasmid. CYP3A4 protein expression level was normalized to corresponding GAPDH level in HepG2 cells, and control group was set as 100%. $***P<0.001$, compared to the negative control ($n=3$ in each group, which refers to the number of independent transfection samples in a representative experiment).

3.4. *CYP3A4 shRNAs alter the chemosensitivity of cells*

Ginkgolic acid (15:1) (GA (15:1)) and ginkgolic acid (17:1) (GA (17:1)), two substrates metabolized by CYP3A4 to more toxic metabolites[18,19], were used to examine the impact of CYP3A4 shRNAs on chemosensitivity. Treatment with GA (15:1) or GA (17:1) (at 5 to 100 μmol/L) for 24 h led to a significant cytotoxicity in HepG2 cells when CYP3A4 was induced by rifampicin[20,21]. Interestingly, the transfection with shRNA plasmid mixture increased the resistance of HepG2 cells to GA (15:1) and GA (17:1), as manifested by a higher IC_{50} value (109 μmol/L and 101 μmol/L, respectively), compared to the transfection with control plasmid (64 μmol/L and 52 μmol/L, respectively) (Fig. 5). A lower cytotoxicity is presumably due to the reduction of CYP3A4 expression by shRNAs (Fig. 4). Take together, these results indicate that shRNA plasmid mixture not only reduces CYP3A4 expression but also affected the sensitivity of cells to drugs metabolized by CYP3A4.

4. Discussion

The cytochrome P450 enzymes (CYP450), which are found in all living organisms, play a predominant role in metabolism. In humans they constitute at least 57 different proteins and are found mainly in the liver. CYPs are the major enzymes involved in drug metabolism and bioactivation and account for about 75% of the total number of different metabolic reactions. CYP3A4 is the most abundant CYP450 in human liver and intestine, and contributes to the metabolism of various drugs[9]. As we know, chemical inhibitors or inhibitory antibodies to CYP3A4 are less specific and always exhibit cross-reactivity between CYP3A4 and other enzymes, especially CYP3A5, which possibly complicates interactions with the substrates[22]. So far, a number of studies using antisense approach to regulate CYP450 have been published in the past decade, which has made great contribution to our understanding of these enzymes. Although the antisense oligonucleotides have received increasing attention, this technology still has formidable problems. In most cases, antisense oligonucleotides degrade rapidly, therefore their effects are temporary and the potency is low[23–25]. Until recently, some progress has been made on the inhibition of CYP450s using RNA interference. The studies by Pan et al.[15] indicated that microRNA-27b (miR-27b) and mouse microRNA-298 (mmu-miR-298) could directly act on the 3′–UTR of CYP3A4. In addition, three different constructs to produce shRNA molecules have been designed and investigated by Chen et al.[14].

In the present study, the vector-based RNAi technology to express shRNA molecules was adopted. As mentioned above, this gene silencing approach has been confirmed to inhibit CYP3A4 expression in Chen et al.'s study. In their research, three oligonucleotides (3A4I, II, and III) were designed, and only CYP3A4III which targeted the 3′–UTR of CYP3A4 (position in gene sequence is 1698–1719) significantly reduced CYP3A4 expression at both mRNA and protein levels, while the other two did not generate obvious inhibitory effects on CYP3A4 expression. As we know, the siRNAs are usually designed to act on the CDS of target genes in order to avoid the interference of regulatory proteins in 5′–UTR or 3′–UTR. Furthermore, this type of siRNAs can be applied to the engineering cell lines which transfected either with the CDS or the full length cDNA of target gene. Therefore, the efficacy of CDS-targeted siRNAs in suppressing CYP3A4 gene expression was important to investigate. For these reasons, according to siRNA design guidelines and the CYP3A4 gene sequence, we chose three novel sites targeting the coding regions whose position in gene sequence were 773–794, 923–944 and 1191–1212, respectively. Up to now, these sites have never been reported and their functions were investigated from three aspects.

We first determined the effective pattern of shRNA expression vectors. Unlike CYP3A4III, which has been selected as the most effective shRNA among the three oligonucleotides in Chen et al.'s research, none of the three kinds of shRNA expression plasmid targeting the coding regions exhibited obvious inhibitory function in CHL cells and HEK293 cells. However, when they were mixed together at a ratio of 1:1:1, CYP3A4 gene was reduced significantly (>50%). These results suggested that the combined shRNAs could be used in further investigation.

The above speculation was derived from the impact of shRNAs on exogenous CYP3A4 which was obtained from recombinant plasmid. In this state, the 3′–UTR of CYP3A4 gene was excluded. As we know, the same siRNA may exhibit great discrepancy of inhibition activity in different conditions. So whether this mixture system possessed similar inhibitory efficiency for endogenous CYP3A4 gene was worthy exploring. The human hepatocellular carcinoma cell line HepG2 is an *in vitro* model system for the study on human CYP gene regulation. Although HepG2 cell line expresses many of the hepatocyte-specific metabolic enzymes that

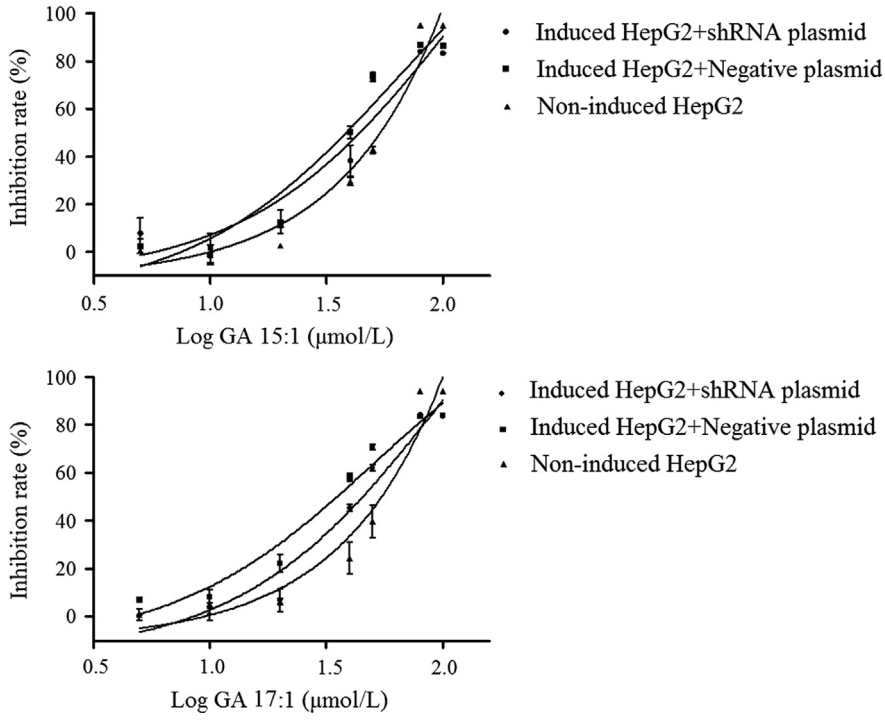

Figure 5 CYP3A4 shRNAs alter the chemosensitivity of cells. Transfection of HepG2 cells with CYP3A4 shRNAs reduces the sensitivity to GA (15:1) or GA (17:1), compared to the cells transfected with control plasmid. Cytotoxicity was determined by the MTT assay. The inhibition rate relative to the control (0%) was calculated for each concentration of drug, and the IC_{50} value was estimated by fitting the data to a Hill equation (Table 2).

characterize the liver, it is seldom adopted because the basal expression level of CYP450s was usually low. In this study, the endogenous CYP3A4 mRNA level was obviously up-regulated by rifampicin, which provided an ideal cell model for CYP3A4 silencing investigations both on gene expression and metabolic activity. RT-PCR and western blot analyses showed that CYP3A4 was suppressed by approximately 50% at both gene and protein levels when CYP3A4 was expressed endogenously. As expected, the inhibition rate was consistent between exogenous and endogenous cell models. Despite the component of the shRNAs mixture was more complicated than the single one, the suppression ratio of CYP3A4 gene remained stable. It was probably because all of the three shRNA constructs were constructed using the same expression vector, so their mixture was homogeneous, possibly suppressing CYP3A4 gene expression in an additive way. As a result, such combined shRNAs could be regarded as a system that owned three different target sites of CYP3A4 coding region. Furthermore, our study demonstrated that the mixture of shRNA expression vectors did not alter CYP3A5 gene expression which exhibited 84% homology with CYP3A4 and similar gene regulation in hepatocytes at mRNA level in HepG2 cells treated with rifampicin. This result revealed that the suppression of CYP3A4 gene by the mixture system was specific and targeted CYP3A4 mRNA only, which further indicated that such shRNA mixture might become a powerful approach for studying CYP3A4 function. For this reason, we applied the mixture of shRNA expression vectors in drug development.

The cytotoxicity assay by MTT method has been applied and proved to be efficient in down-regulating the metabolic activity of CYP450s[19]. GA (15:1) and GA (17:1) are major compounds in ginkgolic acid prepared by our lab and have been proved that the activation of their cytocidal activity requires CYP3A4[19]. Our data demonstrated that shRNA-transfected HepG2 cells were significantly resistant to the drug because of the suppression of CYP3A4 by shRNA expression vectors. As a result, the activation of GA (15:1) and GA (17:1) was reduced. Our study provided confirmatory evidence that CYP3A4 is the major enzyme metabolizing GA (15:1) and GA (17:1). Thus, the use of such shRNA expression vectors targeted the coding regions has important clinical significance because of CYP3A4's important role in manipulating the metabolic fate of existing drugs and the potential substrates. Furthermore, this strategy in combination with other established inhibitors may reduce individual differences in drug metabolism, thereby attenuating organ toxicity. Therefore, the response to therapy will be more predictable[26,27].

To sum up, although the shRNA vector-mediated RNAi for CYP3A4 has been reported by Chen et al., the shRNAs system in the present study still has its own characteristics. First, the three shRNAs expression vectors were designated to the CDS of CYP3A4 which have never been reported. They can be applied to the engineering cell lines transfected either with the CDS or the full length cDNA of CYP3A4 gene, or applied to cell lines which express CYP3A4 endogenously. Therefore, their application range was more extensive. Second, the identification of improved efficiency of mixed shRNAs in silencing CYP3A4 is a very interesting discovery. It may be an additive effect, and the mechanism requires thorough investigation in order to provide alternative strategy for gene silencing. This seems especially true when single siRNA is difficult to screen. According to the literature, some vectors which can express multiple shRNAs have been provided (*e.g.*, pcDNA6.2-GW/miR)[28]. Therefore, the multi-silencing construct could be used in investigations where a gene

with several target sites or where knockdown of multiple genes was required[29]. Such approach may be adopted to avoid carrying out multiple individual shRNA expression vectors and improve transfection efficiency in further research in this area. Third, even though the CYP3A4 gene was not knocked down completely, the activity of CYP3A4 was altered significantly. Thus this shRNAs mixture can be regarded as a specific inhibitor of CYP3A4, which can be utilized to screen the substrates or introducer of CYP3A4.

5. Conclusions

We have achieved specific reduction of CYP3A4 expression using a set of three novel shRNAs that target the CYP3A4 coding region. Our studies demonstrate that a mixture of multiple shRNAs can be used to improve the effectiveness of shRNA-mediated silencing of CYP3A4 expression, and that the usage of RNAi is promising as an approach to inhibit the expression and activity of this important drug metabolism enzyme.

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