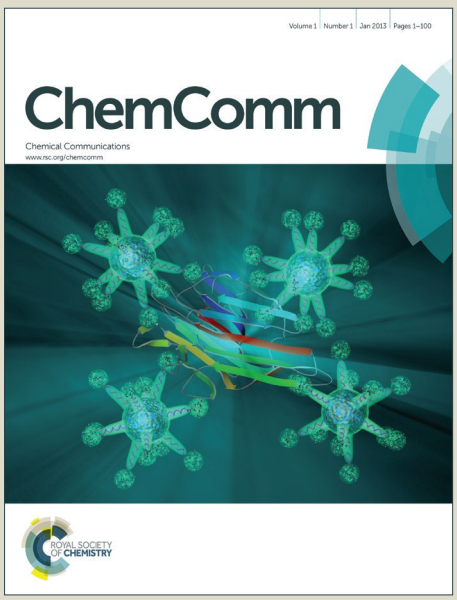
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

ChemComm

RSCPublishing

COMMUNICATION

Crystal structure of 2',4'-BNA^{NC}[N-Me]-modified antisense gapmer in complex with the target RNA

Cite this: DOI: 10.1039/x0xx00000x

Jiro Kondo,*[a] Yusuke Nomura,[b,c] Yukiko Kitahara,[a] Satoshi Obika,*[d] and Hidetaka Torigoe*[b]

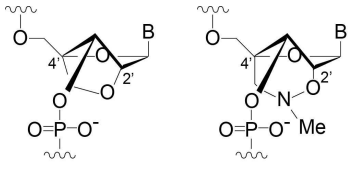
Received 00th October 2015,
Accepted 00th xxxxx 2015

DOI: 10.1039/x0xx00000x

www.rsc.org/

It has been confirmed by our previous studies that 2',4'-BNA^{NC}[N-Me]-modified antisense gapmer displays high affinity and selectivity to the target RNA strand, promising mRNA inhibitory activity and excellent nuclease resistance. Herein, we have obtained a crystal structure that provides insights into these excellent antisense properties.

Since the first antisense drug Fomivirsen (brand name Vitravene) used for cytomegalovirus-induced retinitis was approved by the FDA and appeared on the market in 1998,[1,2] antisense therapy has attracted increasing attention as a promising strategy for treatment of various diseases.[3] However, while a large number of chemically modified antisense oligonucleotides (AONs) have been extensively developed and tested so far,[4] only a few of them have achieved desired antisense activity. In general, AONs bind to viral RNAs or mRNAs produced by disease-causing genes and inhibit their translation. Therefore, AONs should possess high affinity and selectivity to the target RNA strands, high resistance against nuclease and excellent mRNA inhibitory activity *in vivo*. To achieve these goals, our group and Wengel and co-workers independently developed 2'-*O*,4'-*C*-methylene bridged nucleic acids (2',4'-BNA[5] or LNA[6]), in which the ribose ring is locked into the C3'-*endo* conformation by the bridge (Fig. 1).[7] The first-generation 2',4'-BNA/LNA-modified antisense was confirmed to possess higher hybridizing affinity toward both RNA and DNA with complementary sequences compared to natural antisense DNA. However, its RNA selectivity and nuclease resistance were adequate.[5] To solve these issues, a second generation 2',4'-BNA analogue, *N*-methyl substituted 2'-*O*,4'-*C*-aminomethylene bridged nucleic acid (2',4'-BNA^{NC}[N-Me] hereafter), was designed and synthesized by our group (Fig. 1).[8] As expected, AONs containing the 2',4'-BNA^{NC}[N-Me] residues display equal or higher binding affinity against the target RNA, much better RNA selectivity and immensely higher nuclease resistance compared to 2',4'-BNA-modified AONs.[8,9] More recently, promising gene silencing activities of AONs including either the 2',4'-BNA or 2',4'-BNA^{NC}[N-Me] modifications have been confirmed both *in vitro* and *in vivo*.[10] In these tests, 20-mer AONs with nine 2',4'-BNA or 2',4'-BNA^{NC}[N-Me] residues and five DNA gaps were used. These types of oligonucleotides with a DNA region in the window and modified nucleotide regions in the wings, which are called as "gapmers", are generally used as AONs.[11] It has been reported that at least five DNA gaps are essential for a human RNase H1 reaction[11b] and that a short window with five or six DNA gaps improves sequence specificity of mRNA downregulation.[12] Interestingly, a 2',4'-BNA^{NC}[N-Me]-modified gapmer showed earlier gene silencing effect compared to a 2',4'-BNA-modified one. Additional *in vitro*[13] and *in vivo*[14] studies also suggest that the 2',4'-BNA^{NC}[N-Me]-modified AONs can be promising therapeutic agents for several genetic diseases. In the present study, we have performed X-ray analyses to obtain structural bases for the high affinity and selectivity to the target RNA strand, the promising mRNA inhibitory activity and the excellent nuclease-resistance property of the 2',4'-BNA^{NC}[N-Me]-modified gapmer.



2',4'-BNA 2',4'-BNA^{NC}[N-Me]

Fig. 1. Chemical structures of 2',4'-BNA and 2',4'-BNA^{NC}[N-Me].

For crystallization, two 9-mer AONs and their target 9-mer RNA were synthesized; DNA9: d(XTCTTCTTX) (X = 5-methyl-dC), BNA^{NC}9 gapmer with five DNA gaps: d(YZCTTCTZY) (Y = 2',4'-BNA^{NC}[N-Me]-5-methyl-C, Z = 2',4'-BNA^{NC}[N-Me]-T), RNA9: r(GAAGAAGAG). Stabilities of DNA9/RNA9 and BNA^{NC}9/RNA9 duplexes examined by UV melting showed that the T_m value of the BNA^{NC}9/RNA9 duplex (54 °C) increased by 14 °C compared to that of the

DNA9/RNA9 duplex (40 °C) (Fig. S1). Formation abilities of BNA^{NC}9/RNA9 and DNA9/RNA9 duplexes examined by electrophoretic mobility shift assay (Fig. S2) and those of BNA^{NC}10/RNA10 and DNA10/RNA10 duplexes (the same sequence as BNA^{NC}9/RNA9 and DNA9/RNA9 duplexes with an additional 3' single base overhang in both strands) analyzed by BIACORE interaction analysis system (Table S1 and Fig. S3) showed that the binding constant of the 2',4'-BNA^{NC}[N-Me]-modified AON to the target RNA was 500-fold larger than that of the antisense DNA.

Crystallizations were performed by the hanging-drop vapour diffusion method at 20 °C (Table S2). The BNA^{NC}9/RNA9 duplex was crystallized in a condition containing hexammine cobalt chloride (BNA^{NC}9/RNA9-Co, hereafter). The DNA9/RNA9 duplex was crystallized in two different conditions containing either hexammine cobalt chloride or spermine tetrahydrochloride (DNA9/RNA9-Co and DNA9/RNA9-Sp, respectively). Crystal structures of BNA^{NC}9/RNA9-Co, DNA9/RNA9-Co and DNA9/RNA9-Sp solved at resolutions of 1.5, 1.9 and 1.9 Å are deposited in the Protein Data Bank (PDB) with the ID codes 4U6K, 4U6L and 4U6M, respectively (Table S3). A detailed description of materials and methods is included in Supplementary Information.

In the BNA^{NC}9/RNA9-Co crystal, two duplexes are in the asymmetric unit, and three hexammine cobalt molecules are bound to their deep/major grooves (Fig. S4). These duplexes are almost structurally identical (RMSD between them is 1.0 Å). The DNA9/RNA9-Co and DNA9/RNA9-Sp crystals containing one duplex in the asymmetric unit belong to the same space group $P6_1$ with very similar unit cell dimensions. Therefore, the DNA9/RNA9 duplexes observed in these crystals are isomorphous even though major-groove binding of a cobalt hexammine molecule is observed in the DNA9/RNA9-Co crystal. The fact indicates that binding of hexammine cobalt does not affect both overall and local structures of the BNA^{NC}9/RNA9 and DNA9/RNA9 duplexes. Overall structures, side and top views, of one of the two duplexes in the BNA^{NC}9/RNA9-Co crystal and a duplex in the DNA9/RNA9-Co crystal are shown in Fig. 2.

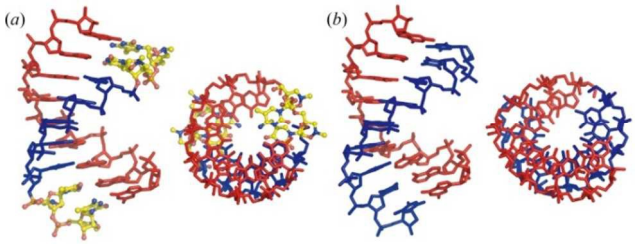


Fig. 2. Overall structures of the BNA^{NC}9/RNA9 (a) and DNA9/RNA9 (b) duplexes, side views (above) and top views (below). The 2',4'-BNA^{NC}[N-Me] residues are illustrated by yellow ball-and-stick model. DNA and RNA residues are represented by blue and red stick model.

Both the BNA^{NC}9/RNA9 and DNA9/RNA9 duplexes take the A-form helix, in which sugar moieties of all DNA and RNA residues adopt the C3'-*endo* sugar puckers (Tables S4 and S5). As we expected, the 2',4'-BNA^{NC}[N-Me] residues introduced at both ends of the BNA^{NC}9 gapmer are locked in the C3'-*endo* conformation as observed in previously reported crystal structures of 2',4'-BNA (LNA),[7] 2',4'-constrained 2'-*O*-methoxyethyl (cMOE) and 2'-*O*-ethyl (cEt) modified DNAs.[15] In general, DNA residues preferentially take the C2'-*endo* sugar pucker, but RNA residues only take the C3'-*endo* conformation. Therefore, hybridization of DNA to RNA is associated with a conformational change from the C2'-*endo* to C3'-*endo* sugar puckers. However, in the case of the 2',4'-BNA^{NC}[N-Me] residues, no energetic cost is needed for binding to the target RNA strand. On the other hand, for hybridization to DNA, the non-modified AON keeps C2'-*endo* conformation to form the B-form duplex, but the modified-AON with the fixed C3'-*endo* conformation forces the target DNA strand to change the sugar pucker from the C2'-*endo* to C3'-*endo* forms. These could be reasons why the 2',4'-BNA^{NC}[N-Me]-modified AON displays high affinity and selectivity to the target RNA strand.

In a situation without crystal structures of the 2',4'-BNA-modified antisense gapmer in complex with the target RNA and DNA, it is difficult to give an answer to the question why the 2',4'-BNA^{NC}[N-Me]-modified AON possesses better RNA selectivity compared to the 2',4'-BNA-modified one. But affinity and selectivity could be related to structure of AON/RNA duplex. Although no significant differences are found in pseudorotation phase angles and local base pair helical parameters of the BNA^{NC}9/RNA9 and DNA9/RNA9 duplexes (Tables S4-S7 and Fig. S5), top views of these duplexes clearly indicate that the DNA9/RNA9 duplex is slightly distorted from the standard A-form conformation compared to the DNA^{NC}9/RNA9 duplex (Fig 2). Therefore, it could be possible that taking the undistorted A-form conformation might be another important factor for AON's affinity and selectivity.

It is clear from the top view of the BNA^{NC}9/RNA9 duplex (Fig. 2a) that a hydrophobic methyl group attached to the bulky 2',4'-BNA^{NC}[N-Me] modification is exposed to the solvent region. As a result, the 2',4'-BNA^{NC}[N-Me] residues are less hydrated compared to the non-modified DNA residues (Fig. 3). On the other hand, the 2',4'-BNA (LNA) residues observed in a crystal structure of a duplex composed of a palindromic 10-mer DNA strand containing one 2',4'-BNA thymine monomer are well hydrated, since the O2' atom involved in the 2',4'-linkage acts as a hydrogen-bonding acceptor.[15] The hydrophobicity around the 2',4'-BNA^{NC}[N-Me] residue may explain its excellent nuclease resistance by disturbing recognition of the AON by DNase. In addition, the hydrophobic methyl group may also affect molecular recognition of the AON/RNA duplex by human RNase H1. In order to obtain a structural basis for the promising gene silencing activities of the 2',4'-BNA^{NC}[N-Me]-modified antisense gapmer, we have produced a structural model of the BNA^{NC}9/RNA9 hybrid duplex in complex with human RNase H1 (Fig. 4). It was revealed by a crystal structure of a complex between DNA/RNA duplex and RNase H1 that four DNA residues are directly recognized by RNase H1 through hydrogen bonds, electrostatic interactions and hydrophobic interactions.[16] Therefore, AONs with less than four DNA gaps may not be recognized by RNase H1 due to steric hindrance between the methyl group and side chains of RNase H1. Thus, at least five DNA gaps may be necessary for RNase H1 recognition of 2',4'-BNA^{NC}[N-Me]/RNA duplex. In the previous *in vitro*[13] and *in vivo*[14] studies, 2',4'-BNA^{NC}[N-Me]-modified AONs with eight[13] and ten[14] DNA gaps showed excellent antisense activity. The number of DNA gaps necessary for RNase H1 recognition is supported by the previous *in vitro*[13] and *in vivo*[14] studies of antisense effect. In addition to the hydrophobicity of the 2',4'-BNA^{NC}[N-Me] residue, its fixed sugar pucker is definitely important for the mRNA inhibitory activity. It has been reported that fully modified AONs with restricted 3'-*endo* furanose do not activate RNase H, and that the activation of RNase H requires AONs

with furanose rings that can adopt O4'-*endo* or C2'-*endo* conformations.[17] These observations agree with the fact that a short (five or six) DNA gaps improves sequence specificity of mRNA downregulation.[12]

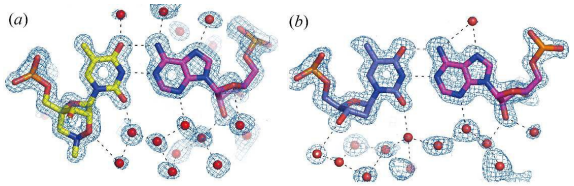


Fig. 3. Local $2|F_o|-|F_c|$ maps at 1 σ contour level and hydration patterns around the 2',4'-BNA^{NC}[N-Me] (*a*) and DNA (*b*) residues observed in the BNA^{NC}9/RNA9 hybrid duplex. Water molecules are represented by red balls.

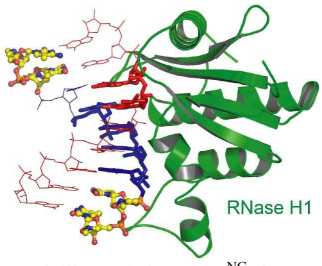


Fig. 4. Molecular modelling of the BNA^{NC}9/RNA9 hybrid duplex in complex with human RNase H1. The model was produced by superimposition of the BNA^{NC}9/RNA9 duplex onto a DNA duplex bound to RNase H1 (PDB-ID = 2QK9).[16] The 2',4'-BNA^{NC}[N-Me] residues are illustrated by yellow ball-and-stick model. DNA and RNA residues are coloured in blue and red, and four DNA residues directly recognized by RNase H1 are represented by stick model. A phosphodiester bond cleaved by RNase H1 is located between two RNA residues represented by stick model.

Conclusions

In the present study, we have solved the first modified-BNA antisense gapmer in complex with the target RNA. The structure provides insights into the bases for the high affinity to the target RNA strand, the promising mRNA inhibitory activity and the excellent nuclease-resistance property of the 2',4'-BNA^{NC}[N-Me]-modified gapmer. Very recently, it has been reported that a short heteroduplex oligonucleotide (HDO) between modified-BNA gapmer and RNA offers a novel concept of nucleic acid-based therapeutics.[18] The structural information obtained in this study is definitely useful for structure-based design of next generation AONs and HDOs.

Notes and references

[a] Department of Materials and Life Sciences, Sophia University, 7-1 Kioi-cho, Chiyoda-ku, 102-8554 Tokyo, Japan.

[b] Department of Applied Chemistry, Faculty of Science, Tokyo University of Science, 1-3 Kagurazaka, Shinjuku-ku, 162–8601 Tokyo, Japan.

[c] Division of Medical Devices, National Institute of Health Science, 1-18-1 Kamiyoga, Setagaya-ku, 158-8501 Tokyo, Japan.

[d] Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, 565-0871 Osaka, Japan.

† Electronic Supplementary Information (ESI) available: Experiments, binding constants of duplex formation (Table S1), crystallization conditions (Table S2), statistics of structure determination (Table S3), structural parameters (Tables S4-S7 and Fig. S5), results of the UV melting analyses (Fig. S1), results of the electrophoretic mobility shift assay (Fig. S2), profiles of the BIACORE interaction analysis system (Fig. S3), and an illustration of crystal structure (Fig. S4). See DOI: 10.1039/c000000x/

†† This work was partly supported by a Strategic Research Foundation Grant-aided Project for Private Universities (No. S1201015) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT). We thank the Photon Factory (No. 2011G630, 2012G147 and 2013G727) and SPring-8 (No. 2011B1999 and 2012A1129) for provision of synchrotron radiation facilities and acknowledge the staff of the BL-5A, BL-17A, AR-NW12 (PF) and BL38B1 (SPring-8) beamlines.

1 C. A. Stein, Y. C. Cheng, *Science*, 1993, **261**, 1004-1012.
2 E. Uhlmann, *Chem. Ztg*., 1998, **32**, 150-160.
3 (a) J. A. Saonere, *J. Med. Genet. Genomics*, 2011, 3, 77-83; (b) V. K. Sharma, R. K. Sharma, S. K. Singh, *Med. Chem. Commun*., 2014, **5**, 1454-1471; (c) A. Goyenvalle, G. Griffith, A. Babbs, S. El Andaloussi, K. Ezzat,A. Avril, B. Dugovic, R. Chaussenot, A. Ferry, T. Voit, H. Amthor, C. Bühr, S. Schürch, M. J. A. Wood, K. E. Davies, C. Vaillend,C. Leumann L. Garcia, *Nat. Med*., 2015, **21**, 270-275.
4 R. P. Iyer, A. Ronald, W. Zhou, K. Ghosh, *Curr. Opin. Mol. Ther*., 1999. **1**. 344-358.
5 (*a*) S. Obika, D. Nanbu, Y. Hari, K. Morino, Y. In, T. Ishida, T. Imanishi, *Tetrahedron Lett*., 1997, **38**, 8735-8738; (*b*) S. Obika, D. Nanbu, Y. Hari, J. Andoh, K. Morino, T. Doi, T. Imanishi, *Tetrahedron Lett*., 1998, **39**, 5401-5404.
6 (*a*) S. K. Singh, P. Nielsen, A.A. Koshkin, J. Wengel. *Chem. Commun*., 1998, 455-456; (*b*) R. N. Veedu, J. Wengel. *Chem. Biodivers*., 2010, **7**, 536-542. (*c*) K. E. Lundin, T. Højland, B. R. Hansen, R. Persson, J. B. Bramsen, J. Kjems, T. Koch, J. Wengel, C. I. Smith, *Adv. Genet*., 2013, **82**, 47-107.
7 M. Egli, G. Minasov, M. Teplova, R. Kumar, J. Wengel, *Chem. Commun*., 2001, 651-652.
8 (*a*) S. M. A. Rahman, S. Seki, S. Obika, T. Imanishi, *Angew. Chem. Int. Ed.*, 2007, **46**, 4306-4309; (*b*) K. Miyashita, S. M. A. Rahman, S. Seki, S. Obika, T. Imanishi, *Chem. Commun*., 2007, **43**, 3765-3767; (*c*) S. M. A. Rahman, S. Seki, S. Obika, H. Yoshikawa, K. Miyashita, T. Imanishi, *J. Am. Chem. Soc*., 2008, **130**, 4886-4896.
9 (*a*) H. Torigoe, S. M. Rahman, H. Takuma, N. Sato, T. Imanishi, S. Obika and K. Sasaki, *Chemistry*, 2011, **17**, 2742-2751; (*b*) H. Torigoe, S. M. Rahman, H. Takuma, N. Sato, T. Imanishi, S. Obika and K. Sasaki, *Nuc. Nuc. Nuc. Acids*, 2011, **30**, 63-81.
10 T. Yamamoto, M. Harada-Shiba, M. Nakatani, S. Wada, H. Yasuhara, K. Narukawa, K. Sasaki, M. Shibata, H. Torigoe, T. Yamaoka, T. Imanishi, S. Obika, *Mol. Ther. Nucleic Acid*, 2012, **1**, e22.
11 (*a*) H. Inoue, Y. Hayase, S. Iwai, E. Ohtsuka, *FEBS Lett*., 1987, **215**, 327-330; (*b*) B. P. Monia, E. A. Lesnik, C. Gonzalez, W. F. Lima, D. McGee, C. J. Guinosso, A. M. Kawasaki, P. D. Cook, S. M. Freier, *J. Biol. Chem*., 1993, **268**, 14514-14522.
12 M. Takagi-Sato, S. Tokuhiro, R. Kawaida, M. Kozumi, *Oligonucleotides*, 2007, **17**, 291-301.
13 T. Yamamoto, H. Yasuhara, F. Wada, M. Harada-Shiba, T. Imanishi, S. Obika, *J. Nucl. Acids*, 2012, **2012**, 707327.
14 T. P. Prakash, A. Siwkowski, C. R. Allerson, M. T. Migawa, S. Lee, H. J. Gaus, C. Black, P. P. Seth, E. E. Swayze, B. Bhat, *J. Med. Chem*., 2010, **53**, 1636-1650.
15 P. S. Pallan, C. R. Allerson, A. Berdeja, P. P. Seth, E. E. Swayze, T. P. Prakash, M. Egli, *Chem. Commun.*, 2012, **48**, 8195-8197.
16 M. Nowotny, S. A. Gaidamakov, R. Ghirlando, S. M. Cerritelli, R. J. Crouch, W. Yang, *Mol. Cell*, 2007, **28**, 264-276.
17 L. Kværnø, J. Wengel, *Chem. Commun*., 2001, 1419-1424.
18 K. Nishina, W. Piao, K. Yoshida-Tanaka, Y. Sujino, T. Nishina, T. Yamamoto, K. Nitta, K. Yoshioka, H. Kuwahara, H. Yasuhara, T. Baba, F. Ono, K. Miyata, K. Miyake, P. P. Seth, A. Low, M. Yoshida, C. F. Bennett, K. Kataoka, H. Mizusawa, S. Obika, T. Yokota. *Nat. Commun*., 2015, **6**, 7969.