Small Interfering RNA-Mediated Silencing of Cytochrome P450 3A4 Gene

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ABSTRACT:
RNA interference (RNAi) is a specific and powerful tool used to manipulate gene expression and study gene function. The cytochrome P450 3A4 (CYP3A4) can metabolize more than 50% of drugs. In the present study, we investigated whether vector-expressed small interfering RNAs (siRNAs) altered the CYP3A4 expression and function using the Chinese hamster cell line (V79) overexpressing CYP3A4 (CHL-3A4). Three different siRNA oligonucleotides (3A4I, 3A4II, and 3A4III) were designed and tested for their ability to interfere with CYP3A4 gene expression. Our study demonstrated that transient transfection of CHL-3A4 cells with the 3A4I siRNAs, but not 3A4I and II, significantly reduced CYP3A4 mRNA levels by 65% and protein expression levels by 75%. All these siRNAs did not affect the expression of CYP3A5 at both mRNA and protein levels in V79 cells overexpressing CYP3A5.

RNA interference (RNAi) is becoming a powerful means to manipulate gene expression experimentally and to probe gene function on a whole-genome scale (Hannon, 2002; Fraser, 2004). It has been used as a research tool to control the expression of specific genes in numerous experimental organisms and has potential as a therapeutic strategy to reduce the expression of problem genes (Clayton, 2004; Mello and Conte, 2004). RNAi is a post-transcriptional gene regulating mechanism by which double-stranded small interfering RNAs (siRNAs) induce degradation of mRNA through an enzyme-mediated process and in a sequence-specific manner (Bernstein et al., 2001).

RNAi involves four major steps: assembly of siRNA with the RNA-induced silencing complex (RISC), activation of the RISC, target recognition, and target cleavage (Meister and Tuschl, 2004). RNAi is initiated by a double-stranded RNA-specific RNase III enzyme known as Dicer that processes long double-stranded RNA into siRNA (Bernstein et al., 2001). These siRNAs are then incorporated into the protein complex RISC, which recognizes and cleaves target RNA molecules (Tuschl, 2002). siRNAs can either be synthesized, in vitro-transcribed, or expressed from a plasmid as RNA hairpin loops (RNA with a self-complementary stem loop). Chemically synthesized siRNAs are widely used for functional gene knockdown studies in mammalian cells, although there is an increasing use of vector-based expression of hairpin RNAs as an alternative for delivery of siRNAs into mammalian cells.

The cytochrome P450s (P450s; EC 1.14.14.1), containing at least 57 genes in humans, are the most important phase I metabolizing enzymes that metabolize a variety of xenobiotics, including environmental procarcinogens and therapeutic drugs, and some important endogenous compounds (Nelson et al., 1996). Among P450s, the
subfamily CYP3A is responsible for the metabolism of approximately 60% of the currently known therapeutic drugs. There are four identified members in the CYP3A subfamily that contribute to overall CYP3A activity: CYP3A4, CYP3A5, CYP3A7, and CYP3A43 (Geller et al., 2001). CYP3A locus also contains two pseudogenes, CYP3A5P1 and CYP3A5P2, as well as several extra exons, which may or may not be included in transcripts produced from this region. Only CYP3A4 and CYP3A5 have been identified unequivocally in adult liver in vivo. CYP3A4 is the most abundant P450 enzyme (~30–40%) in adult liver and metabolizes more than 50% of the clinically used drugs (Shimada et al., 1994; Rendic and Di Carlo, 1997). However, CYP3A5 may also play an important role in drug metabolism because of its considerable contents in some people. However, it has been reported that the CYP3A5 protein does not exceed 17% of the total hepatic CYP3A contents in healthy subjects and accounts for no more than 2% in Caucasians (Sarkar et al., 2003).

Likewise, CYP3A5 mRNA transcripts accounted for less than 4% of the CYP3A transcript pool (Koch et al., 2002; Sarkar et al., 2003), whereas the expression of CYP3A5 had little or no effect on midazolam clearance in vivo (Shih and Huang, 2002). Both CYP3A4 and CYP3A5 are subjected to inhibition and induction by a number of endogenous and exogenous compounds and regulated at transcriptional and translational levels, in particular by the nuclear receptors pregnane X receptor and constitutively activated receptor (Burk et al., 2004).

There are few studies using RNAi to study the function of P450s, probably due to the easy availability of a number of selective chemical inhibitors and inhibitory antibodies for the functional studies in vitro and in vivo. However, these few studies are of poor quality because the authors did not validate the specificity and selectivity of the siRNA used.

In the present study, we intended to investigate whether vector-expressed siRNA could suppress CYP3A4 gene expression and function in transgenic Chinese hamster CHL cell line (V79), which stably overexpresses human liver CYP3A4 (CHL-3A4). Because of the high similarity in the gene sequence (84%) of CYP3A4 with CYP3A5, we also investigated the effects of the vector-expressed siRNAs on the expression of CYP3A5 to confirm the specificity of possible RNAi observed for CYP3A4.

### Materials and Methods

**Chemicals and Reagents.** pSilencer hygro-based siRNA system (cat. nos. 5760 and 5766) for gene knockdown was from Ambion (Austin, TX). Dulbecco’s modified Eagle’s medium, fetal bovine serum, trypsin, and G418 were all from Invitrogen (Guangzhou, China). Lipofectamine 2000, Opti-MEM, and TRizol reagent were all purchased from Invitrogen (Groningen, The Netherlands). M-MLV and Taq enzymes were obtained from Promega Co. (Madison, WI) and Takara Medical Co. (Tokyo, Japan), respectively. Oligonucleotide primers were synthesized by Sangon Co. (Shanghai, China). Antibodies of rabbit anti-human CYP3A4 or CYP3A5 were from CHEMICON International, Inc. (Temecula, CA), and horse radish peroxidase-labeled goat anti-rabbit IgG was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Hyperfilm enhanced chemiluminescence was obtained from Amersham Biosciences (Buckinghamshire, UK). Cyclophosphamide, ifosfamide, nifedipine (mol. wt. = 364.3), and nitrendipine (mol. wt. = 360.4, used as the internal standard) were from Sigma-Aldrich (St. Louis, MO). NAPDH was obtained from Roche Diagnostics (Rotkreuz, Switzerland). All other chemicals used were of HPLC grade or of highest purity available.

**Cell Culture.** The parental Chinese hamster cells V79/MZ (CHL-pIC19h) and the genetically engineered Chinese hamster cells overexpressing human CYP3A4 (CHL-3A4), provided by Dr. Johannes Doehmer (GenPharmTox BioTech AG, Planegg/Martinsried, Germany) (Schneider et al., 1996), have been used for the studies of drugs and other xenobiotics, including cyclophosphamide; teratome A, B, and C; and l-methylpyrene (Schneider et al., 1996; Engst et al., 1999; Philip et al., 1999; Peng et al., 2003, 2006). The CYP3A4-transfected cell line was established using pIC19h plasmids carrying the SV40 early promoter and the SV40 polyadenylation with coexpression of human NADPH-cytochrome P450 reductase (Schneider et al., 1996). This cell line can efficiently catalyze the 6β-hydroxylation of testosterone and the oxidation of midazolam, nifedipine, and aflatoxin B1 (Schneider et al., 1996). CHL-pIC19h and CHL-3A4 cells were grown as a monolayer cell culture in MEM, 10% fetal bovine serum, and G418 (400 µg/ml). CHL-pIC19h cells and CHL-3A4 cultures were grown at 37°C in a humidified atmosphere of 5% CO2 and 95% air. CHL-3A4 cells doubled every 10 ± 1.2 h and were subcultured at a 1:20 ratio approximately every other day. Cells at third passage were routinely used for the experiments.

### Design of Hairpin siRNA Template Oligonucleotides and Cloning of Hairpin siRNAs

Three hairpin siRNA template oligonucleotides (3A4I, II, and III) based on three different parts of the human CYP3A4 gene (GenBank accession no. NM_017460; version 3) were designed using the siRNA Target Finder and Design Tool available at http://www.ambion.com. The hairpin siRNA target sequences and the oligonucleotides designed to produce hairpin RNAs are listed in Table 1. Two complementary oligonucleotides that encoded a hairpin structure with a 19-mer stem deriving from the mRNA target site were cloned into the pSilencer vector of siRNA expression. The pSilencer vector contained compatible restriction sites of BamHI and HindIII to ensure cloning into the correct site of the linearized vector, and the oligonucleotides were annealed and cloned into the vector according to the manufacturer’s recommendations. pSilencer vectors were sequenced with universal primer to confirm that the oligonucleotides were connected to the correct site.

A complete siRNA experiment should include both positive and negative control siRNAs. A negative control pSilencer vector was designed by scrambling the nucleotide sequence of the gene-specific siRNA and conducting a blast search to make sure it lacks homology to any other gene. The negative control insert sequences were provided as follows: 5′-GATTCC GGTATG CTCA AGG GAA CAG GGA CTT TGT TGG AAA-3′ (top strand oligonucleotide template) and 5′-AGCC TTT CCC AAA AAA GGT CCT CCT CCC TGA AAG ATT CTC TCT TGA AGC TTT CAG GGA GGA ACG-3′ (bottom strand oligonucleotide template). The product of the positive control ligation is a pSilencer hygro plasmid containing siRNA template targeting green fluorescent protein (GFP). The GFP control insert sequences were: 5′-GATTCC GGTATG CTCA AGG GAA CAG GGA CTT TGT TGG AAA-3′ (top strand oligonucleotide template) and 5′-G CCAATACGTC-
CTGCCGTAAGTCTCTCAACGCAAGGACATGTAGGGGAAAACCTTC-TGCA-5’ (bottom strand oligonucleotide template). This construct reduced GFP expression of pEGFP-C1 vector. CHO-3A4 cells cotransfected with 400 ng of the mammalian expression vectors with GFP (pEGFP-C1; Invitrogen) and 4 µg of psiSilencer hydro-siGFP. GFP expression was analyzed by fluorescent analysis at regular intervals starting approximately 24 h after transfection.

Plasmid Construction and Transfection of cDNA of Human CYP3A4 to CHO Cells. A full-length cDNA of CYP3A4 gene was cloned, and a recombinant eukaryotic expression plasmid was constructed and then stably transfected in CHO cells established at our laboratory. The plasmids containing full-length CYP3A4 cDNA were constructed by PCR amplification based on the sequence as described previously (Jounaidi et al., 1994). The CYP3A4 cDNA was amplified out of human genomic DNA by PCR with the forward oligonucleotides 5’-ATGGGAAAATAATGTTGGGAAAGC-3’ and 5’-CCGTCGTT-GAAGGTGTCAGAC-3’ (Jounaidi et al., 1994; Krusekopf et al., 2003). The PCR products were cloned using KpnI and Nhel restriction sites to pGL3-Basic (Promega) in front of the luciferase reporter gene. The recombinant plasmid was designated as pGL3-CYP3A4. The identity of the constructs was verified by sequencing. The CYP3A4 sequence amplified from the human liver was found to be fully identical to the sequence (GenBank accession no. NG_000004.2; GI: 21536445).

For transfection, CHO cells were seeded to 24-well plates the day before transfection. The cells were transfected with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s protocol, using 1 µg of plasmid DNA and 2.5 µl of transfection lipid and Opti-MEM I media (Invitrogen). Twenty-four hours after transfection, the media was replaced with serum-free Ham’s F-12 medium, and 10 µM dexamethasone or dimethyl sulfoxide was added when appropriate for 48 h. The luciferase activities for transfected CHO cells were assessed using the luciferase assay system (Promega). The CHO cells, which did not show transcript of CYP3A4, was confirmed using an ultraviolet spectrometer and by gel electrophoresis (BioTek Instruments Inc., Winooski, Vermont) at a wavelength of 490 nm.

Transfection of Plasmids Containing Hairpin siRNAs in CHL-pIC19h, CHL-3A4, CHL-pGL3, and CHL-CYP3A5 Cells. The cells were seeded at a density of 75,000 cells/well in a six-well plate using media conditions as described above and incubated for 24 h. Cells were transfected with hairpin-producing plasmids (4 µg/well) using 10 µl of Lipofectamine 2000 (2 µl/well) and serum-free medium (Opti-MEM) according to the manufacturer’s recommendations. All transfections were done in triplicate, and cells were harvested 48 h post-transfection. Optimal amounts of Lipofectamine 2000 and cell number were determined before transfection of GFP-expressing plasmids. Transfection efficiency was approximately 40 to 60% for vector-based hairpin siRNAs estimated by fluorescence microscopy. Cells were also transfected with the negative control plasmid pGL3 Basic vector.

Reverse Transcription-Polymerase Chain Reaction. Specific intronic PCR primer pairs used in the human CYP3A4 and CYP3A5 genes were designed using their genomic sequences retrieved from GenBank (accession number for CYP3A4: NM_017460; version 3; GI: 13904851; and for CYP3A5: accession no. NG_000004.2; GI: 21536445) (Watkins et al., 1985; Jounaidi et al., 1994; Burk et al., 2004). Exon-intron boundaries of the human CYP3A4 and CYP3A5 genes were defined by comparing genomic sequences with the published mRNA sequences (GenBank accession no. NM_030172.2 for CYP3A4, and GenBank accession no. AY893017.1 and GI: 60654486 for CYP3A5) (Aoyama et al., 1994; Gellner et al., 2001; Strausberg et al., 2002).

Total RNA was isolated from cultured cells using the TRIzol reagent Isolation System kit (Invitrogen) following the instructions from the manufacturer. All RNA was treated with DNase, and purity and integrity of the RNA was confirmed using an ultraviolet spectrometer and by gel electrophoresis before use. PCR reactions using total RNA without prior RT as template did not result in amplification of the products confirmed the absence of contaminating DNA (data not shown). The RT reaction was carried out in a total volume of 25 µl of containing 2 µg of RNA, 0.5 µg random primer (Sangon Co.), 10 mM dNTP mix, M-MLV 5X reaction buffer, 25 U of RNase inhibitor, and 200 U of M-MLV (Promega) at 37°C for 1 h. PCR was performed by using the CYP3A4 primers 5’-AAATCTGAGGCGGAGGAGC-3’ (forward; F) and 5’-TTGCGATGGAATGGGAGAAG-3’ (reverse; R). The PCR conditions were as follows: one cycle of 94°C for 2 min; 30 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 45 s; followed by incubation at 72°C for 7 min. Similar procedures were conducted for the amplification and determination of human CYP3A4 gene, but the primers were 5’-ATGGAAATAATGTTGGGAAACG-3’ (F) and 5’-CCGTCGTTGAAGGTGTCAGAC-3’ (R), as described previously (Krusekopf et al., 2003).

Reverse transcription-polymerase chain reaction (RT-PCR) was also performed using primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene sequences as a control. Primers for GAPDH were 5’-TCCACACCCCTTGTTGTGAG-3’ (F) and 5’-GACCCAGTCCGTAGCATC-3’ (R). The PCR reaction was conducted in a volume of 50 µl containing 1 µl of Taq enzyme (5 U/µl, 4 µl of dNTP at 10 mM, 10 µl of RT product, and 1 µl of each primer at 40 pmol), and 5 µl of 10X PCR reaction buffer. Five microliters of the amplified products were resolved by electrophoresis in a 2.0% agarose gel, and the bands were quantified using GDS-8000 UV photo scanner (Bio-rad, Hercules, CA), and the digital data were collected and processed using the LAB WORK45 Image software (Bio-rad).

Western Blot Analysis. The CYP3A4 and CYP3A5 proteins were detected after separation for 4 h at 125 V on 10% gels by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis as described previously (Laemmli, 1970). The protein bands were homogenized in a buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 1 mM NaF, 1 mM NaN3, 0.2 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. Lysates were centrifuged at 12,000g at 4°C for 10 min. Total protein content was determined in the extracts using the Lowry method using bovine serum albumin as the standard (Lowry et al., 1951). Ten micrograms of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Amersham). The membranes were blotted with antibodies against antibodies of rabbit anti-human CYP3A4 or CYP3A5 (Santa Cruz Biotech Co., Heidelberg, Germany). To assure equivalent protein loading, the membranes were also incubated with mouse anti-human actin monoclonal antibodies (1:2000 in Tris-buffered saline/Tween) and subsequently with a corresponding horseradish peroxidase-conjugated second antibody IgG (Amersham/Pharmacia, Buckinghamshire, UK) and developed using Chemiluminescence Reagent Plus (Perkin Elmer Life Science Inc., Boston, MA). The scan densitometric analysis was carried out using GDS-8000 UV photo scanner and LAB WORK45 Image software.

Cytotoxicity Assay. Cell viability was determined based on the ability of live cells to reduce a tetrazuronium-based compound, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazonium bromide (MTT), to a blue formazan product. Cells in exponential growth phase were washed with Hank’s balanced salt solution. Depending on the cell line and experiment, 5000 to 10,000 cells/well were seeded in duplicate in 96-well plates (Costar, Ann Arbor, MI). The experiment was performed with rapid transfection protocol (i.e., seeding cells and transfecting simultaneously) in a 96-well plate by adding a suspension of cells directly to the well loaded with DNA (0.2 µg) and Lipofectamine 2000 (0.5 µl). Cells adhered to the plate as usual in the presence of DNA (0.2 µg). Lipo- fectamine 2000 (0.5 µl). Drugs (cyclophosphamide or ifosfamide) were added at increasing concentrations 24 h after plating. Control cells were treated with an equal concentration of vehicle alone, usually not exceeding 0.1% (v/v). On the 3rd day, the drug-containing medium was replaced by fresh medium, and aliquots of 20 µl of MT (5 mg/ml) were added to each well. After incubation for 4 h at 37°C, the supernatant was removed, and 150 µl of dimethyl sulfoxide was added. The plates were vigorously shaken for 15 min to solubilize the MTT-formazan product. The absorbance was read using an ELX800 reader (BioTek Instruments Inc., Winoski, Vermont) at a wavelength of 490 nm. Vehicle-treated cells were assigned a value of 100%. Each experiment was performed in eight replicate wells for each drug concentration and carried out independently at least three times in different days. The cytotoxicity was evaluated with reference to the IC50 value that was defined as the concentration needed for a 50% reduction of survival based on the survival curves. IC50 values were calculated from dose-response curves (i.e., cell survival versus drug concentration) obtained in multireplicated experiments.

Preparation of Subcellular S9 Fractions. S9 fractions were prepared from harvested cells as described previously (Zhou et al., 2000). In brief, CHL-pIC19h and CHL-3A4 cells were seeded at 5 × 105/ml and grown in six-well plates for 2 to 4 days until confluent. The cells were washed twice with warm...
(37°C) phosphate-buffered saline (pH 7.4), and harvested by scraping using a policeman. Thereafter, the cells were sonicated in 0.15 M KCl and homogenized using homogenizer at 4°C (Heidolph Instruments GmbH and Co. KG, Schwabach, Germany). The homogenates were centrifuged at 9000g for 20 min at 4°C using an ultracentrifuge with a Type 70 Ti rotor (Beckman Coulter, Inc., Fullerton, CA). After centrifugation, the supernatant fractions (S9s) were rapidly aliquoted and frozen in liquid nitrogen and stored at −80°C. Protein concentration of S9s was measured by the Lowry method using bovine serum album as the standard (Lowry et al., 1951).

In Vitro Metabolism of Nifedipine. The typical incubations were carried out in a total volume of 1.0 ml in the presence of 200 mM NADPH (freshly prepared) and S9s at 0.3 mg in 0.15 M KCl pH 7.4. The mixtures were preincubated for 5 min at 37°C with 100 mM KH₂PO₄ and nifedipine at 5.78, 14.44, and 28.88 μM (i.e., 2, 5, and 10 μg/ml). The reactions were started by the addition of freshly prepared 10 μl of 200 mM NADPH. After incubation for 30 min, the reaction was stopped by adding 3 volumes of ice-cold methanol containing 11.1 μl of the reaction mixture. The samples were vortexed for 2 min, and the supernatant was evaporated using a Speedvac rotary concentrator (TeleChem International, Inc., Sunnyvale, CA). The residues were reconstituted using 50 μl of mobile phase and 25 μl of the solution was injected on the HPLC for nifedipine concentration determination.

HPLC Analysis. For HPLC analysis of nifedipine, a Hypersil BDS C18 reversed-phase column (4.6 × 150 mm, particle size: 5 μm; Dalian Elite Analytical Instruments Co., Ltd., Dalian, China) was used with a Waters (Milford, MA) Alliance HPLC system consisting of a 2487 dual wavelength UV detector, a 1525 Binary HPLC pump, and a 717 plus autosampler. The elution of the analyte was carried out with the mobile phase consisting of acetonitrile/water (50:50, v/v) at pH 3.5 with a flow rate of 1.0 ml/min at ambient room temperature, and the analyte was monitored at a wavelength of 238 nm. The experiments were conducted under dark condition to prevent photodegradation of the nifedipine degradation. The HPLC method for determination of nifedipine was fully validated. The linear range was 0.29 to 57.75 μM (i.e., 0.1–20 μg/ml) (r² = 0.9996), and the limit of quantitation was 0.29 μM. The intra- and inter-run precisions were less than 6.3 and 4.0%, respectively. The extraction recovery was 77.6 to 87.3% using methanol, and the method recovery was 90.5 to 101.7%.

Gene Nomenclature and Statistical Analysis. The genes (CYP3A4 and CYP3A5) investigated in this study were named in accordance with the Human CYPallele Nomenclature Committee (http://www.imm.ki.se/CYPalleles/criteria.html). Genomic sequence number of these two important genes are available at the CYP-allele nomenclature website and GenBank.

All values are expressed as mean ± S.E.M. Statistical comparisons were performed by one-way analysis of variance followed by the Student-Newman-Keuls test. P values less than 0.05 was considered as statistically significant.

Results

Effect of Vector-Based siRNAs on CYP3A4 and CYP3A5 Expression. To identify an efficient RNA sequence targeted by vector-based RNAi, siRNA constructs designed against three different parts of the CYP3A4 and CYP3A5 mRNA sequence were tested by transient transfection for their ability to down-regulate the expression of CYP3A4 and CYP3A5 cells and their respective control cells. Because there is no reliable way to predict a suitable target sequence for RNAi, three different siRNA oligonucleotides (3A4I, 3A4II, and 3A4III) were designed with consideration of the high similarity of both CYP3A4 and CYP3A5 genes with 84% sequence similarity and tested for their ability to interfere with CYP3A4 and CYP3A5 gene expression. As shown in Fig. 1, a 65.4 ± 7.9% decrease in CYP3A4 mRNA level was observed in CHL-3A4 cells with transient transfection of CYP3A4III, whereas the CHL-3A4 cells transfected with CYP3A4I or 3A4II did not show any significant decrease in CYP3A4 mRNA levels as determined by the RT-PCR analysis. Western blot analysis of extracted protein showed that the CYP3A4 protein in CHL-3A4 cells transiently transfected with CYP3A4III decreased by 75.2 ± 8.8%, whereas the same cells transfected with CYP3A4I or 3A4II showed insignificant decreases in CYP3A4 mRNA and CYP3A4 protein levels (Fig. 2). Among the three interfering oligonucleotides tested, only the CYP3A4III significantly reduced the expression of CYP3A4 at both mRNA and protein levels.

As predicted, treatment of CHL-CYP3A5 cells with all three siRNA oligonucleotides (3A4I, 3A4II, and 3A4III) did not show significant effects on the expression of CYP3A5 at both mRNA and protein levels in CHL-3A5 cells (Fig. 3). The parental CHL-pGL3 cells did not express CYP3A5. These results indicated that the marked interfering (inhibitory) effect of CYP3A4III siRNA on the expression of CYP3A4 at both mRNA and protein levels was specific, which did not affect the expression of CYP3A5 despite the 84% of similarity in the sequences of both genes.

We next examined the kinetics of CYP3A4 suppression using CYP3A4III siRNA construct vectors in CHL-3A4 cells. Maximal CYP3A4 suppression was observed after 2 days of siRNA transfection with CYP3A4III, as indicated by a weakest CYP3A4 mRNA and protein expression (Fig. 4). Thereafter, the expression of CYP3A4 mRNA and protein markedly increased on days 3 and 4 and reached to the basal mRNA and protein expression level on the 5th day post-transfection using CYP3A4III siRNA construct vector.

To confirm that CYP3A4III siRNAs diminished CYP3A4 gene expression through a siRNA-mediated mechanism, both positive and negative controls were constructed and tested in our study. The positive control of RNAi was cells transfected with a siRNA targeting GFP gene expression, and the negative control was cells transfected with a siRNA having no homology with any known human genes (both purchased from Ambion Co.). In addition, the CHL-3A4 cells

![Fig. 1. RT-PCR analysis of CYP3A4 after a transient transfection performed with Lipofectamine 2000 in CHL-3A4 and CHL-pIC19h cells. A, after a transient transfection for 48 h, total RNA was isolated and subjected to semiquantitative RT-PCR analysis. The CYP3A4 and GAPDH amplification were performed for 30 and 25 cycles, respectively. PCR products were electrophoresed in a 2% agarose gel and stained with ethidium bromide. Sizes of the PCR products of CYP3A4 and GAPDH were 220 and 450 base pairs (bp), respectively. Lane 1, DG2000 DNA marker; lane 2, CHL-3A4 cells without treatment; lane 3, control CHL-pIC19h cells that did not express CYP3A4; lane 4, CHL-3A4 cells with treatment of Lipofectamine (LP) 2000; lane 5, CHL-3A4 cells transfected with plucifer vectors in which the oligonucleotides were not annealed and cloned into; lane 6, CHL-3A4 mock cells as an RNAi negative control; and lanes 7 to 9, CHL-3A4 cells with different treatment of siRNAs, including CYP3A4III, CYP3A4I, and CYP3A4II, respectively. B, relative CYP3A4 mRNA expression level normalized against GAPDH mRNA expression in CHL-3A4 and CHL-pIC19h cells. The relative expression values are means of at least three independent experiments in triplicate. The CYP3A4/GAPDH mRNA expression ratio in CHL-3A4 was set as 100%. † †, P < 0.01, compared with the control CHL-3A4 cells.](Image 308x538 to 560x737)
cotransfected with pEGFP-C1 (0.4 μg) and pSilencer hygro-siGFP (4 μg), or pSilencer hygro-siGFP-extra, were used as a negative control. After 24-h transient transfection, CHL-3A4 cells were digested by 0.1% trypsin and counted under an inverted microscope, and cell concentration was adjusted to 5 × 10^5/ml in a six-well culture plate. The digested cells were observed and photographed using a fluorescent microscope. Results of photography showed a significant decrease by 70.3 ± 7.6% of GFP gene expression 24 h after transfection with pSilencer hygro-siGFP (Fig. 5). In contrast, cells transfected with the negative control siRNAs showed no significant reduction of CYP3A4 gene expression levels (Figs. 1 and 2). Based on the results from transient transfection using siRNA-expressing vectors, the specific target sequence (CYP3A4III) showing marked interfering effect was selected for the following vector-based RNAi functional experiments.

**Effect of Vector-Based siRNAs on Cytotoxicity of Cyclophosphamide and Ifosfamide.** Lipofectamine 2000 alone did not show any significant cytotoxicity (<5%) in CHL-3A4 and CHL-3A5 cells and the parental control cells (data not shown). Both cyclophosphamide and ifosfamide are activated to form ultimate cytotoxic alkylating mustards via the 4-hydroxylation pathway catalyzed primarily by CYP3A4 (Zhang et al., 2005). Treatment with cyclophosphamide or ifosfamide (both at 25 to 800 μM) resulted in significant cytotoxicity in CHL-pIC19h cells transfected with CYP3A4 cDNAs, with an IC_{50} of 210.2 ± 14.3 and 55.2 ± 6.4 μM, respectively (Fig. 6). Ifosfamide was more (approximately 4-fold) cytotoxic than cyclophosphamide. The parental control CHL-pIC19h cells were considerably resistant to the both cytotoxic drugs, with an IC_{50} >1000 μM (Fig. 6). At any given drug concentrations, the cytotoxicity was more significant in CHL-3A4 cells compared with parental CHL-pIC19h cells. These findings demonstrated that transfection of CHL-3A4 cells with the vectors expressing the 3A4III siRNAs resulted in a significant lower cytotoxicity of cyclophosphamide and ifosfamide compared with that in parental control CHL-pIC19h cells.
Effect of Vector-Based siRNAs on Nifedipine Metabolism. The metabolic ability of CHL-pIC19h, CHL-3A4, and CHL-3A4 cells transfected with vectors expressing the 3A4III siRNAs toward nifedipine was compared by examining the depletion of the substrate. Nifedipine is a known CYP3A4 probe substrate (Galetin et al., 2005). S9 fractions from CHL-pIC19h cells did not significantly metabolize nifedipine at 5.78, 14.44, and 28.88 μM was significantly (P < 0.01) depleted by 100.3 ± 11.2, 40.4 ± 6.5, and 21.8 ± 3.4%, respectively, in S9 fractions from CHL-3A4 cells, compared with the S9s from the parental CHL-pIC19h cells (Fig. 7). The decreased depletion of nifedipine at increased substrate concentration might be due to substrate inhibition. It is noteworthy that the transfection of CHL-3A4 cells with vectors containing CYP3A4III siRNAs almost completely inhibited the CYP3A4-mediated nifedipine metabolism, with the substrate depletion in S9 fractions decreased to 19.2 ± 3.4, 10.2 ± 1.4, and 6.8 ± 7.9% at 5.78, 14.44, and 28.88 μM substrate concentration, respectively (Fig. 7).

Discussion

The identification of P450s responsible for newly developed drugs is important in drug development to predict pharmacogenetics, pharmacokinetics, and potential for drug-drug interactions. Experimental endeavors to overcome the obstacles in the use of pharmacologically conventional inhibitors of P450 enzymes resulted in the development of antisense strategies, inhibitory antibodies, and chemical inhibitor strategies. Inhibitory antibodies to CYP3As are less specific and exhibit cross-reactivity between CYP3A4 and CYP3A5. For most commercially available antibodies, binding to the P450 enzyme does not cause effective inhibition of its catalytic activity. A number of studies using antisense approach to modulate P450 enzymes have been published in the past 7 years and have contributed greatly to our understanding of these important enzymes. Antisense oligonucleotides have long been a problematic technology; in most cases, antisense compounds degrade rapidly, and thus, their effects are transitory and their potency is low. Chemical inhibitors are useful for P450
mechanistic studies and are usually selective rather than P450-specific. However, chemical inhibitors are also problematic with selectivity and possible complicating interactions with the substrates. For example, quinidine not only inhibits CYP2D6 but also inhibits CYP1A1 and CYP1A2 at low concentrations. Molecular biology methods with targeted approaches, however, provide the possibility to distinguish CYP3A4 and CYP3A5 functionally.

In the present study, we used vector-based RNAi, an alternative, novel gene silencing means, to successfully suppress CYP3A4 gene expression and enzyme activity using CHL-3A4 cells. In our study, transfection efficiency was approximately 40 to 60% for vector-based hairpin siRNA. Among the three oligonucleotides (3′AII, II, and III), only CYP3A4II significantly reduced mRNA and protein levels of the CYP3A4 gene. RT-PCR and Western blot analyses showed that in none of the cases the cellular CYP3A4 expression could be reduced completely. A functional utility of this inhibitory approach was presented by way of altering the cytocidal activity of CYP3A4 substrate drugs, cyclophosphamide and ifosfamide, in a predictable manner. Another functional assay using nifedipine as the model CYP3A4 gene sequence and compared the safe use of RNAi in vivo. Removal of the 5′-triphosphate end of the RNAi approach versus chemical approach is the potential ability to distinguish between multiple isoforms of the same enzyme.

Our study has demonstrated successful silencing of CYP3A4 RNAi using viral vectors in vitro. It would be interesting to investigate whether our approach can silence CYP3A4 in vivo. However, difficult delivery is still the main obstacle to achieving in vivo gene silencing by RNAi technologies. In vivo gene silencing with RNAi has been reported by using both viral vector delivery (Scherr et al., 2003) and high-pressure, high-volume intravenous injection of synthetic siRNAs (Song et al., 2003), but these approaches show limitations if used in clinical settings. In vivo gene silencing has also been reported after local, direct administration (intravitreal, intranasal, and intrathecal) of siRNAs to sequestered anatomical sites in models of choroidal neovascularization (Reich et al., 2003), lung ischemia reperfusion injury (Zhang et al., 2004), and neuropathic pain (Dorn et al., 2004). These reported approaches demonstrated the potential of delivery to organs such as the eyes, lungs, and central nervous system. In addition, siRNAs, synthesized using phage polymerases but not by chemical methods, can trigger an antiviral response (activation of interferon pathway) in mammalian cells, potentially complicating the interpretation of RNAi-mediated gene silencing experiments (Gupta et al., 2004; Marques and Williams, 2005). This has raised concerns about the safe use of RNAi in vivo. Removal of the 5′-triphosphate end of an siRNA greatly diminishes interferon induction (Kim et al., 2004). Like other major classes of antisense agents for sequence-specific mRNA knockdowns, including antisense oligonucleotides, ribozymes, and DNAzymes, effective application of RNAi requires efficient delivery, enhanced stability, minimization of off-target ef-
fects, and identification of sensitive sites in the target RNAs (Scherer and Rossi, 2003).

In conclusion, the present study demonstrated the successful and specific inhibition of human CYP3A4 by RNAi approach. This was accomplished by a marked reduction in the metabolic activity of this important enzyme. The use of RNAi to inhibit gene expression in mammalian cells is a promising new tool for the study of cytochrome P450 family gene function.

References


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