2'-*O*-Methyloligoribonucleotide based artificial nucleases (2'-*O*-MeOBANs) cleaving a model of the leukemia related M-BCR/ABL m-RNA

Merita Murtola and Roger Strömberg*

Department of Biosciences and Nutrition, Karolinska Institutet, Novum, S-14157 Huddinge, Sweden
E-mail: Roger.Stromberg@ki.se

This paper is dedicated to Professor Harri Lönnberg on the occasion of his 60th birthday

Abstract
Several 2'-O-methyloligoribonucleotide based artificial nucleases (2'-O-MeOBANs) were developed and evaluated with respect to cleavage of a model of the Leukemia related M-BCR/ABL mRNA. All constructs cleaved the target and the system forming a triadenosine bulge in the target gave the highest rate ($t_{1/2}$ 8.5 h using a 1:1 ratio of 2'-O-MeOBAN to target). The system also displays catalysis with turnover of substrate present in excess.

Keywords: Oligoribonucleotides, artificial nucleases, cleavage

Introduction

Development of oligonucleotide based artificial nucleases (OBANs) is a research area where obtaining high catalytic efficiency and specificity is quite some challenge on its own. When systems become efficient enough there are also many potential uses as tools, as well as in therapeutics. Metal complexes linked to oligonucleotides can be reasonable catalysts for the cleavage of ribonucleic acids.[1] and several studies demonstrate the potential for targeting biologically relevant RNAs with artificial ribonucleases.[2-4]All systems developed so far clearly still have some distance to go in order to achieve rates efficient enough for use in a therapeutic setting. However, in order for efficient systems to evolve for a particular target it seems logical that development of artifical nuclease systems should be moved to a setting that incorporates a

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sequence that is identical to that of a particular target. For more accurate analysis in systems that are under development and still not efficient enough for use in affecting a bioprocess it seems better to first carry out studies on truncated defined model sequences until high enough efficiency is obtained. In our initial approach to developing OBANs[5] we chose a model structure where the 3D structure has been reported[6] and thus provide guidance in the design. This system also have a sequence resemblance to a real potential therapeutic target (i.e., the breakpoint of the Leukemia related M-BCR/ABL mRNA, see below). It is known that RNA bulges are more predisposed to cleavage than fully duplexed RNA[7-10] which implies an advantage for constructs that are designed to form a bulge in the target upon binding. This also provides a pocket for potential interaction with the cleaving agent and/or recognition elements. The initial OBAN model systems have been shown to give cleavage of a target RNA and enzymatic behaviour, *i.e.*, catalysis with turnover.[5] Variation of the target RNA, with respect to the bulge formed, has a substantial influence on cleavage activity, as does the linker position.[5,11] One question was then if the target sequence could be changed at will and adapted to a chosen target as long as the target would form a similar bulge upon formation of the complex.

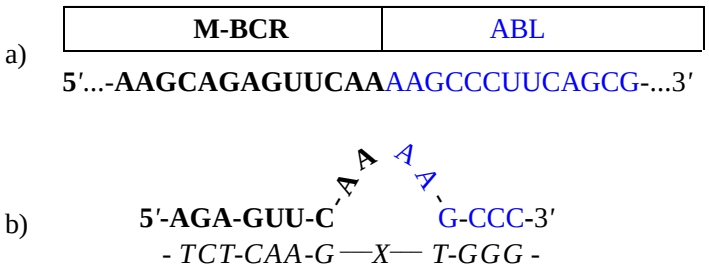

Figure 1. (a) Schematic structure and sequence of the BCR/ABL mRNA fusion site. The sequence in black (and bold) originates from the M-BCR (Major Brakepoint Cluster Region) gene and the blue from the ABL (Abelson tyrosine kinase) gene (b_3a_2 transcript variant). (b) OBAN:BCR/ABL model complex forming a 4 nt bulge.

The M-BCR/ABL mRNA transcript from the Philadelphia chromosome (Ph), t(9;22)[13] has for a long time been associated with human cancer and is the cytogenetic hallmark of Chronic Myeloid Leukemia (CML).[13] As the M-BCR/ABL mRNA is an attractive target for Leukemia treatment[14-16] attempts at suppression of expression of this M-BCR/ABL oncogene, has been done mainly at the mRNA level. Most successful of these approaches has been the use of so called maxizymes which have been shown to effectively cleave chimeric M-BCR/ABL RNA in vitro and in vivo[17], cause apoptosis in cultured leukemic cells with the Philadelphia chromosome[18,19] and to suppress progression of Leukemia in mice[20]. It also has been demonstrated that exogenously delivered chemically synthesized dsRNA (siRNA) directed at the M-BCR/ABL fusion site, is effective in killing leukemic cells.[21] A lentiviral gene transfer strategy resulting in stable expression of BCR/ABL shRNAs has also proven to be of potential therapeutic value.[22] An alternative approach

is development of oligonucleotide based artificial nucleases (OBANs) towards the same target. An OBAN has a built in catalytic activity and can be built to resist degradation, small enough to be taken up by the cells (with the help of a carrier) but has the potential of still being selective and give catalytic cleavage of the mRNA. An OBAN-target complex, forming a tetraadenosine bulge similar to that formed in our initial model system, can be constructed with a part of the junction sequence of the M-BCR/ABL mRNA (the b_3a_2 transcript variant) by choosing the appropriate OBAN sequence. A bulge with four adenosines in this region will then consist of the actual junction point with two of the adenosines originating from the M-BCR part and two from the ABL part (Figure 1). In this report we evaluate a first generation of OBANs targeted against this short model of the junction of BCR/ABL mRNA. Several 2'-*O*-methyloligoribonucleotide based OBANs are synthesized and assessed for their ability to cleave the target RNA.

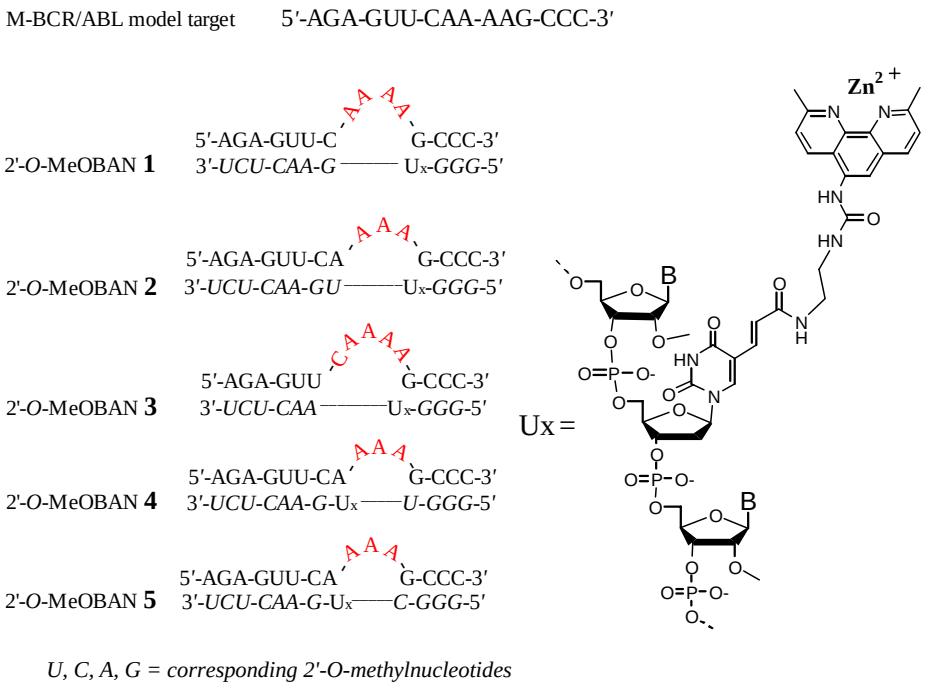

Figure 2. Complexes of 2'-*O*-MeOBANs 1-5 with the M-BCR/ABL RNA model.

Results and Discussion

Five different 2'-*O*-methyloligoribonucleotides were synthesized and conjugated by reaction with N-phenylcarbamoyl-5-amino-2,9-dimethylphenanthroline (5-aminoneocuproine)[11,23] to form 2'-*O*-MeOBAN **1-5** (Figure 2). These are designed to form different complexes with the target RNA thereby exposing 3-5 nt bulges. The thermodynamic stabilities of the different complexes

between 2'-*O*-MeOBAN's and target RNA (without Zn(II) present) were determined by UV thermal melting temperature (T_m) studies. The observed melting points for the 2'-*O*Me based neocuproine complexes were Tm(2'-*O*-MeOBAN **1**)=46 ºC, Tm(2'-*O*-MeOBAN **2**)=46 ºC, Tm(2'-*O*-MeOBAN **3**)=32 ºC, Tm(2'-*O*-MeOBAN **4**)=49 ºC and Tm(2'-*O*-MeOBAN **5**)=57 ºC, which suggests that, except in the case of 2'-*O*-MeOBAN **3**, at 37 ºC and 4 μM each of the 2'-*O*-MeOBAN and RNA, most of the material will be forming a complex.

The efficiency of 2'-*O*-MeOBAN **1**-**5** promoted cleavage of the M-BCR/ABL RNA model was evaluated by determination of the extent of RNA degradation using RP-HPLC analysis. First a 1:1 ratio of target and respective 2'-*O*-MeOBAN was selected (100 μM Zn^{2+}, 10 mM HEPES pH 7.4, 0.1 M NaCl at 37 °C). The products were identified by MS and the pseudo-first-order rate constants for the RNA cleavage were determined by quantifying the areas of the RNA substrate and RNA fragments. It is clear that this target sequence of potential therapeutic use can be cleaved by designed 2'-*O*-MeOBANs. Although the fastest system is somewhat faster than in the early systems[5] it appears that the cleavage rate of the M-BCR/ABL RNA model are of the same order (Table 1). This suggests that, in the target, mainly the bulge part influences the rate and that the sequence in the duplex stems can be selected according the sequence of the target RNA. However, there is some influence since among these system the A_3 bulge is cleaved nearly twice as fast as the A_4 bulge while in the early systems the corresponding bulges were cleaved at more similar rates with the A_4 bulge actually slightly faster ($t_{1/2}$=11h *vs* $t_{1/2}$=14h).

Table 1. Rate constants and half-lives for cleavage of the BCR/ABL RNA model by **1**-**5** at a 1:1 ratio (4 μM of each).[a]

	k_{obs} (10^{-6} s^{-1})	$t_{1/2}$ (h)
2'-*O*-MeOBAN **1**	13.8 ± 0.1	14
2'-*O*-MeOBAN **2**	22.6 ± 0.2	8.5
2'-*O*-MeOBAN **3**	4.2 ± 0.1	46
2'-*O*-MeOBAN **4**	9.6 ± 0.6	20
2'-*O*-MeOBAN **5**	9.0 ± 0.6	21

[a] The experiments were performed in 100 μM Zn^{2+}, 10 mM HEPES buffer, 0.1 M NaCl at pH 7.4, *t* = 37 °C. In the same buffer single stranded BCR/ABL RNA was cleaved at a rate of $2x10^{-6}$ s^{-1}. and rates of cleavage of BCR/ABL RNA in presence of non-conjugated oligos1-5 (1:1) were 1-2 $x10^{-6}$ s^{-1}.

The system most efficient in cleaving the M-BCR/ABL mRNA model also gives the most selective cleavage of target RNA, showing 70% site-selectivity at the bulged out region of the RNA. Careful analysis of the main cleavage site with 2'-*O*-MeOBAN **2** revealed that this is

actually at the junction point of M-BCR/ABL (Figure 3).

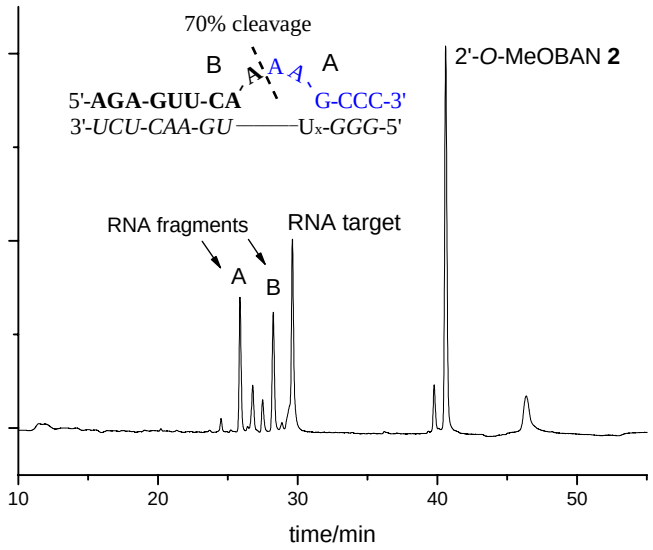

Figure 3. HPLC analysis of the 2'-*O*-MeOBAN **2** promoted cleavage of the BCR/ABL RNA model. The insert shows the most prominent cleavage site as determined by MS of fragments A and B (ESI-TOF MS: A 1857; B 2923)

We then carried out turnover experiments using excess of the M-BCR/ABL model RNA at ratios of 1:2, 1:4 and 1:10 of 2'-*O*-MeOBAN *vs*. target RNA. As single stranded RNA is more reactive, and since RNA is present in excess during the turnover experiments, we lowered the Zn^{2+}-ion concentration in these to reduce possible competing background reaction[6] with free zinc aqua ions. Additional amounts of EDTA and Zn^{2+} (equimolar to each other) were used to eliminate reaction due to possible contamination by Cu^{2+} (that has a higher affinity for EDTA than Zn^{2+}). In all cases this leukemia related target was cleaved by 2'-*O*-MeOBAN **2** and the substrate was almost completely consumed (Figure 4a) thus demonstrating that turnover takes place, also when the RNA is present in excess. We also carried out cleavage studies in the presence of different concentrations of the fastest cleaving system, i.e., using 2'-*O*-MeOBAN **2** showing a clear dependence on concentration of the OBAN (Figure 4b).

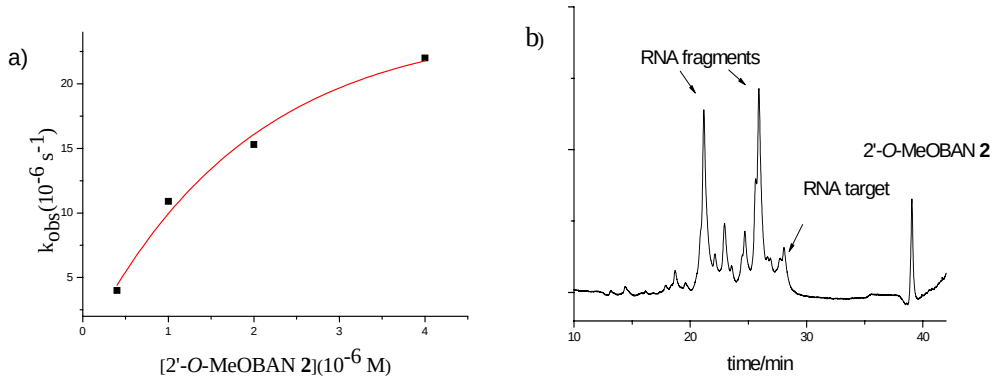

Figure 4. (a) OBAN concentration dependence of the rate constant for cleavage of the BCR/ABL model with 2'-*O*-MeOBAN **2**. (b) HPLC analysis of BCR/ABL cleavage by 2'-*O*-MeOBAN **2** when using a 1:10 ratio of OBAN to substrate (140h reaction time).

We have moved the bulge concept for design of OBANs into systems of 2'-*O*-Me-oligoribonucleotide systems that are directed towards a real potential medical target, that can be developed further to achieve higher efficiency of cleavage and then evaluated for targeting the full m-RNA. The 2'-*O*-MeOBANs catalytically cleaves a truncated version of the Leukemia related M-BCR/ABL sequence. In terms of general OBAN development the results suggest that the sequence in the stems of our general bulge design can be altered to suit a specific target sequence with essentially retained catalytic activity as long as the target sequence is selected to form a similar bulge. Although the sequence is a part of a real medical target there is still quite some improvement needed to be able to move forward to use the systems for affecting gene expression. Cleavage rates for OBANs in general display only a modest distribution, even though different catalytic groups have been used.[1,11] For real use in disease therapeutics the overall rate of RNA cleavage, as catalyzed by any so far developed OBANs, is likely to be insufficient for efficient suppression of gene expression. Clearly something more is needed to take a leap forward in the development of the efficiency of OBANs. For use of OBANs as restriction enzymes in diagnostics or as tools in biomedical investigations, the rates could be sufficient but the specificity of the cleavage has still something left to desire. We are currently working on further development on this Leukemia related and other potential medical target as well as on general development of restriction enzyme system for use as tools. This is done by use of PNA-based systems, other metal ions than zinc and by combining different catalytic groups as well as combining the catalytic group with other modifications that interact with the bulged out region of the RNA substrate, with the aim to influence both selectivity and rate of cleavage.

Experimental Section

General Procedures. 2'-*O*-Me-RNA cyanoethyl phosphoramidite monomers and pre-loaded controlled pore glass (CPG) cartridges were purchased from Glen Research. Solvents and reagents for solid-phase synthesis were synthesis grade from Applied Biosystems. Other solvents were purchased from Merck Eurolab. High-resolution mass spectrometry (HRMS) was performed on a Micromass LCT electrospray time-of-flight (ES-TOF) mass spectrometer in $CH_3CN–H_2O$ 1:1 (v/v) solutions. Molecular weights of the oligonucleotide conjugates were reconstructed from the *m/z* values using the mass deconvolution program of the instrument (Mass Lynx software package). Thermal melting analysis was determined from absorbance *vs* temperature profiles measured at 260 nm on a Varian Cary 300 UV–Vis dual beam spectrophotometer (Varian). All chemicals used in the kinetic experiments were of molecular biology grade.

M-BCR/ABL RNA model

The M-BCR/ABL model RNA substrate was purchased from Dharmacon and was first purified by semi-preparative IE HPLC equipped with an Dionex NucleoPac PA-100 (9 × 250 mm) column. A linear gradient of 0-40 % buffer B over 30 min was used with a flow rate of 4 ml/min (A) 20 mM NaOAc in 30 % aqueous acetonitrile and (B) 20 mM NaOAc, 0.6 M $LiClO_4$ in 30% aqueous acetonitrile. The collected fractions were lyophilized and then purified with RP HPLC using ODS Hypersil (250 × 10 mm, 5μm) column. A linear gradient of 0-23 % buffer B over 30 min was used with a flow rate of 3 ml/min (A) 50 mM triethylammonium acetate in water (pH 6.5) and (B) 50 mM triethylammonium acetate (pH 6.5) in 50 % aqueous acetonitrile. Purifications of the M-BCR/ABL RNA model were performed at 40 °C. The RNA was lyophilized three times before use and stored as frozen solution. ES-TOF: mass calculated for the M-BCR/ABL RNA model C144H179N61O99P14 [M], 4782; found, 4783.

2'-*O*-MeOBANs 1-5

The 2'-*O*-Me based OBANs were synthesized from the aminolinker containing oligonucleotide precursors by reactions with phenyloxycarbonyl- 5-amino-2,9-dimethyl-1,10-phenanthroline[11,23]. These precursors are named **Oligo1** to **Oligo5** (see below). 2'-*O*-MeOBAN **1** was purified by RP HPLC using a ODS Hypersil (250 × 4,6 mm, 5μm) column. A linear gradient of 100% buffer A for 5 min, then 0-40 % buffer B over 35 min at a flow rate of 1 ml/min (A) 50 mM triethylammonium acetate in water (pH 6.5) and (B) 50 mM triethylammonium acetate (pH 6.5) in 50 % aqueous acetonitrile. Purification was performed at 50 °C. 2'-*O*-MeOBANs **2-5** were purified by RP HPLC using a ODS Hypersil (250 × 10 mm, 5μm) column. A linear gradient of 100% buffer A for 5 min, then 0-30 % buffer B over 35 min at a flow rate of 3 ml/min (A) 50 mM triethylammonium acetate in water (pH 6.5) and (B) 50 mM triethylammonium acetate (pH 6.5) in 50 % aqueous acetonitrile. Purifications were performed at 50 °C. HPLC retention times: 2'-*O*-MeOBAN **1**, rt 30 min; 2'-*O*-MeOBAN **2**, rt 39 min; 2'-*O*-MeOBAN **3**, rt 39 min; 2'-*O*-MeOBAN **4**, rt 39 min;

2'-*O*-MeOBAN **5**, rt 39 min. The Oligonucleotides were lyophilized three times before use and stored as frozen solutions. MS analysis (ES-TOF): mass calculated for 2'-*O*-MeOBAN **1** C135H169N47O77P10 [M], 3992; found, 3992; 2'-*O*-MeOBAN **2** C145H182N49O85P11 [M], 4312; found, 4312; 2'-*O*-MeOBAN **3** C124H155N42O70P9 [M], 3633; found, 3632; 2'-*O*-MeOBAN **4** C145H182N49O85P11 [M], 4312; found, 4312; 2'-*O*-MeOBAN **5** C145H183N50O84P11 [M], 4311; found, 4309.

Oligonucleotide precursors (Oligo 1-5)

Oligo1 was purchased from TriLink Biotechnologies and purified using IE and RP HPLC. The crude oligo was purified by semi-preparative IE HPLC equipped with an Dionex NucleoPac PA-100 (9 × 250 mm) column. A linear gradient of 0-20 % buffer B over 35 min was used with a flow rate of 4 ml/min at 50 °C (A) 20 mM NaOAc in 30 % aqueous MeCN and (B) 20 mM NaOAc, 0.6M $LiClO_4$ in 30% aqueous MeCN. RP HPLC purification was performed as for oligos2-5 (see below). All other amino-linker containing 2'-*O*-Me oligonucleotides (**oligo2-5**) were synthesized on an Applied Biosystems A392 DNA/RNA synthesizer. Monomers for the solid-phase synthesis were dried in vacuo in the presence of P_2O_5 prior to use. Oligonucleotides were assembled on pre-loaded CPG cartridges (2'-*O*-Me-U-RNA-CPG) using 2-cyanoethyl phosphoramidite chemistry 5'-DMT-dN-3'-P(OCE)N*i*Pr_2 2'-*O*-Me dN = U, A^{Bz}, C^{Ac}, G^{DMM}, Amino-Modifier C2-dT at 1.0 μmol scale using the manufacture's protocols with 10 min coupling time. After synthesis was the resin treated with AMA reagent (1:1 (v/v) 32% aqueous ammonia : 40% aqueous methylamine) for 10 min at room temperature. The CPG was then removed by filtration and filtrate was kept at 65 °C for 15 min in a tightly sealed flask. The AMA reagent was evaporated and the crude oligo was lyophilized. Purifications were performed using IE and RP HPLC. The crude oligo was first purified by semi-preparative IE HPLC equipped with an Dionex NucleoPac PA-100 (9 × 250 mm) column. A linear gradient of 0-32 % buffer B over 35 min was used with a flow rate of 4 ml/min at 50 °C (A) 25 mM Tris (pH=8.3) in 30 % aqueous methanol and (B) 25 mM Tris (pH=8.3), 0.36M $LiClO_4$ in 30% aqueous methanol. The collected fractions were lyophilized and then purified with RP HPLC using ODS Hypersil (250 × 10 mm, 5μm) column. A linear gradient of 100% buffer A for 5 min, then 0-30 % buffer B over 35 min was used with a flow rate of 4 ml/min at 50 °C. (A) 50 mM triethylammonium acetate in water (pH 6.5) and (B) 50 mM triethylammonium acetate (pH 6.5) in 50 % aqueous acetonitrile. HPLC retention times: **Oligo1** IE rt 32 min, RP rt 32 min; **Oligo2** IE rt 34 min , RP rt 34 min; **Oligo3** IE rt 30 min, RP rt 34 min; **Oligo4** IE rt 35 min , RP rt 34 min; **Oligo5** IE rt 33 min , RP rt 34 min. The Oligonucleotides were lyophilized three times before use and stored as frozen solutions. ES-TOF: mass calculated for amino-linker containing 2'-*O*-Me oligonucleotides. **Oligo1** C119H158N44O76P10 [M], 3743; found, 3743; **Oligo2** C130H171N46O84P11 [M], 4063; found, 4062; **Oligo3** C109H144N39O69P9 [M], 3383; found, 3384; **Oligo4** C130H171N46O84P11 [M], 4063; found, 4062; **Oligo5** C130H172N47O83P11 [M], 4062; found, 4060.

Thermal melting analysis

Absorbance *vs* temperature profiles were measured at 260 nm on a Varian Cary 300 UV–Vis dual beam spectrophotometer (Varian). The samples were prepared by mixing two oligonucleotide strands in a 1:1 ratio, 4 μM of each, in a 10 mM phosphate buffer containing 100 mM NaCl and 0.1 mM EDTA at pH 7.0. Extinction coefficients were calculated from the nearest-neighbour approximation.[24] A termostattable (peltier) multicell (6 + 6) block was used to simultaneously monitor samples. The samples were rapidly heated to 90 ºC, left for 5 min and then allowed to cool to 10 ºC. After equilibration for 10 min at the starting temperature, the dissociation was recorded by heating to 90 ºC at rate of 0.2 ºC/min and data points were collected every 0.1 °C. The Varian Cary WinUV software, version 3 was used to determine the melting temperatures (T_m) by fitting the melting profile to two-state transition model.[24] with linearly sloping lower and upper baselines. Reported values are the average of at least two experiments.

Kinetic measurements. Concentrations of RNA substates and 2'-O-MeOBANs were determined by UV spectroscopy using the extinction coefficients calculated with the nearest-neighbour approximation.[24] The reactions were carried out in sealed tubes immersed in a thermostatted water bath (t = 37 °C). The Kinetic experiments were performed in 100 μM Zn^{2+}, 10 mM HEPES (Sigma) buffer, 0.1 M NaCl at pH 7.4, except for turnover experiments which were carried out in 90 μM Zn^{2+}, 40 μM EDTA, 10 mM HEPES (Sigma) buffer, 0.1 M NaCl at pH 7.4.

Reactions with equimolar concentrations of substrate RNA and OBAN were performed with 4 μM OBAN and 4 μM substrate RNA. Turnover experiments were done with 0.4, 1 or 2 μM OBAN while keeping the concentration of the substrate RNA at 4 μM. For these experiments stock solutions (conc. 1.25 times the final concentration used) of Zn^{2+}–HEPES–NaCl–EDTA were mixed and the pH was adjusted to 7.4 with NaOH (*aq*). Appropriate amounts of substrate RNA, OBAN and water were added to achieve the final concentrations (i.e., with 100 μM Zn^{2+} for the 1:1 experiments and effectively 50 μM accessible Zinc ions for the turnover experiments) after which the reaction vials were incubated at 37 °C in a water bath. Immediately after addition of all components and at appropriate time intervals 40 μL aliquots were withdrawn from the reactions. The aliquots from the 2'-*O*-MeOBAN reactions were quenched by addition of a chelating resin (Sigma Chelex 100, iminodiacetic acid). Samples were shaken and left on the resin for 10 min after which they were filtered through a Millipore Millex-GV (4 mm) syringe driven filter unit to remove traces of particles and chelating resin. The aliquots were then diluted to 100 μL with buffer A (see below) before RP-HPLC analysis.

The aliquots from the kinetics experiments were analysed by RP-HPLC. The buffers used were buffer A: 50 mM triethylammonium acetate, 10 μM EDTA, pH 6.5 and buffer B: buffer A containing 50% MeCN. The gradient was 0–5 min: 100% A; 6–40 min 0–30% B; flow: 0.43 mL min^{-1} at 50 °C. A Genesis AQ 120A 4 μm (150 × 3.0 mm) column was used and UV detection was carried out at 260 nm.

First-order rate constants for the OBAN induced cleavage of RNA substrates were obtained by quantification of remaining RNA and the sum of all formed fragments from the HPLC analysis. The natural logarithm of the reverse of the remaining fraction of substrate RNA was plotted against time and the first-order rate constants (*k*) were obtained by least-squares linear fitting.

Acknowledgements

We thank the Swedish Research Council and the Swedish Foundation for Strategic Research for financial support.

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