Reduction of Bilirubin by Targeting Human Heme Oxygenase-1 Through siRNA

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Neonatal hyperbilirubinemia is a common clinical condition caused mainly by the increased production and decreased excretion of bilirubin. Current treatment is aimed at reducing the serum levels of bilirubin. Heme oxygenase-1 (HO-1) is a rate-limiting enzyme that generates bilirubin. In this study we intended to suppress HO-1 using the RNA interference technique. Small interfering RNA (siRNA)-A, -B, and -C were designed based on human HO-1 (hHO-1) mRNA sequences. siRNA was transfected into a human hepatic cell line (HL-7702). hHO-1 transcription and protein levels were then determined. In addition, the inhibitory effect of siRNA on hHO-1 was assessed in cells treated with hemin or transfected with an hHO-1 plasmid. siRNA-C showed the most potent suppressive effect on hHO-1. This inhibition is dose and time dependent. Compared with control, both hemin and hHO-1 plasmids up-regulated hHO-1 expression in HL-7702 cells. However, the up-regulation was significantly attenuated by siRNA-C. Furthermore, the decrease in hHO-1 activity was coincident with the suppression of its transcription. Finally, siRNA-C was shown to reduce hHO-1 enzymatic activity and bilirubin levels. Thus, this study provides a novel therapeutic rationale by blocking bilirubin formation *via* siRNA for preventing and treating neonatal hyperbilirubinemia and bilirubin encephalopathy at an early clinical stage. Exp Biol Med 232:495–502, 2007

Key words: heme oxygenase; RNA-mediated interference; small interfering RNA; bilirubin; hyperbilirubinemia

Introduction

Neonatal hyperbilirubinemia, a common clinical condition found in newborns, is caused by the increased production and decreased excretion of bilirubin (1). Approximately 60% of full-term infants develop hyperbilirubinemia within 1 week of birth, and approximately 10% of those neonates require clinical intervention (2). In most circumstances neonatal hyperbilirubinemia has a benign course. However, severe cases may result in bilirubin encephalopathy (kernicterus). The incidence of bilirubin encephalopathy has risen in the past 15 years (3).

Heme oxygenase (HO) is a rate-limiting enzyme that metabolizes heme into bilirubin *via* an energy-consuming process and simultaneous release of 1 molecule each of CO and Fe^{3+} (1). HO has 3 isoenzymes among which HO-1 can be up-regulated by many factors including hemin (4). The expression of HO-1 usually increases in hyperbilirubinemic neonates (5). Nevertheless, when the expression or enzymatic activity of HO-1 is reduced, nonredox heme is generally excreted by the liver through the biliary duct system (6).

RNA-mediated interference (RNAi), a newly emerging technique, can target specific mRNA *via* duplex RNA, silencing the corresponding gene. RNAi has been demonstrated as a highly specific and effective gene inhibitor. As the second generation of therapeutic RNA, small interfering RNA (siRNA) is more specific and stable and has become a promising candidate for therapeutic gene targeting (7–9). To date, there have been no reports of applying siRNA to reduce bilirubin levels for prevention or treatment of neonatal hyperbilirubinemia.

In this study we used the RNAi technique to design and synthesize 3 pairs of siRNA based on the human HO-1 (hHO-1) mRNA sequence. We examined the inhibitory effect of siRNA on hHO-1 expression in a human hepatic cell line, HL-7702. hHO-1 transcription and protein levels were determined by reverse transcriptase polymerase chain

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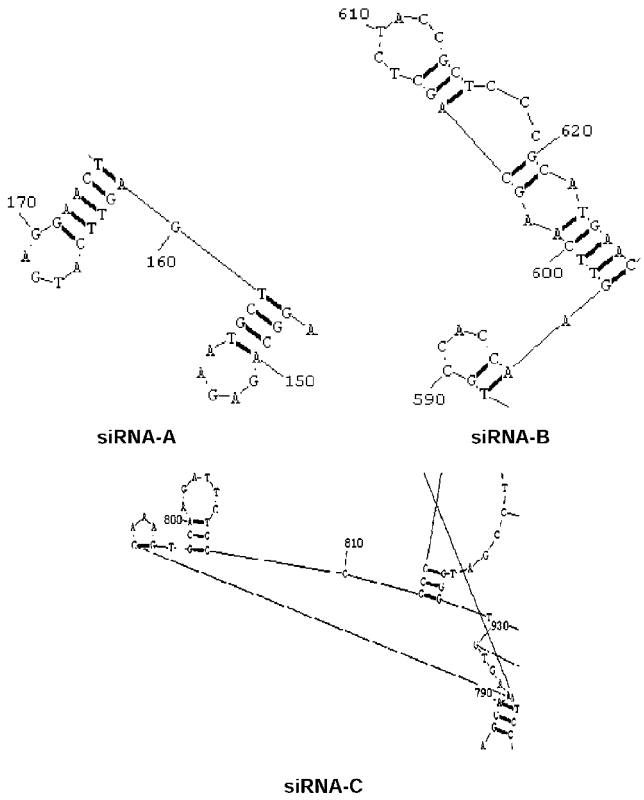


Figure 1. Three pairs of siRNA target the sequences of hHO-1 mRNA loop regions with low energy of RNA secondary structure.

reaction (RT-PCR) and by performing a Western blot, respectively. An hHO-1 enzymatic assay was performed to evaluate whether siRNA could effectively reduce the activity of HO-1 and lower the production of bilirubin.

Materials and Methods

Cell Culture. A human hepatic cell line (HL-7702) was provided by the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Gibco-BRL, Grand Island, NY) at 37°C under an atmosphere of 5% CO_2.

Experimental Treatments. HL-7702 cells were treated with hemin (Sigma-Aldrich, St. Louis, MO) at 0, 10, 30, or 50 μg/ml for 24, 48, and 72 hrs. The cells were then harvested for determination of hHO-1 expression in the different experimental settings.

Construction and Delivery of siRNA. Three pairs of siRNA were designed according to the sequences of hHO-1 mRNA loop regions with low energy of RNA secondary structure (Fig. 1, Table 1). The oligonucleotides were synthesized by the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Transfection of siRNA into the cells was carried out using Oligofectamine reagent (Invitrogen, Carlsbad, CA). Briefly, the cells were split into 12-well plates the day before the transfection at 50%–70% confluency. siRNA duplexes diluted in Opti-Mem I were incubated with Oligofectamine2000 in Opti-Mem I at room temperature for 25 mins to form a liposome-siRNA complex. Meanwhile, the same transfection method was used for blank and the nonspecific siRNA control groups. The liposome complex was added to cells in serum-free media for subsequent experiments.

Table 1. Sequences of 3 Pairs of siRNA Molecules Targeting hHO-1 mRNA

		Sequences
siRNA-A	154+	5′-UGC UGA GUU CAU GAG GAA CdTdT-3′
	154−	5′-GUU CCU CAU GAA CUC AGC AdTdT-3′
siRNA-B	591+	5′-CCA AGU UCA AGC AGC UCU AdTdT-3′
	591−	5′-UAG AGC UGC UUG AAC UUG GdTdT-3′
siRNA-C	786+	5′-GCA ACA AAG UGC AAG AUU CdTdT-3′
	786−	5′-GAA UCU UGC ACU UUG UUG CdTdT-3′

Assessment of Transfection Efficiency. Sterile cover glasses were placed in a 6-well plate. HL-7702 cells were seeded on the cover glasses for 24 hrs. The cells were transfected with fluorescein isothiocyanate (FITC)-labeled nonspecific siRNA for 48 hrs, followed by nucleus staining using propidium iodide. The fluorescence of siRNA (green) and nucleus (blue) was analyzed by fluorescent microscopy.

Construction and Transfection of pcDNA3.1h-HO-1. pcDNA3 plasmid was purchased from Invitrogen. pcDNA3.1hHO-1 was constructed as described previously (10). One hundred, 200, or 400 ng of the plasmid diluted in Opti-Mem I was incubated with Oligofectamine2000 for 25 mins at room temperature to form a liposome-plasmid complex. The liposome complex was then added to the cells in serum-free media for an hHO-1 expression assay.

RT–PCR Analysis. Total RNA from HL-7702 cells was isolated using TRIzol reagent (Invitrogen) at 24 hrs after siRNA transfection. RT-PCR was performed using the following primers: hHO-1: forward 5′-GGT GAT AGA AGA GGC CAA GAC TGC-3′, reverse 5′-TGT AAG GAC CCA TCG GAG AAG C-3′; hHO-2: forward 5′-CCA CCA CGG CAC TTT ACT TCA-3′, reverse 5′-GCT GGG CAT TGT CCA CAT TCT-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5′-GTC GTG GAG TCT ACT GGC GTC TT-3′, reverse 5′-CAG TCT TCT GAG TGG CAG TGA TGG-3′ (all primers were synthesized by Shanghai BioAsia Biotechnology Co., China). The PCR conditions were 94°C for 4 mins to denature the RNA/cDNA hybrid; 35 cycles at 94°C for 1 min; 56°C (hHO-1), 59°C (hHO-2), and 58°C (GAPDH) for 30 seconds; 72°C for 30 seconds; and final extension at 72°C for 10 mins. PCR products were analyzed on a 4.5% polyacrylamide gel.

Western Blot Analysis. The cells were harvested and centrifuged at 6000 rpm for 5 mins. Twenty μl of 2X SDS loading buffer was added to the cell lysates, and then the lysates were boiled for 10 mins. The samples were again centrifuged at 10,000 rpm for 3 mins. Aliquots of cell lysates containing 50 μg of protein were separated by a 12%

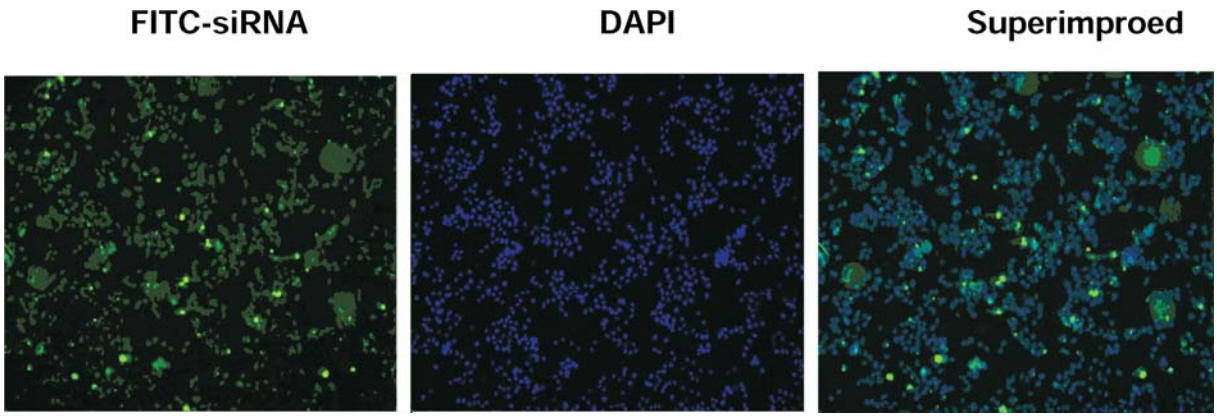


Figure 2. Assessment of siRNA transfection efficiency. FITC-labeled nonspecific siRNA was used to transfect the HL-7702 cells. Twenty-four hrs later the nucleus was stained with DAPI. The fluorescence microscope was employed to analyze the efficacy of siRNA transfection in HL-7702 cells. More than 90% cells were FITC positive. These data are representative of 3 separate experiments.

SDS-polyacrylamide gel, and the proteins were then transferred to a nitrocellulose membrane. The membrane was incubated with anti-hHO-1 IgG (Sigma-Aldrich, St. Louis, MO) at a 1:200 dilution overnight, followed by the addition of horseradish peroxidase–linked anti-mouse IgG (Rockland Immunochemicals, Inc., Gilbertville, PA) at a 1:5000 dilution. hHO-1 bands were visualized by ECL (Amersham, Arlington Heights, IL).

Assessment of HO-1 Enzymatic Activity. The cells were seeded in a 10-cm plate and transfected with 200 n*M* siRNA-C. Four hrs after siRNA transfection these cells were cultured with 30 μg/ml hemin for 48 hrs. In addition, some of the cells were cotransfected with 400 ng of pcDNA3.1hHO-1 for 24 hrs. The cells were lysed at 4°C and then centrifuged at 14,000 rpm for 10 mins. Ten grams of fresh Sprague-Dawley rat tissue was added into 20 ml of 0.1 *M* potassium phosphate buffer (pH 7.4), homogenized, and centrifuged at 40,000 rpm for 1 hr at 4°C. The middle-level aqueous phase containing biliverdin reductase was collected, and the protein concentration was measured using the BAC kit (Pierce, Rockford, IL) according to the manufacturer's instructions. The enzyme-catalyzed system included 10 n*M* hemin, 20 n*M* β-nicotinamide adenine dinucleotide phosphate hydrogenase (Sigma-Aldrich), 1 U/μl glucose-6-phosphate dehydrogenase (Sigma-Aldrich), 1.17 *M* glucose-6-phosphate (Sigma-Aldrich), 25 m*M* $MgCl_2$, an aliquot of biliverdin reductase, and the cell supernatant. The activity of hHO-1 was determined by spectrophotometric measurement of bilirubin production at OD_{464}. One unit of hHO-1 enzymatic activity was equivalent to 1 nmol bilirubin production per hour (11).

Statistical Analysis. Statistical comparisons were made between the means of data obtained from experimental groups and control groups using analysis of variance with Fisher's post hoc analysis.

Results

Efficacy of siRNA Transfection in HL-7702 Cells. Since the liver is a major organ for bilirubin metabolism, we selected a human hepatic cell line (HL-7702) for the study. After labeling, transfection, and staining, the intracellular distribution of siRNA in HL-7702 cells was analyzed by fluorescent microscopy. The fluorescent signals of siRNA (green) and the nucleus (blue) were superimposed to determine the location of siRNA in the cells. As illustrated in Figure 2, green siRNA fluorescence was localized around the nucleus. Furthermore, a majority of the cells expressed green fluorescence, and siRNA transfection efficiency in HL-7702 cells was estimated up to 90% (Fig. 2).

Down-Regulation of hHO-1 Gene Expression and Protein Production by siRNA. Subsequently we transfected HL-7702 cells for 48 hrs with siRNA-A, -B, and -C specifically designed to target hHO-1. RT-PCR and Western blots were performed to determine hHO-1 transcription and its protein level, respectively. GAPDH was used as an internal control for the experiment. Figure 3 shows that the expression of the hHO-1 gene and its protein decreased markedly at 48 hrs after siRNA transfection. Among the 3 types of siRNA probed, siRNA-C was the most potent suppressor of hHO-1 expression (Fig. 3A and B).

Dose-Dependent Down-Regulation of hHO-1 by siRNA. Based on the above finding, we chose siRNA-C for the following experiments. HL-7702 cells were transfected with 0, 100, 200, and 400 nM siRNA-C, respectively. Forty-eight hrs later total RNA and protein were extracted. RT-PCR and Western blots were employed to examine the changes in hHO-1 levels. As shown in Figure 4A and B, the decrease in hHO-1 mRNA expression and protein levels was correlated with the increase in siRNA-C concentration. Furthermore, siRNA-C did not alter GAPDH expression. These data indicate that the inhibitory effect of siRNA is dose dependent.

Time-Dependent Down-Regulation of hHO-1 by siRNA. Next we tested whether siRNA has a persistent inhibitory effect on hHO-1 expression. A time-course study was conducted in the HL-7702 cells transfected with 200

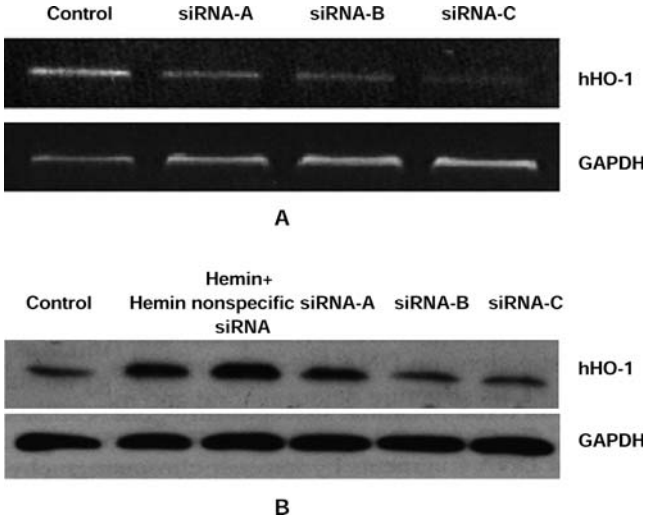


Figure 3. Comparison of the inhibitory efficacy of siRNA-A, -B, and -C specifically targeting hHO-1. HL-7702 cells were transfected with siRNA-A, -B, and -C, respectively. hHO-1 transcription was determined by RT-PCR. Compared with the control group without specific siRNA, siRNA-C caused the most marked suppression of hHO-1 transcription (A); hHO-1 protein production was determined by Western blot analysis. Compared with control group without any stimulation, hemin (30 μg/ml) and hemin (30 μg/ml) with nonspecific siRNA induced hHO-1 production. However, hHO-1 protein levels were decreased by the transfection of siRNA-A, -B, and -C, and siRNA-C exhibited the most potent inhibition on hHO-1 expression (B). These data are representative of 3 separate experiments.

n*M* siRNA-C. At 24, 48, and 72 hrs after transfection the cells were harvested for total RNA and protein extraction. hHO-1 mRNA and protein were detected by RT-PCR and Western blot, respectively. As shown in Figure 5A, siRNA-C caused the most profound inhibition of hHO-1 at 24 hrs after transfection. This inhibition was gradually diminished by 72 hrs. A similar suppressive pattern by siRNA-C was observed in hHO-1 protein production (Fig. 5B), suggesting that siRNA-mediated hHO-1 inhibition is time dependent.

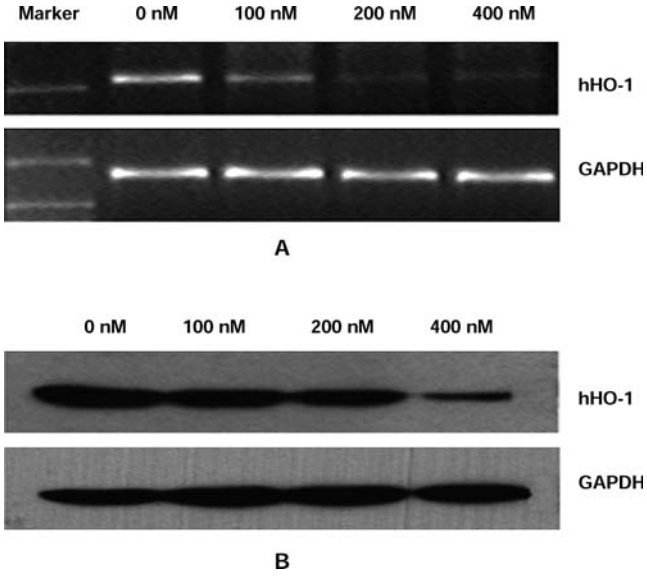


Figure 4. Dose-dependent down-regulation of hHO-1 transcription and protein production by siRNA-C. HL-7702 cells were transfected with siRNA-C of various concentrations. hHO-1 transcription was determined by RT-PCR. siRNA-C inhibited hHO-1 transcription in a dose-dependent manner (A); hHO-1 protein levels were determined by Western blot analysis. hHO-1 production was adversely correlated with the increase of siRNA-C concentrations (B). These data are representative of 3 separate experiments.

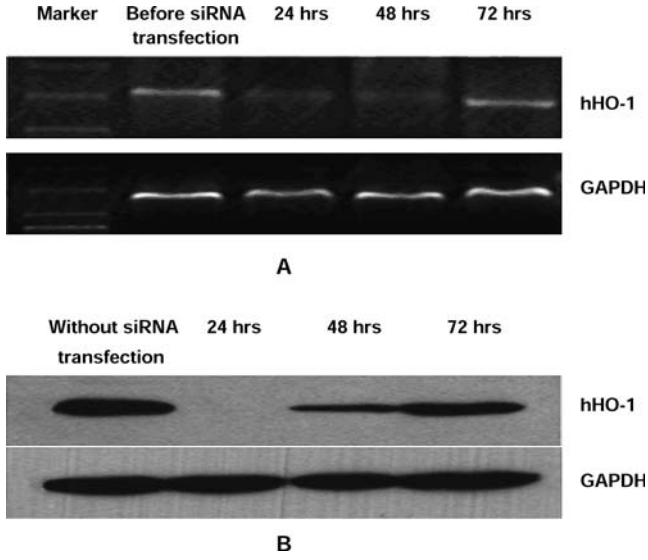


Figure 5. Time-dependent down-regulation of hHO-1 transcription and protein production by siRNA-C. HL-7702 cells were transfected with siRNA-C and harvested at 24, 48, and 72 hrs after transfection. hHO-1 transcription was determined by RT-PCR. siRNA-C abolished hHO-1 transcription. This inhibition of hHO-1 by siRNA-C was diminished by 72 hrs (A). hHO-1 protein levels were determined by Western blot analysis. siRNA-C blocked hHO-1 translation at 24 hrs. However, the inhibition was diminished by 72 hrs (B). These data are representative of 3 separate experiments.

Effects of Hemin and pcDNA3.1hHO-1 on hHO-1 Expression. To examine the potential effect of siRNA-C on hHO-1 expression induced by its regulators and exogenous HO-1, we first determined whether hemin was able to up-regulate hHO-1 in HL-7702 cells. The hepatic cells were treated with 0, 10, 30, and 50 μg/ml hemin, respectively. In a separate experiment HL-7702 cells were incubated with 30 μg/ml hemin for 24, 48, or 72 hrs. The level of hHO-1 protein was determined to assess the effective dose and acting duration of hemin. The experiment showed that hemin induced hHO-1 production in a dose-dependent manner (Fig. 6A). While in the presence of the same dose of hemin (30 μg/ml), the level of hHO-1 protein reached a peak level at 24 hrs and decreased afterward (Fig. 6B).

Finally, the production of exogenous HO-1 was analyzed by the transfection of pcDNA3.1hHO-1 at the different doses and durations. HL-7702 cells were transfected with 0, 100, 200, and 400 ng of pcDNA3.1hHO-1, respectively. A time-course study was also performed in the cells transfected with 400 ng of pcDNA3.1hHO-1 at 24, 48, and 72 hrs. hHO-1 levels increased in proportion to the concentration of pcDNA3.1hHO-1 (Fig. 6C), and the production of hHO-1 protein reached a peak level at 24 hrs and then decreased by 72 hrs (Fig. 6D).

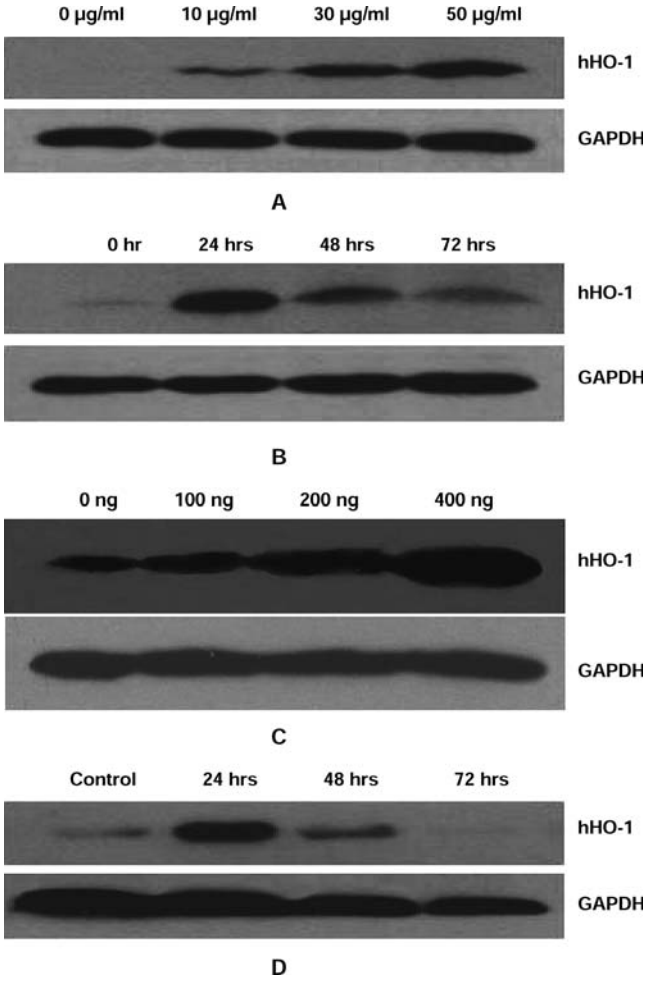
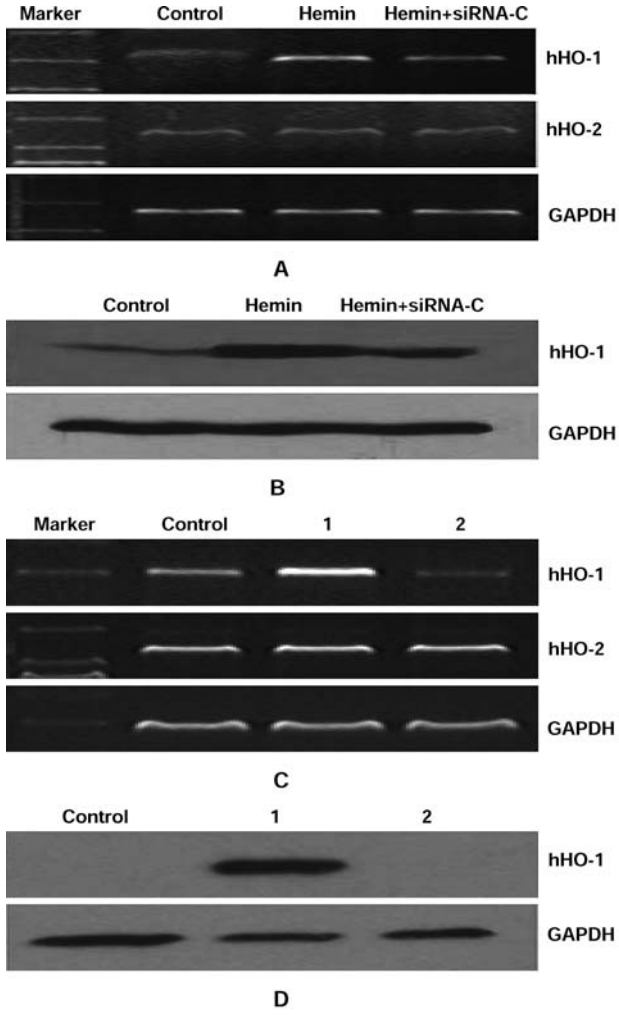


Figure 6. Effects of hemin and pcDNA3.1hHO-1 on hHO-1 level. (A) Dose-dependent induction of hHO-1 production by hemin. HL-7702 cells were treated with hemin of various concentrations. hHO-1 production was determined by Western blot. Hemin induced hHO-1 production in a dose-dependent manner. (B) Time-dependent up-regulation of hHO-1 by hemin. HL-7702 cells were treated with 30 μg/ml hemin. The level of hHO-1 protein was determined at 24, 48, and 72 hrs after the stimulation. hHO-1 production reached a peak level at 24 hrs and decreased afterward. (C) The production of hHO-1 protein in HL-7702 cells transfected with pcDNA3.1hHO-1. HL-7702 cells were transfected with pcDNA3.1hHO-1 of various concentrations. hHO-1 levels were determined by Western blot. pcDNA3.1h-HO-1 enhanced the production of exogenous hHO-1 in a dose-dependent manner. (D) Time-dependent production of hHO-1 in HL-7702 cells transfected with pcDNA3.1hHO-1. HL-7702 cells were transfected with 400 ng of pcDNA3.1hHO-1. The production of hHO-1 protein was determined at 24, 48, and 72 hrs after transfection. The exogenous hHO-1 production reached a maximal level at 24 hrs and diminished by 72 hrs. These data are representative of 3 separate experiments.



1. pcDNA3.1hHO-1 400 ng, 2. pcDNA3.1hHO-1 400 ng+siRNA-C 200 nmol/L

Figure 7. Inhibitory effects of siRNA-C on the up-regulation of hHO-1 mediated by hemin and pcDNA3.1hHO-1 transfection. The attenuation of hemin-induced hHO-1 expression and protein level by siRNA-C. HL-7702 cells were transfected with or without siRNA-C in the presence of hemin. hHO-1 transcription was analyzed by RT-PCR. Compared with control group, hemin enhanced the expression of hHO-1. Hemin-mediated hHO-1 expression was attenuated by siRNA-C. The inhibitory effect of siRNA-C was hHO-1 specific as it did not alter hHO-2 expression (A). hHO-1 protein levels were determined by Western blot analysis. Compared with control group, hemin enhanced hHO-1 production, which was alleviated by siRNA-C (B). The suppression of exogenous hHO-1 expression and protein production by siRNA-C. HL-7702 cells transfected with pcDNA3.1h-HO-1 were treated with or without siRNA-C. hHO-1 transcription was analyzed by RT-PCR. Compared with control group, pcDNA3.1hHO-1 enhanced the expression of hHO-1. This exogenous hHO-1 was attenuated by siRNA-C. The inhibitory effect of siRNA-C was hHO-1 specific as it did not alter hHO-2 expression (C). hHO-1 protein levels were determined by Western blot analysis. Compared with control group, pcDNA3.1hHO-1 augmented hHO-1 production. The expression of exogenous hHO-1 was abrogated by siRNA-C (D). These data are representative of 3 separate experiments.

Inhibitory Effects of siRNA on the Up-Regulation of hHO-1 Mediated by Hemin and pcDNA3.1h-HO-1 Transfection. After we demonstrated that the endogenous hHO-1 was inducible by hemin, we examined whether siRNA-C was able to suppress the hemin-augmented and/or exogenous hHO-1. The cells were transfected with 200 n*M* siRNA-C, and 4 hrs later 30 μg/ml hemin was added into the culture medium. After 48 hrs of incubation, the cells were collected for determination of the effect of siRNA-C on hemin-induced expression of hHO-1. Transfection of siRNA-C significantly inhibited hemin-induced hHO-1 expression at both transcriptional and translational levels (Fig. 7A and B). We also used hHO-

Table 2. The Enzymatic Activity of hHO-1 After Hemin Induction and siRNA-C Transfection

	Control ($n = 6$)	Hemin ($n = 6$)	Hemin + siRNA-C ($n = 6$)
Enzymatic activity (units)	6270 ± 150	7430 ± 250	7030 ± 210
Specific activity (units/mg)	199,518.8 ± 4,863.4	952,588.4 ± 32,250.6*	311,805.5 ± 9,228.6
Activity ratio	1	4.77	1.56

*$P < 0.05$ was considered statistically significant.

2 as a control to determine the specificity of siRNA-C inhibition. The expression of hHO-2 was not altered by siRNA-C, suggesting that the inhibition by siRNA is hHO-1 specific.

Next HL-7702 cells were cotransfected with 200 n*M* siRNA-C and 400 ng of pcDNA3.1hHO-1. Twenty-four hours later total RNA and cellular protein were collected to test the extent of siRNA-C-mediated inhibition on exogenous hHO-1. Similar to its effect on hemin-induced hHO-1 expression, siRNA-C specifically inhibited the transfected hHO-1 at both transcriptional and protein levels (Fig. 7C and D). In contrast, it did not affect hHO-2 expression. As discussed previously, HO-1 is often up-regulated in neonates with hyperbilirubinemia. These results imply that siRNA is a potential effective means to block induced HO-1.

Effect of siRNA-C on the Enzymatic Activity of hHO-1. Lastly, we tested whether the suppression of HO-1 by siRNA-C ultimately led to reduction of bilirubin. The homogenates from HL-7702 cells transfected with and without siRNA-C were used to measure bilirubin production in an HO-1 enzymatic assay. As summarized in Tables 2 and 3, the enzymatic assay showed that the activity of hHO-1 was increased by hemin induction and pcDNA3.1hHO-1 transfection, while the siRNA-C intervention significantly inhibited hHO-1 action and bilirubin production.

Discussion

As a common clinical condition during early infancy, neonatal hyperbilirubinemia results mainly from both overproduction and undersecretion of bilirubin. Approximately two-thirds of neonates develop this condition. The incidence of severe hyperbilirubinemia is 10.5% in full-term and 25.3% in premature babies (12). In East Asia the incidence and severity are much higher with relative risks at 1.37 and 1.70, respectively (13). Despite the fact that the majority of neonatal hyperbilirubinemia is physiologic in nature, it can evolve into a pathologic process if left without any intervention. Pathologic hyperbilirubinemia is potentially neurotoxic and can cause bilirubin-toxic encephalopathy, which has high mortality and morbidity.

Hemoglobin, myoglobin, and heme-containing enzymes release heme, which is further converted to bilirubin by HO. Three-quarters of bilirubin originates from hemoglobin, and 1 g of hemoglobin is able to produce 34 mg of bilirubin. Because of the lysis of a large quantity of fetal red blood cells (RBC), a normal full-term baby initially can produce 6–10 mg of bilirubin per kg body weight per day. This is 2–3 times the amount produced by adults. The production of bilirubin usually returns to the adult level 10–14 days after birth (14). As a lipid-soluble molecule, unconjugated bilirubin has the cytotoxic effects of suppressing mitochondrial enzyme activity, interfering with DNA synthesis, breaking DNA strands, inhibiting protein synthesis, and phosphorylation (15, 16). Due to its high affinity for cell membrane phospholipids, bilirubin can inhibit cellular absorption of tyrosine, a synaptic transmitter, and block ion channels of N-methyl-D-aspartate receptor. Thus, it is feasible to postulate that bilirubin impairs nerve conduction, especially in the acoustic nerve, by interfering with the signal transduction of neuron cells. Furthermore, a recent study showed that bilirubin can inhibit ion exchange and water transport in kidney cells. This finding could explain the mechanism of neural edema in bilirubin-toxic encephalopathy (17).

At present phototherapy and exchange transfusion are the mainstream treatments for severe neonatal hyperbilirubinemia (15, 18). Discovered 40 years ago, phototherapy is known to effectively reduce serum levels of bilirubin. It is convenient to perform, and the side effects are generally mild. Therefore, it became a first-line therapy widely used for severe neonatal hyperbilirubinemia. However, some patients respond poorly to the phototherapy with an increased risk of developing bilirubin encephalopathy. In these cases, exchange transfusion is often used to rapidly displace toxic materials in the blood such as bilirubin,

Table 3. The Enzymatic Activity of hHO-1 After pcDNA3.1hHO-1 Transfection and siRNA-C Intervention

	Control ($n = 6$)	pcDNA3.1hHO-1 ($n = 6$)	pcDNA3.1hHO-1 + siRNA-C ($n = 6$)
Enzymatic activity (units)	6500 ± 200	7600 ± 100	6400 ± 260
Specific activity (units/mg)	919,946.9 ± 28,306.1	1,566,171 ± 20,607.5*	820,165.8 ± 33,905.5
Activity ratio	1	1.7	0.89

*$P < 0.05$ was considered statistically significant.

antibodies, and sensitized RBC, thereby lowering serum bilirubin levels and preventing toxic encephalopathy. However, complications associated with exchange therapy are more common and severe (19). Moreover, both phototherapy and exchange transfusion are unable to block the production of bilirubin prior to its formation. Hence, these treatments fail to prevent the development of hyperbilirubinemia in its early stage.

HO is a rate-limiting enzyme of heme metabolism and bilirubin generation. Inhibiting HO activity can effectively reduce the production of bilirubin. When the hepatic function of processing bilirubin remains immature during early infancy, it may be more feasible to block the excessive production of bilirubin using an HO inhibitor rather than delay the intervention until excessive bilirubin is produced. HO inhibitors usually contain biocompatible metals, which are not degradable in many tissues. In addition, they are unable to generate photochemical reactions. Sn-mesoporphyrin (SnMP) is a structural analog of heme. Circulating SnMP can be rapidly absorbed by the liver, spleen, and kidney, which possess the highest HO-1 activity. SnMP inhibits the function of HO-1 by specifically binding to the catalytic sites of the enzyme (1). However, SnMP does not affect the conjugation or excretion of unconjugated bilirubin and glucuronate in the liver. When the activity of HO-1 is inhibited by SnMP, unchanged heme is usually excreted *via* an alternative excretory pathway from the biliary system into the intestine (6). A single dose of SnMP (<6 μmol/kg) can lower serum bilirubin levels for 7–10 days and effectively reduce the incidence of neonatal hyperbilirubinemia. Other synthetic heme analogs such as cobalt-protoporphyrin have been found to inhibit HO-1 *in vivo*, although they may inhibit the enzymatic activity of HO-1 due to their biochemical and pharmacologic properties. Likewise, zinc- and chromium-protoporphyrin possess histotoxicity (6); thus, they are not suitable for treating neonatal hyperbilirubinemia. Lingering concerns relate to potential long-term adverse effects such as suppression of the cytoprotective effects of HO-1 against oxidative stress and inflammation in critically ill neonates (20, 21) and the timing of HO inhibition, which may coincide with peak exposure to inflammation and oxidative stress in the critically ill neonatal patient. It is unclear how SnMP and other metalloporphyrins are metabolized in the body, and metalloporphyrins may have long-lasting effects. Clinical trials are currently under way in the United States to further assess the safety and efficacy of SnMP, but the outcome of these trials is not yet known. Therefore, SnMP is currently not recommended for routine clinical practice, and further study is needed (18, 22).

Many drugs can bind to albumin, replace out bilirubin, and interfere with the phosphoglycoprotein function, leading to entry of unbound bilirubin into the brain and increasing the risks of neurotoxicity. For that reason, conventional pharmacologic therapy for neonatal hyperbilirubinemia is potentially associated with many side effects (18). The novel siRNA is a double-stranded RNA with 21–23 base pairs. It uses the mechanism of RNA interference to inactivate gene transcriptional and inhibit the protein synthesis by degrading sequence-specific mRNA target. Therefore, it is becoming a new powerful tool for gene functional studies and gene therapy.

RNA interference has several unique features: (i) it has high specificity and only targets the corresponding sequence-specific genes; (ii) RNAi is highly stable, especially the dsRNA at the hanging 3′ TT base pairs, and unlike antisense RNA, RNAi requires no extensive chemical modifications to enhance its half-life; (iii) RNAi is also very efficient—a low dose of siRNA is sufficient to exert a remarkable inhibitory effect; and (iv) RNAi can be delivered efficiently. Due to its small size, siRNA molecules can easily enter the cells. In light of these characteristics, siRNA is considered as a novel therapeutic method with a promising application prospect.

In the present study, we used RNAi technology to inhibit HO-1, the rate-limiting enzyme of bilirubin metabolism in hope of reducing the bilirubin production. We designed three pairs of siRNA targeting hHO-1 using auxiliary computer analysis. Our study found that siRNA targeting the different gene loci of *HO-1* displayed various inhibitory capacities. This difference is mainly determined by nucleotide sequence of a target site. A small variation of several base pairs at the target site is sufficient to cause marked difference in the inhibitory effect of siRNA. Furthermore, the efficacy of inhibition is also related to the secondary structure of mRNA and bonding proximity of base pairs at the action loci (23). Compared with the base pairs at both ends, the base pairs in the middle of targeted mRNA are more essential for siRNA effect (24). Therefore, we selected a pair of siRNA (siRNA-C) with the best inhibitory effect for the subsequent experiments. Our result demonstrated that the inhibition of HO-1 expression was proportional to the concentration of siRNA. This indicates that siRNA action is dose-dependent. This finding is in agreement with other published studies (25). Meanwhile, when the human liver cell line was transfected with the identical concentration of siRNA, the effect of siRNA also depends upon the timing of transfection. The inhibition of HO-1 expression diminished over time after siRNA transfection, and the level of HO-1 recovered gradually in 48 hrs. This time-dependent effect is consistent with other reports (26).

When neonatal hyperbilirubinemia occurs, HO-1 in the liver and spleen is up-regulated to degrade overproduced heme from hemolysed fetal RBC. In order to mimic the *in vivo* change, we used both hemin and HO-1 plasmid to enhance HO-1 expression. Here, we showed that siRNA was able to inhibit the HO-1 up-regulated by hemin induction and plasmid transfection. Thus, it offers a possibility that siRNA can inhibit induced HO-1 *in vivo*.

Finally, we showed that both hemin and plasmid transfection increased intracellular HO-1 activity. siRNA-C

markedly attenuated the enzymatic activity of HO-1 by inhibiting HO-1 protein level. This further illustrates that siRNA can ultimately alleviate the production of bilirubin. This result lays a foundation for future study to test the approach of siRNA in the animal model of neonatal hyperbilirubinemia (27). A recent study utilized lipoid embedding technique to successfully target rat hepatocytes (28). Systemic injection of hHO-1 siRNA specifically interferes with *hHO-1* gene expression to reduce the levels of bilirubin. However the reports thus far of siRNA *in vivo* use systemic delivery of the siRNA and/or require the use of a transfection chemical, which potentially raises concerns for toxicity if used clinically. Furthermore, although systemically delivered siRNA can be detected in multiple organs, it is clear that the biologic activity of systemically administered siRNA is not equally effective in all organs. Therefore, more studies will be performed to clarify the features of siRNA. However, our study and others provide an insight into the development of novel and effective strategy for treating neonatal hyperbilirubinemia and preventing bilirubin encephalopathy at an early clinical stage.

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