Asymmetric siRNA targeting the bcl-2 gene inhibits the proliferation of cancer cells *in vitro* and *in vivo*

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Abstract. Small interfering RNAs (siRNAs) are valuable reagents for efficient gene silencing in a sequence-specific manner via the RNA interference (RNAi) pathway. The current synthetic siRNA structure consists of symmetrical duplexes of 19-21 base pairs (bp) with 2 nucleotide (nt) 3' overhangs. In this study, we report that an asymmetric siRNA (asiRNA) consisting of 17 bp duplex region (17 bp asiRNA) exhibited potent activity in inhibiting bcl-2 gene expression and cancer cell proliferation *in vitro*. Importantly, this asiRNA structure significantly reduced off-target silencing by the sense strand. To improve the stability of the 17 bp asiRNA, we synthesized a series of chemically modified 17 bp asiRNAs. Further experiments showed that in comparison with the 17 bp asiRNA, the 17 bp asiRNA-M2, one of the modified 17 bp asiRNAs, exhibited a comparable gene silencing activity and an improved stability *in vitro*. Furthermore, the 17 bp asiRNA-M2 with a proteolipid micelle delivery system can effectively suppress the growth of H22 and BGC 803 tumors *in vivo*. These results suggest that the chemically modified asiRNAs may have potential as an effective therapeutic approach for cancer gene therapy in the future.

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Introduction

The Bcl-2 family proteins are central regulators of programmed cell death (1). Among the family, Bcl-2 is capable of inhibiting cell apoptosis and is overexpressed in several types of cancer, such as lung, breast, gastrointestinal and bladder cancer (2-7). Therefore, the inhibition of Bcl-2 protein expression is an attractive strategy for cancer gene therapy.

RNA interference (RNAi) is a powerful method to specifically suppress the expression of target genes in a homology-dependent manner and is therefore widely used for experimental as well as therapeutic purposes. Current RNAi technology usually relies on small interfering RNA (siRNA) consisting of symmetrical and complementary duplexes of 19-21 base pairs (bp) with 2 nucleotide (nt) 3' overhangs (8,9). However, this siRNA structure triggers several non-specific effects posing challenges to the application of RNAi therapeutics in clinical practice (10,11). Recent studies have shown that the asymmetric RNA duplexes are capable of effectively silencing target gene expressions (12-14). The asymmetric siRNA (asiRNA), similar to siRNA, is incorporated into the RNA-induced silencing complex (RISC) and mediates the sequence-specific cleavage of the target mRNA. In addition, asiRNA can reduce off-target silencing by the sense strand and reduce the saturation of the cellular RNAi machinery. Compared with conventional siRNA, asiRNA possesses certain advantages in RNAi. However, whether asiRNA targeting the bcl-2 gene can effectively silence the target gene expression has not yet been investigated.

The poor stability and inefficient delivery of siRNA are two major obstacles for the therapeutic application of siRNA following systemic administration. Chemical modification is an effective method for improving the stability of siRNA and several studies have reported that some siRNAs with chemical modifications show durable activities in RNAi assays (15-17). To effectively deliver siRNA *in vivo*, various delivery systems have been developed, including lipids, polymers and nanoparticles, with varying degrees of success (18-20).

Our previous study demonstrated that siRNA targeting bcl-2 inhibited gene expression and induced the apoptosis of tumor cells *in vitro* and *in vivo* (21). In the present study, we synthesized a series of asiRNAs ranging from the 13-17 bp duplex region (13-17 bp asiRNAs) targeting bcl-2 and evaluated their activities in cancer cells. The results showed that 17 bp asiRNA had potent activity in downregulating bcl-2 gene expression and inhibiting tumor proliferation *in vitro*. Furthermore, chemically modified 17 bp asiRNA improved the stability of the molecule and effectively inhibited tumor growth *in vivo* when delivered through proteolipid micelles.

Materials and methods

Design and synthesis of siRNA and asiRNAs. The siRNA sequence targeting the bcl-2 gene was selected from GenBank (accession no. M14745, nt sequences 607-625). This sequence was shown to significantly suppress bcl-2 gene expression in our previous study (21). The structures of the asiRNAs consisted of duplex strands ranging from 13-17 bp. The 17 bp asiRNA was chemically modified with cholesterol, 2'-O-methylation (2'-O-Me), 2'-fluoro-uridine (2'-FU) and phosphorothioate and was termed M1-M7. All the siRNA, asiRNAs and chemically modified asiRNAs were synthesized and purified by RiboBio Co. Ltd. (Guangzhou, China). The siRNA and asiRNAs were dissolved in sterilized and RNase-free water to a final concentration of 20 μM.

Animals and tumor cells. Male BABL/c nude mice (grade SPF, 18-20 g), male and female ICR mice (grade SPF, 19-21 g) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). Male Kunming mice, aged 6-8 weeks (20±2 g), were obtained from the Qinglongshan Animal Center (Nanjing, China). The animals were maintained in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication 80-23, revised 1996) and the experimental protocols were approved by the Nanjing University Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

HeLa B2 cell lines which expressed high levels of Bcl-2 protein were kindly provided by Professor Chen Lin (Cell Center of Chinese Academy of Military Medicine, Beijing, China) and were used to evaluate the siRNA suppression of bcl-2 gene expression *in vitro*. The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with 10% fetal bovine serum (FBS, Gibco-BRL), 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37˚C. The HuH-7 human hepatoma cell line was provided by the Shanghai Cancer Institute (Shanghai, China). H22 murine hepatoma cells were supplied by the Shanghai Academy of Medical Industry (Shanghai, China). BGC 803 gastric cancer cells were obtained from Jiangsu Provincial Institute of Materia Medica (Nanjing, China).

Cell transfection. One day prior to transfection, HeLa B2 cells were seeded in 2 ml of complete medium without antibiotics in a 6-well plate so that the cells reached 30-50% confluency at the time of transfection. A total 5 μl of siRNA or asiRNA (20 μM stock) was added to 250 μl of Opti-MEM (Invitrogen, Carlsbad, CA, USA), and 2.5 μl of Lipofectamine 2000 (Invitrogen) was diluted in the same amount of medium. After incubation for 5 min at room temperature, the diluted siRNA or asiRNA were mixed gently with the diluted Lipofectamine 2000, and incubated for 20 min at room temperature. The mixture was then added to the plates with 1.5 ml of serum-free and antibiotic-free DMEM. The final concentration of siRNA or asiRNA was 33 nM.

Quantitative real-time RT-PCR (qRT-PCR). HeLa B2 cells transfected with 33 nM siRNA or asiRNA were harvested 48 h after transfection. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was synthesized using the PrimeScript RT reagent kit (Takara Bio Inc., Shiga, Japan) followed by diluting the concentration of the reverse transcription products to 1:20. qRT-PCR was performed using SYBR-Green real-time PCR master mix according to the manufacturer's instructions in a 7300 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). The PCR reaction program was as follows: 1 min at 95˚C followed by 40 cycles of 15 sec at 95˚C, 15 sec at 57˚C and 45 sec at 72˚C. β-actin was used as the internal control. The sequences of the specific primers were: 5'-GGTCATGTGTGTGGAGAGC-3' (forward) and 5'-GATCCAGGTGTGCAGGTG-3' (reverse) for bcl-2; 5'-AGTTGCGTTACACCCTTTC-3' (forward) and 5'-CCT TCACCGTTCCAGTTT-3' (reverse) for β-actin. The data were analyzed using 7300 system SDS software v1.3.3. The relative levels of individual mRNA transcripts to the control β-actin were calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments were carried out in triplicate.

Flow cytometric analysis. The relative expression levels of the Bcl-2 protein in HeLa B2 cells treated with siRNA or asiRNA were detected by flow cytometry. HeLa B2 cells transfected with 33 nM siRNA or asiRNA were harvested 72 h later and fixed with 4% paraformaldehyde (PFA) for 20 min, followed by washing once with PBS. Subsequently, 0.1% saponin was added and incubated for 15 min before the cells were stained with PE conjugated mouse anti-human bcl-2 monoclonal antibody (BD Biosciences, Franklin Lakes, NJ, USA) at 4˚C for 30 min. Cells were analyzed by the BD FACS Arial Flow Cytometer and data were collected using BD FACSDiva software (BD Biosciences).

Cell proliferation assay. The effect of siRNA or asiRNA on the proliferation of HeLa B2 cells was measured using the Cell Counting Kit-8 (CCK-8). HeLa B2 cells were plated in 96-well microplates and transfected with 33 nM siRNA or asiRNA. The medium was replaced with 100 μl fresh DMEM together with 10 μl CCK-8 (Dojindo, Kunamoto, Japan) per well 72 h after the transfection. After incubating the microplate at 37˚C with 5% CO_2 for 2 h, the absorbance at 450 nm was measured using a microplate reader (Atobio Diagnostics Co. Ltd., Zhengzhou, China). The cell viability (%) was calculated using the following equation:

$$\text{cell viability (\%)} = \frac{A_s - A_b}{A_c - A_b} \times 100$$

A_s is the absorbance measurement from a well treated with siRNA or asiRNA transfection samples, A_c is the absorbance

measurement from a well treated with transfection reagent and A_b is the absorbance measurement from a blank well.

EGFP reporter assay. DNA oligonucleotides corresponding to the sense and antisense strands of siRNA targeting bcl-2 were cloned into the 3' untranslated region of the pEGFP-C1 plasmid (Clontech Laboratories, Mountain View, CA, USA) by PCR. The DNA oligonucleotide sequences were as follows: Bcl-2 sense target, 5'-AAAGGCATCCCAGCCTCCG-3', 3'-TTTC CGTAGGGTCGGAGGC-5'; Bcl-2 antisense target, 5'-CGGA GGCTGGGATGCCTTT-3', 3'-GCCTCCGACCCTACGG AAA-5'.

Serum stability assay in vitro. For the stability assay, 2 μl (40 pmol) of 17 bp asiRNA or chemically modified 17 bp asiRNA-M2 were incubated in 8 μl of FBS for 2, 4, 8 and 24 h at 37˚C, respectively. At different time-points, 2 μl loading buffer was added and 10 μl of each sample was loaded onto a 2% agarose gel followed by ethidium bromide staining. Equal amounts of asiRNA prior to serum incubation (0 h) were loaded as the control.

In vivo bio-distribution assay. In all the *in vivo* experiments carried out in this study, a proteolipid micelle was used to deliver asiRNA. The proteolipid micelle (termed PDE) was composed of polyethylene glycol-phosphatidylethanolamine (PEG-PE), phospholipid dioleylphosphatidylserine (DOPS) and rh-endostatin. The preparation and characterization of PDE were described in our previous study (22). The asiRNA/PDE complexes were prepared freshly and the mass ratio of asiRNA, rh-endostatin, PEG-PE and DOPS was 1:10:2.5:2.5. BABL/c nude mice were inoculated with HuH-7 hepatocarcinoma cells ($3x10^6$ cells/0.2 ml) into the right flank subcutaneously. When the tumor size grew to 150 mm^3, the mice were administered a single injection of 300 μl Cy5.5-labeled naked 17 bp asiRNA-M2 (40 μg/mouse) or Cy5.5-labeled 17 bp asiRNA-M2 (40 μg/mouse)/PDE complexes via the tail vein, respectively. The mice were anesthetized and photographed using the *in vivo* imaging system (Maestro, Cambridge Research & Instrumentation Inc., Woburn, MA, USA) at 1, 12, 24 and 48 h. At 1 and 24 h, the mice were sacrificed and the major tissues and tumors were excised and imaged using the *in vivo* imaging system. The fluorescence intensities in the excised organs and tumors were quantified by the analysis software of the *in vivo* imaging system.

In vivo tumor models. The H22 hepatoma cell lines and BGC 803 gastric cancer cell lines were chosen for the evaluation of the effect of asiRNA on tumor inhibition *in vivo*. The H22 tumor model was established by a subcutaneous injection of H22 tumor cells ($1x10^6$ cells/0.2 ml) into the right flank of Kunming mice. The mice were weighed and divided randomly into five groups (ten per group) 24 h after inoculation (day 0). The mice in each group were injected with normal saline [0.4 ml, intravenously (i.v.), on days 1-14], 30 mg/kg CTX [intraperitonealy (i.p.), on days 1-7], naked 17 bp asiRNA-M2 (1 mg/kg asiRNA, 0.4 ml, i.v., on days 1-14), PDE (0.4 ml, i.v., on days 1-14), or 17 bp asiRNA-M2/PDE complex (1 mg/kg asiRNA, 0.4 ml, i.v., on days 1-14). The tumors were assessed every five days for up to 20 days post-treatment using calipers. The volumes of the tumors was calculated using the formula: tumor volume = $(\text{width})^2$ x length x 0.52. The BGC 803 gastric tumor model was established by a subcutaneous injection of BGC 803 tumor cells ($1x10^6$ cells/0.2 ml) into the right flank of BLBA/c nude mice. When the tumor volume reached approximately 5 mm^3, the nude mice were divided randomly into four groups (six per group), and injected with normal saline, CTX, PDE and 17 bp asiRNA-M2/PDE complex, respectively, as mentioned above. The mice were sacrificed on day 18 and the tumors were excised, weighed and measured. The tumor growth inhibition rate (IR) was determined by the following formula:

$$\frac{\text{tumor weight/volume of control group - tumor weight/volume of treatment group}}{\text{tumor weight/volume of control group}} \times 100$$

Statistical analysis. The data are presented as the means ± SD. The difference among groups was analyzed by one-way ANOVA and using the Student's t-test in both groups and the ANOVA test in multiple groups. The comparisons between multiple groups were performed using the Student-Newman-Keuls (SNK) q-test. P-values <0.05 were considered to indicate statistically significant differences.

Results

Silencing bcl-2 gene expression by siRNA or asiRNAs. In order to compare the gene silencing activity of siRNA and asiRNAs, a series of asiRNAs with the sense strand from 13 to 17 nt with 2 nt 3' overhangs (dTdT) were designed, and the antisense strand contained 19 nt with 2 nt 3' overhangs (Fig. 1A). These asymmetrical siRNAs were termed 13-17 bp asiRNAs.

The effect of siRNA or asiRNAs on the downregulation of bcl-2 mRNA expression was determined by qRT-PCR. As shown in Fig. 1B, the relative levels of bcl-2 mRNA were reduced by approximately 65 and 60% by 17 bp asiRNA and siRNA, respectively, while the relative levels of bcl-2 mRNA from the cells treated with 13-16 bp asiRNAs were reduced by approximately 20% compared with the control group. Though all the siRNA and asiRNAs inhibited bcl-2 mRNA expression, 17 bp asiRNA was the most effective.

Flow cytometric analysis was performed to measure the Bcl-2 protein expression levels. As shown in Fig. 1C, the relative levels of Bcl-2 protein from the cells treated with 13-17 bp asiRNAs and siRNA were reduced by 35, 44, 52, 54, 76 and 68%, respectively. Consistent with the downregulation of bcl-2 mRNA levels, 17 bp asiRNA downregulated Bcl-2 protein expressions the most efficaciously. These results demonstrated that 17 bp asiRNA targeting bcl-2 exhibited more potent gene silencing activity than that of conventional siRNA.

Inhibition of cell proliferation by siRNA or asiRNAs. The effect of siRNA or asiRNAs on the proliferation of HeLa B2 cells was determined using CCK-8. As shown in Fig. 1D, 13-16 bp asiRNAs did not inhibit the cell proliferation while 17 bp asiRNA and siRNA had a significant inhibitory effect ($P<0.05$), with a 40 and 32% IR, respectively. The results demonstrated that 17 bp asiRNA targeting bcl-2 exhibited a cell proliferation inhibition activity comparable to that of the conventional siRNA.

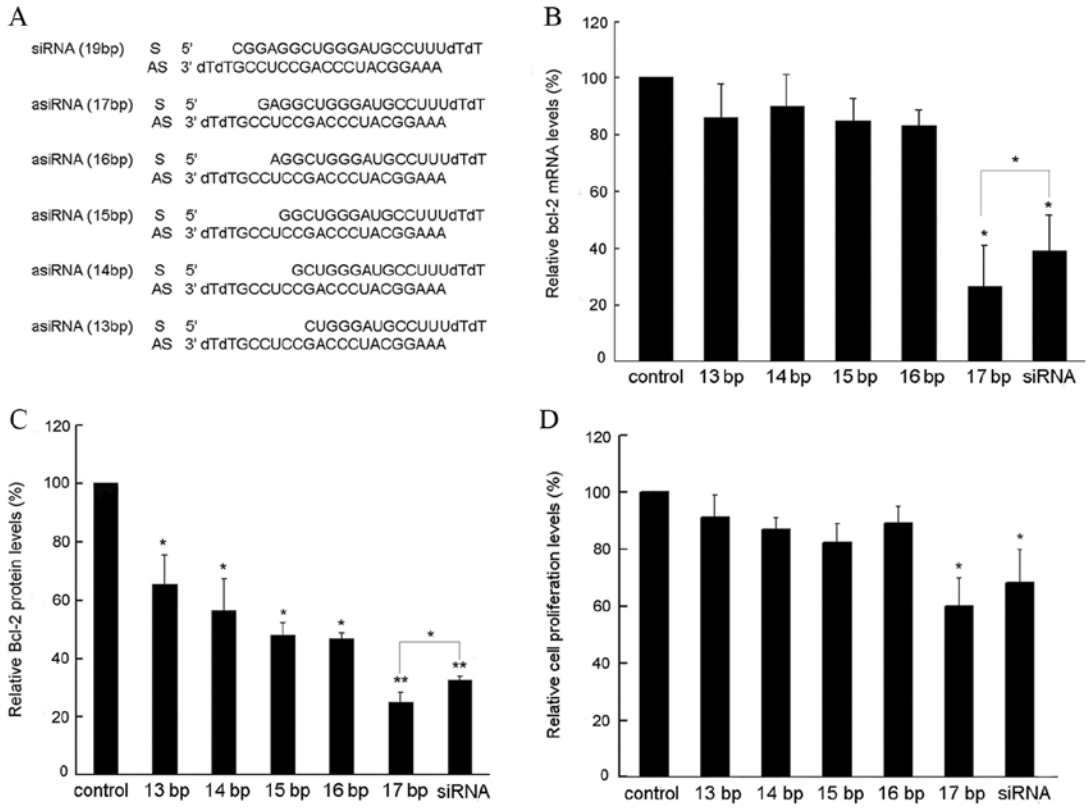

Figure 1. Effect of siRNA and asiRNAs on silencing bcl-2 expression and inhibiting the proliferation of HeLa B2 cells *in vitro*. (A) The sequences of siRNA and asiRNAs targeting bcl-2. (B) Bcl-2 mRNA expression level in HeLa B2 cells transfected with siRNA or asiRNAs. The relative levels of bcl-2 mRNA to control β-actin were determined by qRT-PCR. (C) Effect of siRNA and asiRNAs on bcl-2 protein expression levels in HeLa B2 cells. The relative bcl-2 protein levels were determined by flow cytometric analysis. (D) The siRNA and asiRNAs targeting bcl-2 inhibited the proliferation of HeLa B2 cells. The cell viabilities were determined by CCK-8 assay. All data in the graph represent the mean ± SD values of three independent experiments. The control group was cells only treated with transfection reagent. $^{*}P<0.05$; $^{**}P<0.01$.

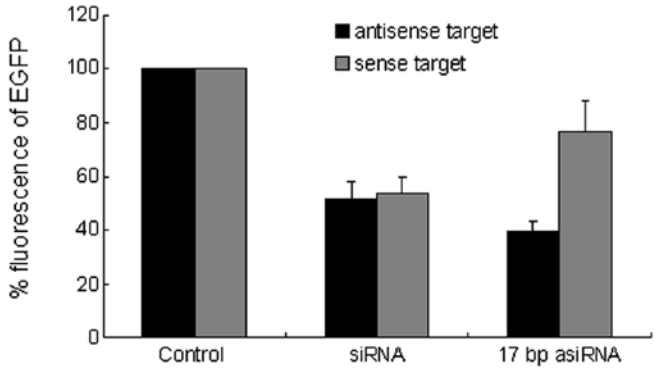

Figure 2. Reduced sense strand-mediated off-target gene silencing by asiRNA. HeLa cells were transfected with the EGFP reporter plasmid containing the antisense or sense target sequence with or without siRNA or 17 bp asiRNA targeting bcl-2. EGFP expression was measured 48 h after transfection by flow cytometry.

asiRNA reduces off-target silencing activity. Several studies have demonstrated that asymmetric RNA structure significantly reduces off-target silencing by the sense strand (12,13). To investigate whether asiRNA targeting bcl-2 also reduces off-target silencing mediated by the sense strand, we constructed an expression plasmid encoding EGFP mRNA containing either the sense or antisense sequence of bcl-2 in the 3' untranslated region. The EGFP expression plasmid and siRNA or 17 bp asiRNA were then co-transfected into HeLa cells and EGFP expression was analyzed by flow cytometry. Both siRNA and asiRNA showed strong silencing activity toward the antisense target. siRNA exhibited a silencing effect toward the sense target, while 17 bp asiRNA significantly reduced silencing activity toward the sense target (Fig. 2). These results demonstrated that 17 bp asiRNAs targeting bcl-2 reduced off-target silencing activity mediated by the sense strand.

Downregulation of bcl-2 expression by chemically modified 17 bp asiRNA. As 17 bp asiRNA was shown to have potent gene silencing activity, we further investigated this effect with different chemical modifications and selected a stable and active modified 17 bp asiRNA for *in vivo* study. The 17 bp asiRNA was chemically modified with partial phosphorothioate backbone, 2'-O-Me and 2'-FU on the sense or antisense strands, and the 5' end of sense strands were all conjugated with a cholesterol molecule. These chemically modified 17 bp asiRNAs were termed M1-M7. The detailed modification methods and sequences are shown in Fig. 3A. The efficacy of individual chemically modified 17 bp asiRNA in downregulating the expression of Bcl-2 protein was determined by flow cytometric assay. As shown in Fig. 3B, the IRs of Bcl-2 protein expression in the cells treated with modified asiRNAs M1-M7 were generally lower than those of the original asiRNA, and 17 bp asiRNA-M2

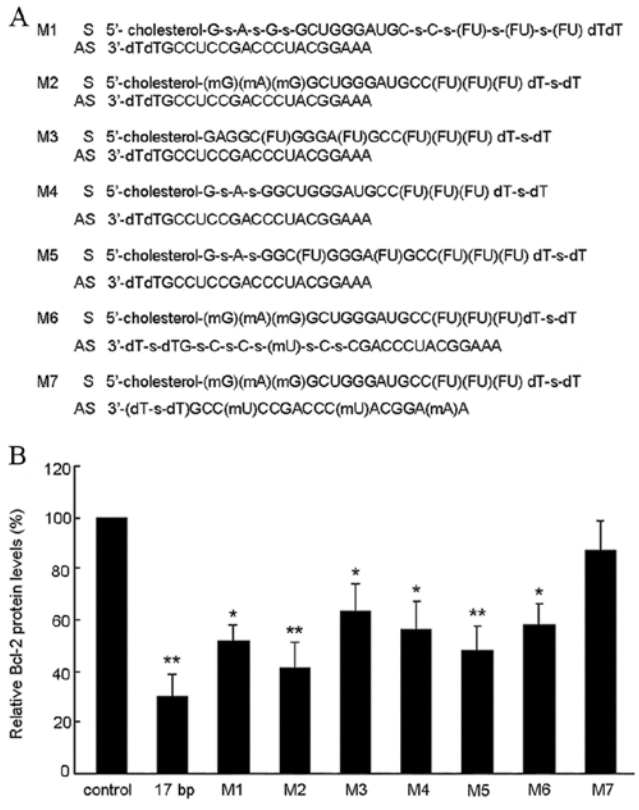

Figure 3. *In vitro* gene silencing activity of chemically modified 17 bp asiRNA. (A) The sequences and structures of chemically modified 17 bp asiRNAs. Lowercase s between nucleotides, phosphorothioate modification; lowercase m, 2'-O-methylation modification; FU, 2'-fluoro-uridine modification. (B) Effect of chemically modified 17 bp asiRNAs on the downregulation of the expression of the Bcl-2 protein. Unmodified or chemically modified 17 bp asiRNA were transfected into HeLa B2 cells for 72 h. The Bcl-2 protein levels were analyzed by flow cytometric assay. The control group was cells only treated with transfection reagent. *P<0.05; **P<0.01.

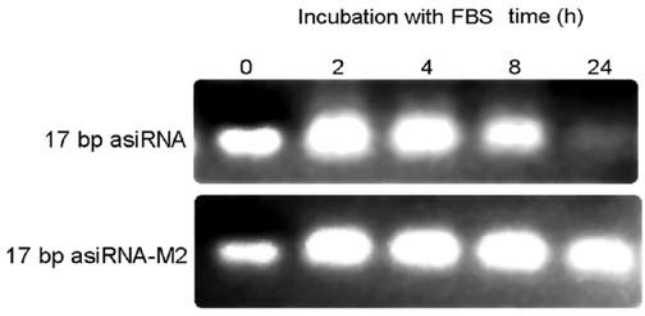

Figure 4. Comparison of the serum stability of 17 bp asiRNA and chemically modified 17 bp asiRNA-M2 *in vitro*. Unmodified and modified 17 bp asiRNA was incubated at 37˚C in FBS for 2, 4, 8 and 24 h. Each sample was loaded onto a 2% agarose gel and visualized by ethidium bromide staining.

showed the best gene silencing effect among all the chemically modified asiRNAs. There was no significant difference between the 17 bp asiRNA and 17 bp asiRNA-M2 groups (P>0.05).

Serum stability of chemically modified and unmodified asiRNA in vitro. To evaluate the stability of the asiRNAs in serum, 17 bp asiRNA and 17 bp asiRNA-M2 were incubated in FBS and separated by gel electrophoresis and subsequently visualized by ethidium bromide staining. As shown in Fig. 4, unmodified 17 bp asiRNA degraded rapidly in the presence of serum, with the band becoming undetectable at 24 h. By contrast, there was a little detectable degradation of 17 bp asiRNA-M2, even after 24 h. The results showed that the chemically modified 17 bp asiRNA-M2 enhanced nuclease resistance of the asiRNA and significantly increased its serum stability.

Distribution of 17 bp asiRNA-M2/PDE complex in vivo. The effectiveness of siRNA therapeutics is hindered by limited blood stability and the successful delivery system of siRNA *in vivo*. In our previous study, a micelle PDE was successfully used to deliver siRNA (22). In this study, to investigate the real-time bio-distribution and the durability of a chemically modified 17 bp asiRNA either naked (17 bp asiRNA-M2) or delivered by PDE (17 bp asiRNA-M2/PDE complex) *in vivo*, we intravenously administered naked Cy5.5-labeled 17 bp asiRNA-M2 or Cy5.5-labeled 17 bp asiRNA-M2/PDE complex to HuH-7 tumor-bearing mice. As shown in Fig. 5A, the 17 bp asiRNA-M2/PDE complex-treated mice exhibited a substantial fluorescence intensity (over their whole bodies) for up to 48 h, while the fluorescence intensity in the naked 17 bp asiRNA-M2-treated mice was significantly reduced at 24 h. As shown in Fig. 5B and C, fluorescence signals were detected in all organs and tumors. Compared with the naked 17 bp asiRNA-M2-treated group, the 17 bp asiRNA-M2/PDE complex-treated group showed a stronger signal, which was particularly obvious in tumors.

Treatment with chemically modified asiRNA targeting bcl-2 inhibits the growth of tumors in vivo. To investigate the therapeutic effect of 17 bp asiRNA-M2 on the growth of tumors *in vivo*, H22 tumor cells were subcutaneously inoculated into Kunming mice. The mice were treated with normal saline, CTX, naked 17 bp asiRNA-M2, PDE and 17 bp asiRNA-M2/PDE complexes continuously for 14 days, respectively. The volumes of the tumors were assessed every five days. Twenty days after the treatment, the inhibitory rate of the 17 bp asiRNA-M2/PDE complex-treated group was 64.3% (P<0.01) compared with the normal saline group, while that of the naked 17 bp asiRNA-M2-treated group was only 32.4% (P>0.05) (Fig. 6A). The results demonstrated that the 17 bp asiRNA-M2/PDE complex significantly inhibited the H22 tumor growth *in vivo*.

BGC 803 gastric tumor cells were subcutaneously inoculated into BLBA/c nude mice. The mice were treated with normal saline, CTX, PDE and 17 bp asiRNA-M2/PDE complexes continuously for 14 days, respectively. On day 18 post the initiation of the treatment, the mice were sacrificed and the tumors were excised, measured and weighed. As shown in Fig. 6B and C, the tumor inhibitory rate of 17 bp asiRNA-M2/PDE complex-treated group was 68.2% (P<0.01) by volume and 64.3% (P<0.01) by weight compared with the normal saline group. When compared with the PDE group, the inhibitory rate of 17 bp asiRNA-M2/PDE complex-treated group was 60.6% (P<0.01) by volume and 53.1% (P<0.01) by weight. The results suggested that 17 bp asiRNA-M2/PDE complexes were similarly effective in inhibiting BGC 803 tumor growth *in vivo*.

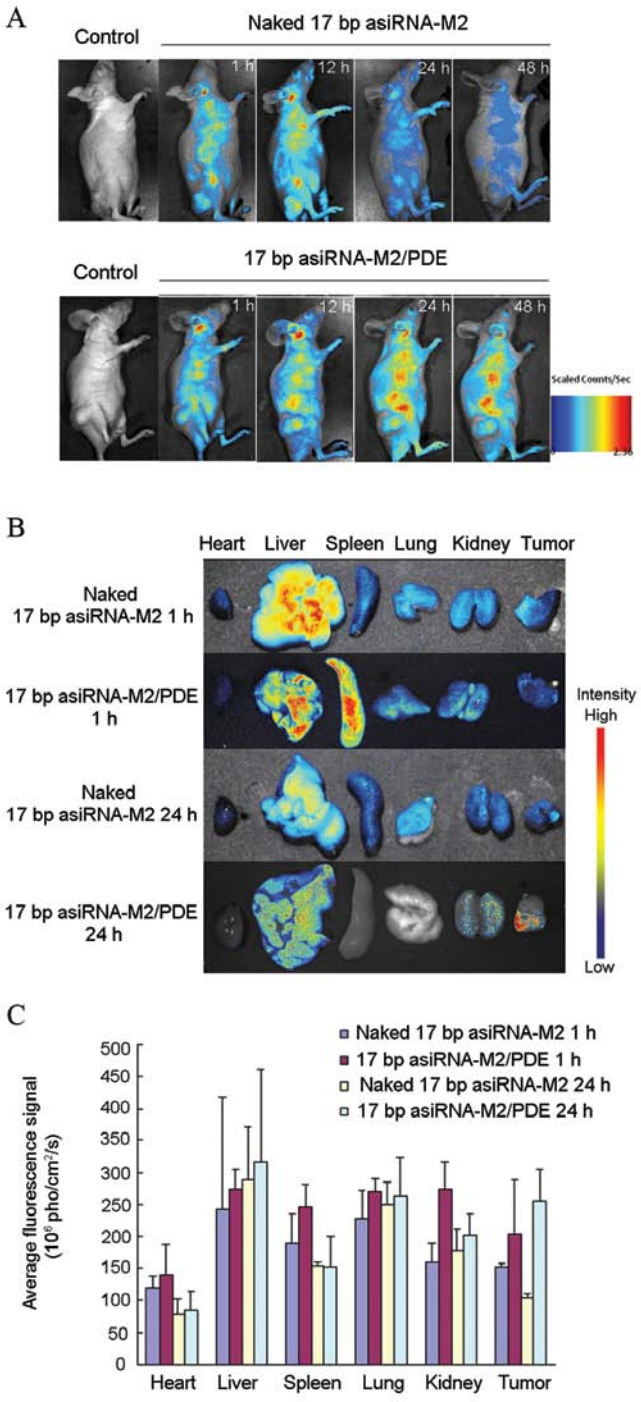

Figure 5. *In vivo* 17 bp asiRNA-M2/PDE complex distribution. (A) Whole-body distribution of 17 bp asiRNA-M2/PDE complex *in vivo*. Mice were injected intravenously with 40 μg Cy5.5 labeled 17 bp asiRNA-M2 either naked or mixed with PDE. (B) *In vivo* distribution of naked 17 bp asiRNA-M2 or 17 bp asiRNA-M2/PDE complexes in the organs and tumors. (C) Quantitative analysis of the distribution of naked 17 bp asiRNA-M2 or 17 bp asiRNA-M2/PDE complex in isolated organs and tumors (mean ± SD, n=3).

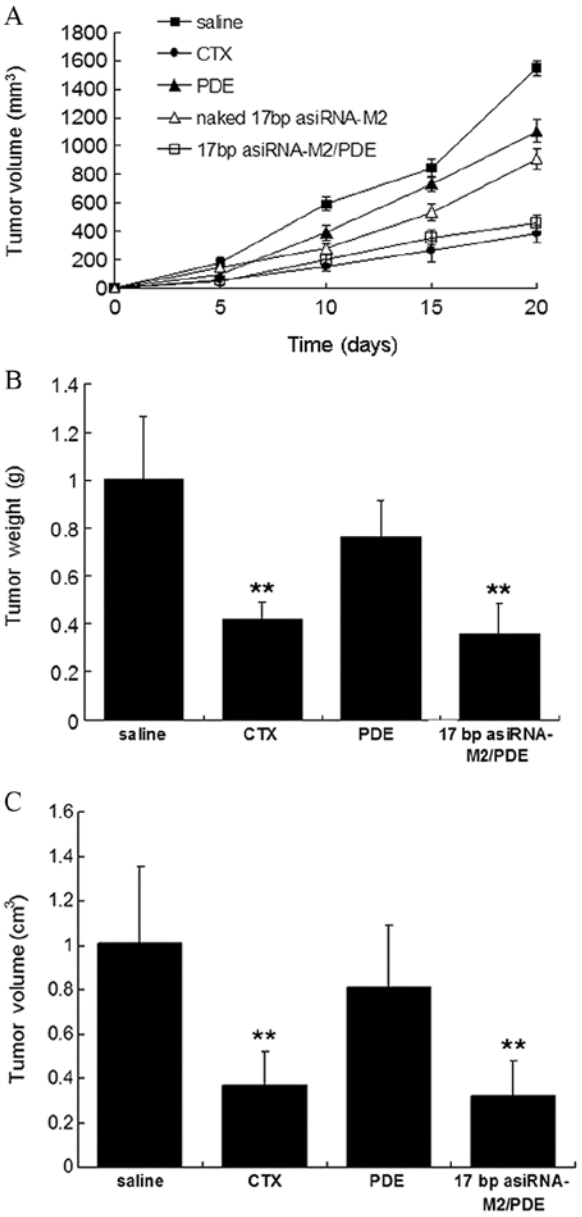

Figure 6. Effects of the 17 bp asiRNA-M2/PDE complex on the growth of H22 and BGC 803 tumors *in vivo*. (A) The growth curves of H22 tumors in different groups after treatment (mean ± SD, n=10). Tumors were measured every five days after the treatment and the tumor volumes were calculated. (B and C) The average tumor weight and tumor volume in different groups are shown (mean ± SD, n=6). **P<0.01.

Discussion

In this study, we designed and synthesized asiRNAs targeting bcl-2 with truncated siRNA backbone structures with duplex regions shorter than 19 bp and the results showed that 17 bp asiRNA downregulated bcl-2 expression more effectively than that of symmetrical siRNA and its other asymmetrical counterparts. Previous studies have demonstrated that asiRNA is effective in silencing the target gene expression. Sun *et al* (12) designed asymmetric RNA duplexes of various lengths with overhangs at the 3' and 5' ends of the antisense strand to target CTNNB1 and 15 bp asiRNA showed more potent silencing efficacy. Chang *et al* (13) reported that two structures with duplexes shorter than 19 bp, 17+2A and 16+3A, efficiently reduced the expression of laminA/C, survivin and integrin mRNAs. Our data in this study are consistent with these reports and demonstrate that a symmetrical scaffold of siRNA is not necessarily required for the activation of RNAi. However, the optimal structure of asiRNA may depend on sequences and target genes.

Off-target gene silencing is a major obstacle to the therapeutic application of siRNA. One mechanism of off-target gene silencing is the incorporation of sense strand into RISC (11). Previous studies have demonstrated that the strand selection of siRNAs depends on the thermodynamic stabilities of their 5'-ends (23,24). However, Sano *et al* (25) reported that terminal structures of siRNAs duplexes are the predominant determinants of the strand selection rather than thermodynamic asymmetry at the ends of siRNAs. In the current study, we demonstrated that asiRNA with asymmetric RNA duplexes significantly reduced the off-target gene silencing mediated by the sense strand, which may be a possible reason of the potent RNAi activity of asiRNA. Our results further demonstrated that structural asymmetry is another crucial determinant of RISC loading and provide useful guidelines for optimal siRNAs design.

The therapeutic application of siRNA has been hampered due to its limited stability, poor cellular uptake and the lack of an appropriate delivery method *in vivo* (26,27). Chemical modification is the principal strategy used to improve the nuclease resistance of siRNA. Sugar modification at the 2'-OH and phosphate linkage modification have been demonstrated to improve the stability of siRNA (16,17,28) and conjugation of cholesterol at termini of the strands could enhance the siRNA cell uptake (29,30). In this study, we introduced 2'-O-Me, 2'-FU, phosphorothioate and cholesterol modification to 17 bp asiRNA and evaluated their gene silencing activity *in vitro*. Although the activity of all the chemically modified 17 bp asiRNAs was affected by the modification, 17 bp asiRNA-M2 retained comparable activity comparing with its original counterpart and was the most active one among the others. Furthermore, 17 bp asiRNA-M2 was shown to be more stable than unmodified asiRNA *in vitro*. Our data are consistent with previous reports that the stability of chemically modified siRNA can markedly improve, while the gene silencing activity usually decreases (16,28). Generally, increasing interference efficiency is not the main objective of chemical modification and maintaining the potency of unmodified siRNA while increasing its serum stability is sufficient. In our study, although the silencing activity of modified 17 bp asiRNA-M2 was slightly reduced with no statistical significance, its stability was obviously improved. However, for systemic application of siRNA *in vivo*, chemical modification is not enough because most target cells are not permeable to naked siRNAs. A number of approaches have been developed to deliver siRNA to the targeted cells after systemic injections (19,20). In this study, the PDE which was developed in our previous study for siRNA delivery was used to deliver 17 bp asiRNA-M2 targeting bcl-2. The *in vivo* distribution analysis showed that the 17 bp asiRNA-M2/PDE complex maintained longer fluorescence signals in the whole body and stronger fluorescence intensity in the organs and tumors compared with the naked 17 bp asiRNA-M2 (Fig. 5). These results demonstrated the high stability of the 17 bp asiRNA-M2/PDE complex *in vivo*. Compared with the naked asiRNA, significant signals were detected in the tumor by use of PDE 24 h after injection, suggesting 17 bp asiRNA-M2 could be delivered to tumor by PDE-mediated systemic administration. The average particle size of the 17 bp asiRNA-M2/PDE complex was approximately 147 nm (data not shown), so the nano-sized complex may accumulate in the tumors via the enhanced permeability and retention (EPR) effect (31,32). In addition, endostatin can bind to membranes containing acidic phospholipids, phosphatidylserine (PS) or phosphatidylglycerol (PG) and many tumors cells have been found to have aberrantly exposed PS-rich domains on the cell surface (33-35), which may be another possible mechanism for tumor accumulation of the complexes. Furthermore, the 17 bp asiRNA-M2/PDE complex was shown to result in significant tumor regression without toxic or side-effects *in vivo*.

In conclusion, synthesized 17 bp asiRNA was more potent in inhibiting bcl-2 expression and tumor cell proliferation *in vitro*. When 17 bp asiRNA was appropriately modified and delivered by a suitable system, it proved to be very stable and significantly inhibited tumor growth *in vivo*. Our study suggests that asiRNA targeting bcl-2 may be a promising approach for cancer therapy.

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