Antisense activity of 2′,4′-BNA targeted to bcl-xL gene in HepG2 cell

Somjing Roongjiang1, Kurena Takahashi1, Myunji Park1, Satoshi Obika1,2 and Takeshi Imanishi1
1Graduate School of Pharmaceutical Science, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan and 2PRESTO, JST, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan

ABSTRACT
Introduction of the 2′,4′-BNA monomer into oligonucleotides significantly enhanced binding affinity toward ssRNA and resistance to nuclease degradation. Here, we evaluated the antisense activity of 2′,4′-BNA oligonucleotides against bcl-xL in HepG2 cells. Our data revealed that 2′,4′-BNA antisense oligonucleotides remarkably inhibited the expression of bcl-xL in HepG2 cells compared to the natural DNA antisense oligonucleotide. The inhibitory effect of 2′,4′-BNA antisense oligonucleotide was dose-dependent and highly sequence-specific.

INTRODUCTION
Antisense oligonucleotides are a rather new class of therapeutic agents that have a great potential to provide effective therapies for a wide variety of diseases. In recent years, a variety of modified antisense oligonucleotides have been synthesized and were examined their stability in biological media and their ability to bind specifically to a target RNA.1

In our laboratory, a number of novel nucleic acid analogues bearing a conformationally restricted sugar moiety, i.e. Bridged Nucleic Acids (BNAs) have been developed to date.2 One of the most promising BNA is 2′,4′-BNA/LNA3, which contains a methylene bridge that connects the 2′-oxygen to the 4′-carbon, thereby locking the conformation of the furanose ring in an RNA-like endo conformation (Figure 1). Introduction of 2′,4′-BNA monomer into an oligonucleotide improved the affinity for complementary RNA and DNA.3,4 In addition, 2′,4′-BNA modification also has been demonstrated to protect an oligonucleotide from endonucleolytic and exonucleolytic degradations.5

The Bcl-2 family proteins have an essential role in the mitochondrial pathway of apoptosis. This family includes both pro-apoptotic and anti-apoptotic members. Bcl-xL, a potent anti-apoptotic member, is over-expressed in numerous types of cancers. This over-expression of Bcl-xL is associated with decreased apoptosis in tumors, resistance to chemotherapeutic drugs and a poor clinical outcome.6 Here we describe the antisense effect of 2′,4′-BNA oligonucleotides against bcl-xL gene in HepG2 cells.

MATERIALS AND METHODS
Cell culture.
HepG2 (human hepatoma) cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, L-glutamine, and antibiotics (penicillin/streptomycin), at 37°C in a 5% CO2 atmosphere. All culture reagents were purchased from Invitrogen. The day before antisense oligonucleotides transfection, HepG2 cells were cultured in 6-well plates at an initial density of 6 x 104 cells/well in a volume of 2 ml.

Oligonucleotides.
The sequences of the 18-mer antisense oligonucleotides complementary to the ATG region of bcl-xL (3′-GGCCAA CGAGACTCTGTGA-5′) were as follows. DNA AS: 5′-CCGGTTGCTCTAGACAT-3′
BNA 1: 5′-CCGGTTGCTCTAGACAT-3′
BNA 2: 5′-CCGGTTGCTCTAGACAT-3′
The following oligonucleotide sequences were used as scramble controls:
DNA scramble: 5′-CTGATCGGAGTCCTTTAG-3′
BNA 1 scramble: 5′-CTGATCGGAGTCCTTTAG-3′
BNA 2 scramble: 5′-CTGATCGGAGTCCTTTAG-3′ (C and T: 2′,4′-BNA 5-methyl cytosine and 2′,4′-BNA thymine, respectively).

Transfection.
The antisense oligonucleotides were mixed with 13 µl of Lipofectamine 2000 in 500 µl of Opti-MEM. This mixture was added to HepG2 cells and incubated at 37°C for 4 h. The composite transfection mixture was removed and replaced with DMEM. The cells remained in culture for 24 h before being harvested to analyze for mRNA expression.

RT-PCR.
At 24 h post-treatment, the medium was removed and the cells were directly lysed in the well. Total RNA was extracted using ISOGEN reagent following the manufacturer’s instructions. The expression level of bcl-xL mRNA was evaluated using RT–PCR. Briefly, the first strand cDNA was synthesized from 3 µg total RNA using Superscript II reverse transcriptase and random dT (12-18 mer) in 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 25 mM MgCl2, 10 mM DTT and 0.5 mM dNTPs. The reaction was allowed to proceed for 50 min at 42°C and stopped by incubation of the reaction mixture at 70°C for 15 min. Reverse transcription reaction products subsequently
underwent PCR amplification for the detection of specific genes. Aliquots of RT reaction products were amplified separately for bcl-xL and GAPDH by PCR in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 μM each of the gene-specific primers and Taq DNA polymerase. The primer pair for bcl-xL was 5’-ttggacaagtgtcctg-3’/5’-ctgggttacatgcacagt-3’ (304 bp amplified product) and for GAPDH was 5’-gatgcaagaggtgtgcttcgtctagc-3’ (150 bp amplified product). DNA amplification was performed using a GeneAmp PCR system 2700 under the following conditions: 30 s at 94°C, followed by 30s at 59°C and 30 s at 72°C for 25 or 27 cycles. Amplified products were electrophoresed in a 2.0% (w/v) agarose gel stained by ethidium bromide. Levels of relative band intensity, corrected on the basis of the GAPDH level, were quantitated using a digital camera (FAS III; TOYOB0).

RESULTS AND DISCUSSION

The effects of 2’,4’-BNA oligonucleotides on the expression level of bcl-xL gene in HepG2 cells were evaluated. At 24 h after transfection, total RNA was extracted and the expression of bcl-xL was analyzed by RT-PCR. The natural DNA antisense oligonucleotide showed no inhibition of bcl-xL expression. In the case of 2’,4’-BNA antisense oligonucleotides, BNA 2, containing six 2’,4’-BNA monomers inhibited bcl-xL expression by ~80%. Moreover, BNA 1, containing nine 2’,4’-BNA monomers inhibited completely (100% inhibition) the expression of bcl-xL, compared with Lipofectamine alone (No oligo). Conversely, 2’,4’-BNA scramble oligonucleotides did not reduce the bcl-xL mRNA expression level (Figure 2). These data indicate that 2’,4’-BNA antisense oligonucleotides significantly inhibited bcl-xL expression, compared with

![Figure 2](https://example.com/image2.png)

**Figure 2** The expression of bcl-xL in HepG2 cells treated with Lipofectamine alone (No oligo), 100 nM DNA AS, BNA 1, BNA 2 and their scramble oligonucleotides for 24 h. Total mRNA was extracted and bcl-xL expression was analyzed by RT-PCR. Expression levels were normalized using GAPDH.

Figure 3 Dose-dependent inhibition of bcl-xL mRNA expression in HepG2 cells by BNA 1. Cells were treated with increasing concentrations of BNA 1(0, 20, 40, 60, 80 nM) for 24 hours. Total RNA extraction, cDNA synthesis and RT-PCR quantification were performed as described in Materials and Methods.

natural DNA antisense oligonucleotide and with scramble oligonucleotides.

Next, we investigated the dose-dependent inhibition of 2’,4’-BNA antisense oligonucleotides. The bcl-xL mRNA expression was significantly reduced upon increasing the concentration of 2’,4’-BNA antisense oligonucleotides, while the human cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression level was not changed upon increasing the concentration of 2’,4’-BNA antisense oligonucleotides (Figure 3). These findings indicate that the inhibitory effect of 2’,4’-BNA antisense oligonucleotides were dose-dependent and specific to bcl-xL translation.

CONCLUSION

We have found that 2’,4’-BNA antisense oligonucleotide was efficient for inhibition of the target bcl-xL gene expression at the translational level. Moreover, the 2’,4’-BNA antisense oligonucleotide also showed the dose-dependent and sequence-specific inhibition. In future, we believe that the 2’,4’-BNA antisense oligonucleotide could be a powerful tool for in vivo applications and therapeutic purposes.

REFERENCES