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TITLE PAGE:

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

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Running Title Page

a) Running title: Silencing of *CYP3A4* gene by RNAi

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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 3A4III siRNAs, but not 3A4I and II, significantly reduced *CYP3A4* mRNA level by 65% and protein expression level by 75%. All these siRNAs did not affect the expression of *CYP3A5* at both mRNA and protein levels in V79 cells overexpressing CYP3A5. Transfection of CHL-3A4 cells with 3A4III siRNAs significantly diminished the cytotoxicity of two CYP3A4 substrate drugs, cyclophosphamide and ifosfamide, in CHL-3A4 cells, with the IC_{50} increased from 55–210 μM to >1000 μM. Nifedipine at 5.78, 14.44, and 28.88 μM was significantly ($P < 0.01$) depleted by about 100%, 40%, and 22%, respectively, in S9 fractions from CHL-3A4 cells, compared to parental CHL-pIC19h cells. In addition, transfection of the CHL-3A4 cells with vectors expressing the 3A4III siRNAs almost completely inhibited CYP3A4-mediated nifedipine metabolism. This study, for the first time, demonstrated the specific suppression of *CYP3A4* expression and function using vector-based RNAi technique. The use of RNAi is a promising tool for the study of cytochrome P450 family function.

INTRODUCTION:

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 dsRNA into siRNA (Bernstein et al., 2001). These siRNAs are then incorporated into the protein complex RISC that 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, while there is an increasing use of vector-based expression of hairpin RNAs as an alternative for delivery of siRNAs into mammalian cells.

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The cytochrome P450s (CYPs, 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 CYPs, the subfamily CYP3A is responsible for the metabolism of about 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 (Gellner 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 CYP 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 due to 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 Caucasian (Sarkar et al., 2003). Similarly, *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 particularly by the nuclear receptors, pregnane X receptor and constitutively activated receptor (Burk et al., 2004).

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There are few studies using RNAi to study the function of CYPs, 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 over-expresses 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*.

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MATERIALS AND METHODS:

Chemicals and Reagents

p*Silencer*™ hygro-based siRNA system (Cat #5760, 5766) for gene knockdown was from Ambion (Woodward, Austin, USA). DMEM, fetal bovine serum, trypsin and G418 were all from Invitrogen (Guangzhou, China). Lipofectamine 2000, OPTIMEM, and Trizol Reagent were all purchased from Invitrogen (Groningen, NL). 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 horseradish peroxidase labeled goat anti-rabbit IgG was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Hyperfilm ECL was obtained from Amersham Biosciences (Buckinghamshire, UK). Cyclophosphamide, ifosfamide, nifedipine (MW = 364.3) and nitrendipine (MW = 360.4, used as the internal standard) were from Sigma-Aldrich (St. Louis, MO). NADPH 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 V79MZ (CHL-pIC19h) and the genetically engineered Chinese hamster cells overexpressing human CYP3A4 (CHL-3A4) were provided by Dr. Johannes Doehmer (GenPharmTox BioTech AG, Fraunhoferstr. 9, 82152 Planegg/Martinsried, Germany) (Schneider et al., 1996), which have been used for the studies of drugs and other xenobiotics, including cyclophosphamide, territrem A, B

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and C, and 1-methylpyrene (Schneider et al., 1996; Engst et al., 1999; Philip et al., 1999; Peng et al., 2003; Peng et al., 2006). The *CYP3A4*-transfected cell line was established using pIC19h plasmids carrying the SV40 early promoter and the SV40 polyadenylation with co-expression 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^{o}C in a humidified atmosphere of 5% CO_2 and 95% air. CHL-3A4 cells doubled every 10 ± 12 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 (Accession No. NM_017460, version 3 [GenBank]) 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 which encoded a hairpin structure with a 19-mer stem deriving from the mRNA target site were cloned into the p*Silencer*™ vector of siRNA expression. The p*Silencer*™ vector contained compatible restriction sites of *Bam*H I and *Hin*d III to ensure cloning into the correct site of the linearized vector and the oligonucleotides were

annealed and cloned into the vector according to the manufacturers recommendations. p*Silencer*™ vector were sequenced with universal primer in order 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 p*Silencer*™ 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 follow: 5'- GAT CCG TTC CTC CCT GAA AGA TTC TTC AAG AGA GAA TCT TTC AGG GAG GAA CTT TTT TGG AAA-3' (Top strand oligonucleotide template), and 5'-AGC TTT TCC AAA AAA GTT CCT CCC TGA AAG ATT CTC TCT TGA AGA ATC TTT CAG GGA GGA ACG -3' (Bottom strand oligonucleotide template). The product of the positive control ligation is a p*Silencer* hygro plasmid containing siRNA template targeting green fluorescent protein (GFP). The GFP control insert sequences were: 5'-GATCC GGTTATG TACAGG AACGCA TTCAAGAGATGCGTTC CTGTACATAACC TTTTTGGAAA-3' (Top strand oligonucleotide template), and 3'-G CCAATACATGTCCTTGCGT AAGTTCTCT ACGCAAGGACATGTATTGG AAAAACCTTTTCGA-5' (Bottom strand oligonucleotide template). This construct reduced GFP expression of pEGFP-C1 vector. CHL-3A4 cells cotransfected with 400 ng of the mammalian expression vectors with GFP (pEGFP-C1, from Invitrogen) and 4 μg of p*Silencer* hygro-siGFP. GFP expression was analyzed by fluorescent analysis at regular intervals starting about 24 hr after transfection.

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Plasmid Construction and Transfection of cDNA of human *CYP3A5* to CHO cells

A full-length cDNA of *CYP3A5* gene was cloned, and a recombinant eukaryotic expression plasmid was constructed, then stably transfected in CHO cells were established at our laboratory. The plasmids containing a full length of *CYP3A5* cDNA were constructed by PCR amplification based on the sequence as described previously (Jounaidi et al., 1994). The *CYP3A5* cDNA was amplified out of human genomic DNA by PCR with the forward oligonucleotides, 5′-ATGGAAAAATGTGGGGAACG-3′ and 5′-CGCTGGTGAAGGTTGGAGAC-3′ (Jounaidi et al., 1994; Krusekopf et al., 2003). The PCR products were cloned using *Kpn*I and *Nhe*I restriction sites to pGL3-Basic (Promega, Madison, WI) in front of the luciferase reporter gene. The recombinant plasmid was designated as pGL3-*CYP3A5*. The identity of the constructs was verified by sequencing. The *CYP3A5* sequence amplified from the human liver was found to be fully identical to the sequence (accession no. NG_000004.2; GI: 21536445) [GenBank].

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, Singapore). Twenty-four hours after transfection, the media was replaced with serum-free Ham's F-12 medium, and the 10 µM dexamethasone or dimethyl sulfoxide was added when appropriate for 48 h. The luciferase activities for transfected CHO cells were measured using the luciferase assay system (Promega, Madison, WI). The CHO cells, which did not show transcript of *CYP3A5* gene, with recombinant plasmid pGL3-*CYP3A5*, resulted in CHL-CYP3A5 cell

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line, and transfection of CHO cells with the parental pGL3-Basic vector served as control CHL-pGL3 cell line. The stable expression of CYP3A5 was confirmed by Western blotting analysis using antibody against CYP3A5.

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 6-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 lipofectamine 2000 (LP2000) and serum-free medium (OPTI-MEM) according to the manufacturers recommendations. All transfections were done in triplicate and cells were harvested 48 h post-transfection. Optimal amount of LP2000 and cell number were determined prior to transfection of GFP expressing plasmids. Transfection efficiency was about 40–60% for vector-based hairpin siRNAs estimated by fluorescence microscopy. Cells were also transfected with the negative control and positive control plasmids.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

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 [GenBank]; and for *CYP3A5*: accession no. NG_000004.2; GI: 21536445) [GenBank]) (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

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sequences (accession no. NM_033017.2 for *CYP3A4* [GenBank], and AY893017.1 and GI: 60654486 for *CYP3A5* [GenBank]) (Aoyama et al., 1989; Gellner et al., 2001; Strausberg et al., 2002).

Total RNA was isolated from cultured cells using the TRIzol™ reagent Isolation System kit (Invitrogen, Guangzhou, China) 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 containing 2 μg RNA, 0.5 μg/l random primer (Sangon Co, China), 10 mM dNTP mix, M-MLV 5 × Reaction buffer, 25 unit RNase inhibitor and 200 unit M-MLV (Promega Co., Madison, WI) at 37˚C for 1 h. PCR was performed by using the *CYP3A4* primers, 5'AAATCTGAGGCGGGAAGC3' (forward, F) and 5'TTGGGATGAGGAATGGAAAG 3' (reverse, R). The PCR conditions were as follows: 1 cycle of 94°C for 2 min; 30 cycles of 94°C for 45 sec, 55°C for 45 sec and 72°C for 45 sec; followed by incubation at 72°C for 7 min. Similar procedures were conducted for the amplification and determination of human *CYP3A5* gene, but the primers were 5′-ATGGAAAAATGTGGGGAACG-3′ (F) and 5′-CGCTGGTGAAGGTTGGAGAC-3′ (R), as described previously (Krusekopf et al., 2003).

RT-PCR was also performed using primers for glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) gene messages as a control. Primers for GAPDH were 5'-TCCACCACCCTGTTGCTGTAG -3' (F) and 5'- GACCACAGTCCATGACATCACT -3' (R). The PCR reaction was conducted in a volume of 50 μl containing 1 μl Taq enzyme (5 U/μl, 4 μl dNTP at 10 mM, 10 μl RT product, and 1 μl of each primer at 40 pmol), and 5 μl 10 × 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 UVP photo scanner (Bio-rad, Hercules, CA) and the digital data were collected nd processed using the LAB WOEK45 Image software.

Western Blotting 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 cellular protein 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 Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Lysates were centrifuged at 12, 000 × *g* at 4^{o}C for 10 min. Total protein content was determined in the extracts using the Lowry method using bovine serum album 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 (1:2,000 in TBS/Tween),

and subsequently with a corresponding horseradish peroxidase-conjugated second antibody IgG (Amersham /Pharmacia, UK) and developed using Chemiluminescence Reagent Plus (Perkin Elmer Life Science, Inc). The scan densitometric analysis was carried out using GDS-8000 UVP photo scanner and LAB WOEK45 Image software (Bio-rad).

Cytotoxicity Assay

Cell viability was determined based on the ability of live cells to reduce a tetrazonium-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, 5,000–10,000 cells/well were seeded in duplicate in 96 wells 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)-lipofectamine 2000 (0.5 μl). Cells adhered to the plate as usual in the presence of DNA (0.2 μg)-Lipofectamine 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 third day, the drug-containing medium was replaced by fresh medium and aliquots of 20 μl MTT (5 mg/ml) were added to each well. After incubation for 4 h at 37°C, the supernatant was removed and 150 μl DMSO 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., Winooski,

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Vermont) at a wavelength of 490 nm. Vehicle-treated cells were assigned a value of 100%. Each experiment was performed in 8 replicate wells for each drug concentration and carried out independently at least 3 times in different days. The cytotoxicity was evaluated with reference to the IC_{50} value that was defined as the concentration needed for a 50% reduction of survival based on the survival curves. IC_{50} values were calculated from dose-response curves (i.e., cell survival *vs*. drug concentration) obtained in multi-replicated experiments.

Preparation of Subcellular S9 Fractions

S9 fractions were prepared from harvested cells as described previously (Zhou et al., 2000). Briefly, CHL-pIC19h and CHL-3A4 cells were seeded at 5×10^5/ml and grown in 6-well plate for 2-4 days until confluent. The cells were washed twice with warm (37^oC) phosphate-buffered saline (PBS, pH 7.4), and harvested by scraping using a policeman. Subsequently, the cells were sonicated in 0.15 mol/l KCl and homogenized using homogenizer at 4^oC (Heidolph Instruments GmbH & Co. KG, Germany). The homogenates were centrifuged at $9{,}000 \times g$ for 20 min at 4^oC 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^oC. 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

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200 mM NADPH (freshly prepared) and S9s at 0.3 mg in 0.15 mol/l KCl pH 7.4. The mixtures were pre-incubated for 5 min at 37^{o}C with 100 mM KH_2PO_4 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 μM (i.e. 4.0 μg/ml) of nitrendipine. 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 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 C_{18} reversed-phase column (4.6 mm × 150 mm, particle size: 5 μm) Dalian Elite Analytical Instruments Co., Ltd., Dalian, China) was used with a Waters (Milford, MA, USA) 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 nifedipine degradation. The HPLC method for determination of nifedipine was fully validated. The linear range was 0.29–57.75 μM (i.e. 0.1–20 μg/ml) (r^2=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 77.6–87.3% using methanol and the method recovery was 90.5-101.7%.

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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.htm). Genomic sequence numbers 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 (ANOVA) followed by the Student-Newman-Keuls test. A *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 THE respective gene and the corresponding protein in CHL-3A4 and CHL-3A5 cells and their respective control cells. Since 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 decrease 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 fifth 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 co-transfected 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

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and counted under inverted microscope, and cell concentration was adjusted to 5×10^5/ml in a 6-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 p*Silencer* hygro- siGFP (**Fig. 5**). In contrast, cells transfected with the negative control siRNAs showed no significant reduction of *CYP3A4* gene expression levels (**Fig. 1** & **Fig. 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 (about 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

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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 towards 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 due to the lack or minimal activity of CYP3A4. 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 to 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. Notably, 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**).

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DISCUSSION:

The identification of CYPs 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 CYP 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 CYP enzyme does not cause effective inhibition of its catalytic activity. A number of studies utilizing antisense approach to modulate CYP enzymes have been published in the past seven years and have contributed greatly to our understanding of these important enzymes. Antisense oligonucleotides have long been a problematic technology, in most case, 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 about 40–60% for vector-based hairpin siRNA. Among the three oligonucleotides (3A4I, II and III), only CYP3A4III 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 substrate also demonstrated the efficient inhibition of CYP3A4 activity in CHL-3A4 cells.

Our study demonstrated the three siRNAs tested did not generate any inhibitory effects on *CYP3A5* expression at both mRNA and protein levels in CHL-3A5 cells. This result indicated that the observed suppression of *CYP3A4* expression and function by CYP3A4III was specific targeting *CYP3A4* mRNA only. This provides further evidence that RNAi is a powerful approach to study gene function, in particular when other approached are hard to distinguish similar genes' (e.g. genes from the same family or superfamily) relative contribution in vivo and in vitro.

These results are consistent with previously reported functional studies for both CYP3A4 and CYP3A5. Although the amino acid sequences of CYP3A4 and

CYP3A5 are 84% identical, their functional differences indicate that key differences may exist in their active sites. Key differences can be seen in amino acid residues in substrate recognition sites regions in CYP3A4 and CYP3A5 that confer functional competence and/or divergence, novel catalytic specificities and activities, and specific regio- and stereo-selective targeting of a given substrate (Wang et al., 1998). Thus, the differences in the substrate recognition sites regions between CYP3A4 and CYP3A5 appear to critically influence the metabolism of some their common substrates such as territrems B and C (Peng et al., 2006).

Transfection of synthetic- or in vitro transcribed siRNAs causes only transient knockdown of target genes and is often limited to cell lines that are easily transfected (Bernstein et al., 2001). Our study demonstrated that the vector-based knockdown of *CYP3A4* gene could be maintained for up to 4 days. We selected siRNA target sites at different positions of the *CYP3A4* gene sequence and compared the potential target sites to the appropriate genome database (human, mouse, rat, etc.) and eliminate any target sequences with more than 16–17 contiguous base pairs of homology to other coding sequences by using BLAST.

The suppression in cytocidal activities of CYP3A4 substrate drugs cyclophosphamide and ifosfamide, when coadministered with CYP3A4III plasmids, has important clinical implication in chemotherapy. Both cyclophosphamide and ifosfamide requires CYP3A4-mediated 4-hydroxylation for activation of its cytocidal activity. Our data

indicate that siRNA-transfected CHL-3A4 cells were significantly resistant to the both prodrugs due to the inhibition of CYP3A4 by RNAi and reduced activation. The use of RNAi technology for CYP3A4 inhibition has important clinical significance with regards to manipulating the metabolic fate of existing drugs and others under development that are CYP3A4 substrates. It is likely to attenuate the organ (e.g. kidney, intestine, and bone marrow) toxicity using RNAi technology. Furthermore, RNAi strategy in combination with established drugs is likely to reduce inter-individual variation of drug metabolism thereby resulting in a more predictable response to therapy.

RNAi may have potential application in drug development. Our study provided confirmative evidence that CYP3A4 are the major enzyme metabolizing cyclophosphamide, ifosfamide and nifedipine. The inhibition of specific enzymes of this family by RNAi can be used for unequivocal identification of the CYPs involved in the metabolism of a particular compound and significantly alter the disposition and toxicity of substrate drugs. An important distinguishing feature 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 ischaemia 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, DNAzymes, effective application of RNAi requires efficient delivery, enhanced stability, minimization of off-target effects 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 accompanied with 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.

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FOOTNOTES SECTION:

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Figure Legends:

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 semi-quantitative RT-PCR analysis. The *CYP3A4* and *GAPDH* amplification were performed for 30 cycles and 25 cycles, respectively. PCR products were electrophoresed in a 2.0% agarose gel and stained with ethidium bromide. Sizes of the PCR products of CYP3A4 and GAPDH were 220 and 450 bp, respectively. Lane 1, DL2000 DNA Marker; lane 2, CHL-3A4 cells without treatment; lane 3, control CHL-pIC19h cells which did not express CYP3A4; lane 4, CHL-3A4 cells with treatment of lipofectamine (LP) 2000; lane 5, CHL-3A4 cells transfected with p*Silencer* vectors in which the oligonucleotides were not annealed and cloned into; lane 6, CHL-3A4 mock cells as an RNAi negative control; and Lanes 7–9, CHL-3A4 cells with different treatment of siRNAs, including CYP3A4III, CYP3A4II, and CYP3A4I, 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.

Fig. 2. Western blot analysis of CYP3A4 after a transient transfection performed with Lipofectamine 2000 in CHL-3A4 and CHL-pIC19h cells. All experiments were performed 48 h after treatment with three different siRNA molecules and control treatments. **A:** Lane 1, control CHL-pIC19h cells which did not express CYP3A4 protein;

lane 2, CHL-3A4 cells without treatment; lane 3, CHL-3A4 cells with treatment of lipofectamine (LP) 2000; lane 4, CHL-3A4 cells transfected with p*Silencer* vectors in which the oligonucleotides were not annealed and cloned into; lanes 5–6, CHL-3A4 mock cells as RNAi negative controls; and Lanes 7–9, CHL-3A4 cells with treatment of different siRNAs, including CYP3A4I, CYP3A4II, and CYP3A4III, respectively. **B:** Relative CYP3A4 protein expression level normalized against β-actin protein expression in CHL-3A4 and CHL-pIC19h cells. The CYP3A4/β-actin protein expression ratio in CHL-3A4 cells was set as 100. The relative expression values are means of at least three independent experiments in triplicate. $^{**}P < 0.01$, compared with the control CHL-3A4 cells.

Fig. 3. Effects of various siRNAs on the expression of *CYP3A5* at RNA (A & B) and protein levels (C & D) in the control CHL-pGL3 and *CYP3A5* cDNA-transfected CHL-3A5 cells. RT-PCT and Western blotting assay procedures for *CYP3A5* mRNA and protein level determination were largely similar to those for *CYP3A4* CHL-3A4 and CHL-pIC19h cells. A: Lane 1, DL2000 DNA Marker; lane 2, CHL-3A5 cells without treatment; lane 3, parental CHL-pGL3 cells which did not express CYP3A5; lane 4, CHL-3A5 cells treated with lipofectamine (LP) 2000; lane 5, CHL-3A5 cells transfected with p*Silencer* vectors in which the oligonucleotides were not annealed and cloned into; lane 6, CHL-3A5 mock cells as the RNAi negative control; and Lanes 7–9, CHL-3A5 cells treated with different siRNAs, including CYP3A4III, CYP3A4II, and CYP3A4I, respectively. **B** (bar graph): Relative *CYP3A5* mRNA expression level normalized against *GAPDH* mRNA expression in CHL-3A5 and CHL-pGL3 cells. The relative expression

values are means of at least three to five independent experiments in triplicate. The *CYP3A5/GAPDH* mRNA expression ratio in CHL-3A5 was set as 100%. **C**: Western blot analysis of CYP3A5 after a transient transfection performed with lipofectamine 2000 in CHL-3A5 and CHL-pGL3 cells. All experiments were performed 48 h after treatment with three different siRNA molecules and control treatments. Lane 1, CHL-3A4 cells without treatment; lane 2, CHL-pGL3 cells which did not express CYP3A5 protein; lane 3, CHL-3A5 cells with treatment of lipofectamine (LP) 2000; lane 4, CHL-3A5 cells transfected with p*Silencer* vectors in which the oligonucleotides were not annealed and cloned into; lanes 5–6, CHL-3A5 mock cells as the RNAi negative controls; and Lanes 7–9, CHL-3A5 cells with treatment of different siRNAs, including CYP3A4I, CYP3A4II, and CYP3A4III, respectively. **D**: Relative CYP3A5 protein expression level normalized against β-actin protein expression in CHL-3A5 and CHL-pGL3 cells. The CYP3A5/β-actin protein expression ratio in CHL-3A5 cells was set as 100. The relative expression values are means of at least three independent experiments in triplicate.

Fig. 4. A kinetic study of *CYP3A4* expression in CHL-3A4 cells transfected with vectors expressing CYP3A4III siRNAs over 5 days. **A**: mRNA expression; and **B**: protein expression level. CHL-3A4 cells were seeded at a density of 75,000 cells/well in a 6-well plate and incubated for 24 h. Cells were transfected with hairpin-producing plasmids (4.0 μg/well) using 10 μl lipofectamine 2000 (LF2000) and serum-free medium. All transfections were done in triplicate and cells were harvested 2–5 days post-transfection. The MRNA and protein were determined by RT-PCR and Western blotting analysis, respectively.

Fig. 5. Typical fluorescent microscopic pictures of CHL-3A4 cells at 24 h after transient transfection performed with lipofectamine 2000 in CHL-3A4 cells in a 6-well culture plate. CHL-3A4 cells were digested with 0.1% trypsin and counted under inverted microscope, and the cell density was adjusted to 5×10^5/ml in a 6-well culture plate. **A**: untransfected CHL-3A4 cells as a control for the fluorescent signal; **B**: CHL-3A4 cells transfected with EGFP expression vectors (pEGFP-C1) as a positive control; **C**: CHL-3A4 cells cotransfected with 400 ng of pEGFP-C1 and p*Silencer* hygro-siGFP-extra as the negative control for the siRNA experiment; and **D**: CHL-3A4 cells cotransfected with 400 ng of pEGFP-C1 and 4 μg of p*Silencer* hygro- siGFP.

Fig. 6. Survival curves of CHL-pIC19h, CHL-3A4, and CHL-3A4 cells transfected with siRNA-expressing vectors with CYP3A4III when treated with cyclophosphamide (**A**), or ifosfamide (**B**). CHL-pIC19h, CHL-3A4 and CHL-3A4 cells transfected with siRNA-expressing vectors with CYP3A4III were incubated with increasing concentrations of either drug for 24 h. Cytotoxicity was determined by the MTT assay and the percentage survival of cells relative to controls was calculated for each concentration of drug to determine the IC_{50}.

Fig. 7. Suppression of CYP3A4-mediated nifedipine metabolism by RNA interference in CHL-pIC19h and CHL-3A4 cells. Nifedipine at 5.78, 14.44, and 28.88 μM was incubated with 0.3 mg of S9 homogenates from CHL-pIC19h, or CHL-3A4 cells, in the

presence of an NADPH generating system in 100 mM KH_2PO_4 at pH 7.4 and 37^{o}C for 30 min with a total volume of 1.0 ml. The reaction was stopped by adding 3 volumes of ice-cold methanol containing 11.1 μM nitrendipine, which was used as the internal standard. The samples were vortexed for 2 min and the supernatant was evaporated using a Speedvac rotary concentrator. The residues were reconstituted using 50 μl mobile phase and 25 μl of the solution was injected onto the HPLC for nifedipine concentration determination. $^{**}P < 0.01$ compared with the control CHL-3A4 cells ($n = 6$).

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TABLE:

TABLE 1

Sequences of Small Interfering RNA oligonucleotides used in this study.

hairpin siRNA	Target sequence	Sense strand (5'-3')	Antisense strand (5'-3')	Position in gene sequence
CYP3A4I	AAAGACGTCTTTGGGGCCTAC	5'-GATCCAGACGTCTTTGGGGCCTAC TTCAAGAGAGTAGGCCCCAAAGAC GTCTTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAAAAAGACGTCTT TGGGGCCTACTCTCTTGAAGTAGGCC CCAAAGACGTCT G-3'	621-642
CYP3A4II	AAGAAATCTGTGCCTGAGAAC	5'-GATCCGAAATCTGTGCCTGAGAAC TTCAAGAGAGTTCTCAGGCACAGAT TTCTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAAAAGAAATCTGT GCCTGAGAACTCTCTTGAAGTTCTCA GGCACAGATTTC G-3'	1647 -1658
CYP3A4III	AACTCTGAAATGAAGATGGGC	5'-GATCCGCTCTGAAATGAAGATGG GCTTCAAGAGAGCCCATCTTCATTT CAGAGTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAAAACTCTGAAAT GAAGATGGGCTCTCTTGAAGCCCATC TTCATTTCAGAG CG-3'	1698 -1719

Figure 1

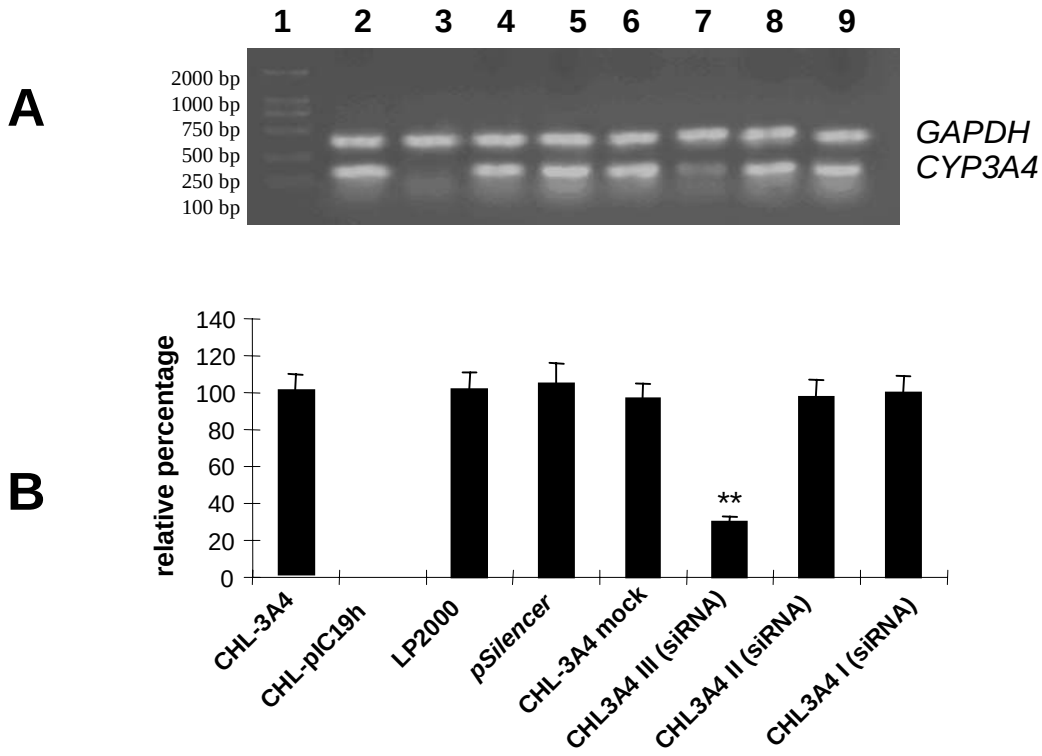

Figure 2

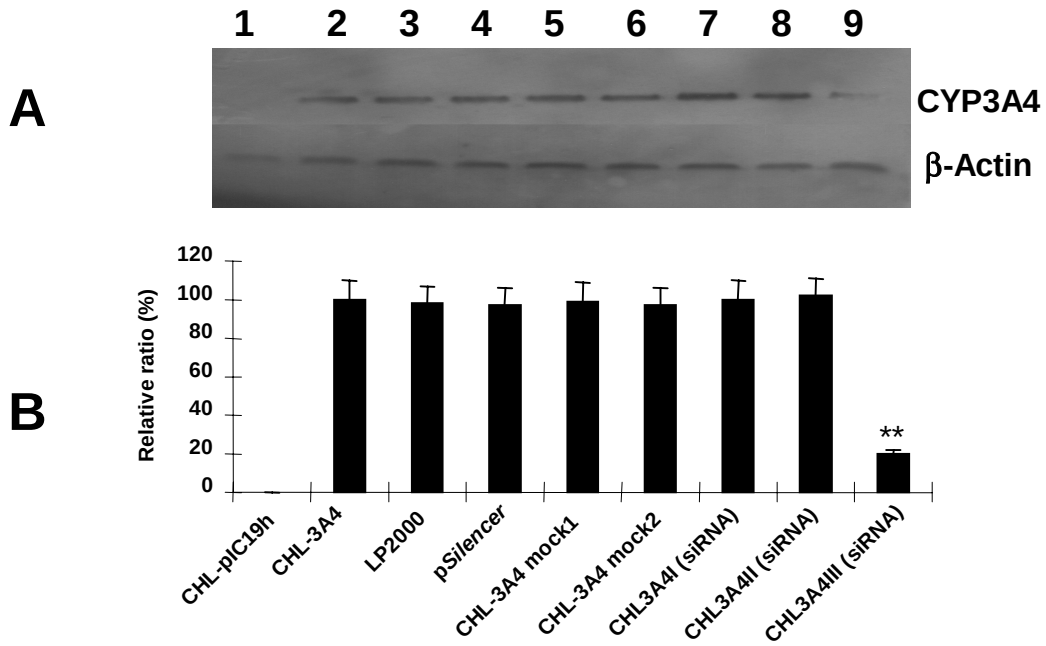

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Figure 3

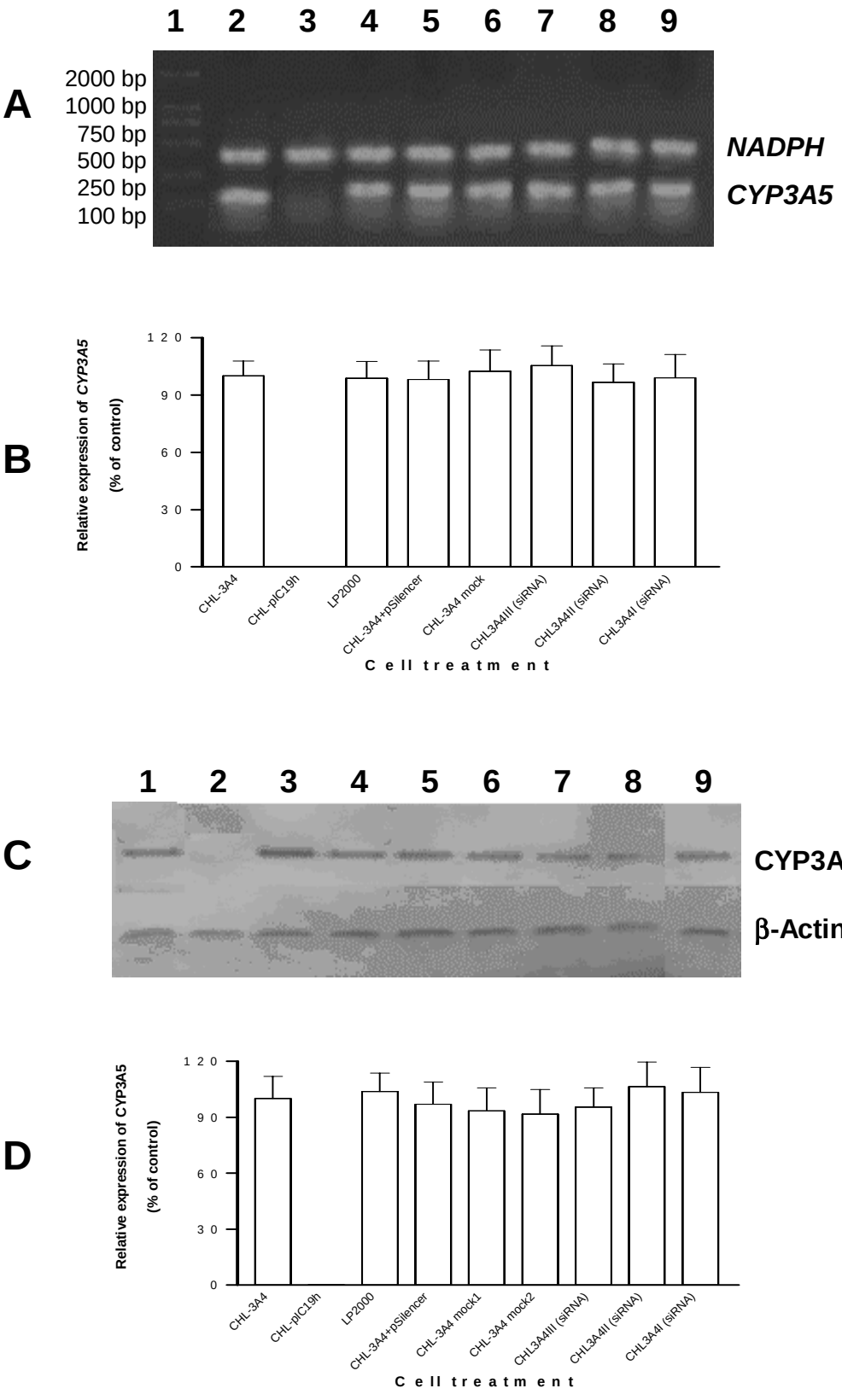
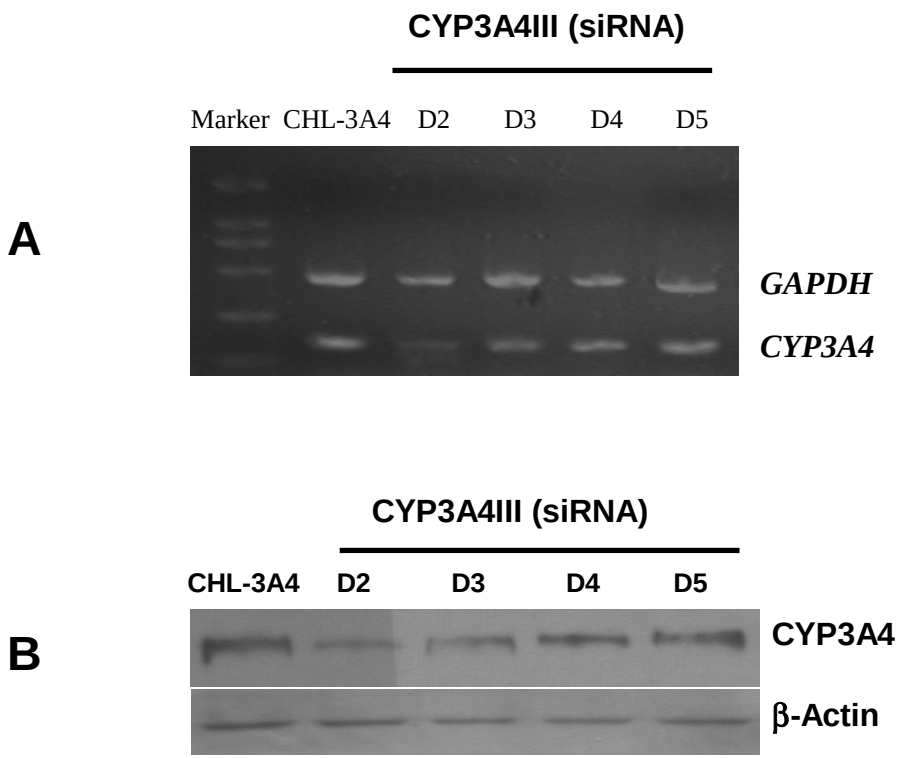


Figure 4



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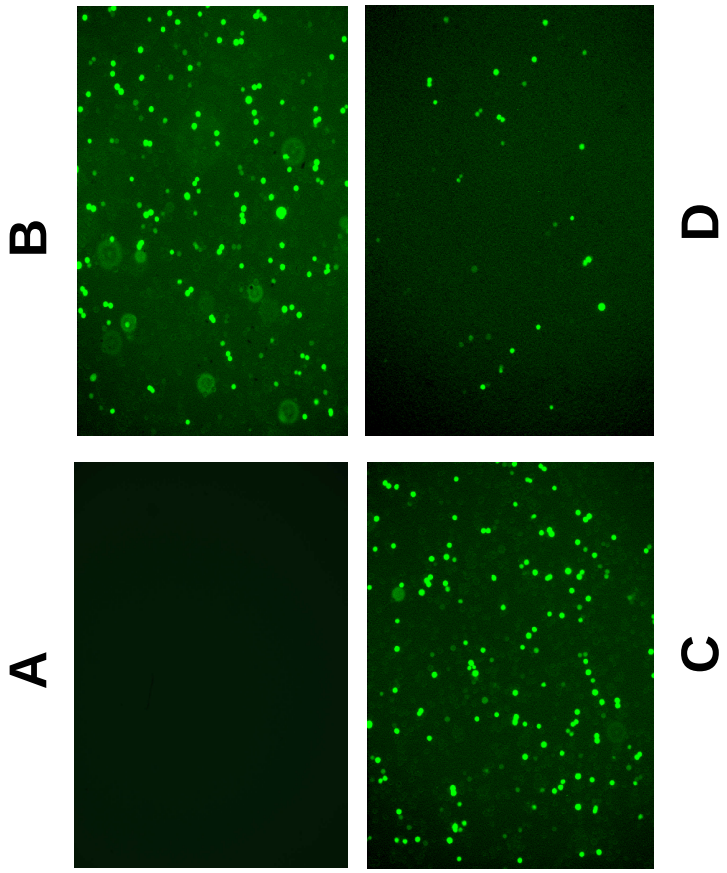

Figure 5

Figure 6

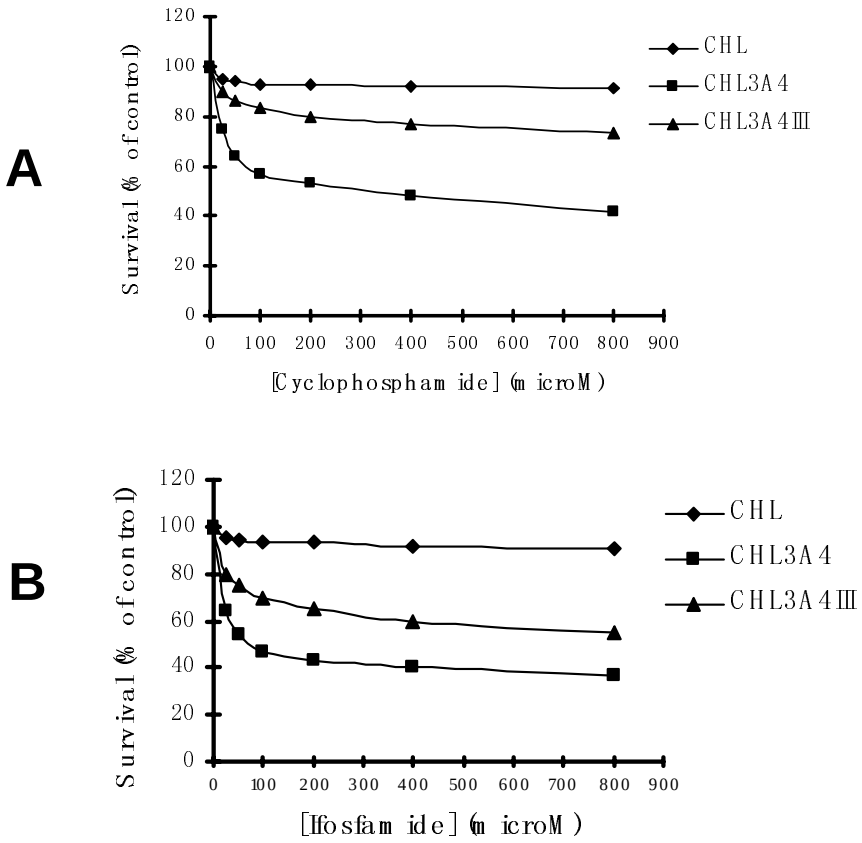

Figure 7