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Oligonucleotide based artificial nuclease (OBAN) systems. Bulge size dependence and positioning of catalytic group in cleavage of RNA-bulges

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Three zinc ion dependent oligonucleotide based artificial nucleases (OBANs) have been synthesized. These consist of 2′-*O*-methyloligoribonucleosides connected to 5-amino-2,9-dimethylphenanthroline *via* a urea function to a linker extending either from C-5 of deoxyuridine or from the 2′-position of uridine moieties. Both types of linkers are placed centrally in the modified sequence and in addition one OBAN carries the C-5 modified dU as an additional nucleoside unit at the 5′-end. All three OBANs are shown to cleave target oligoribonucleotides selectively. The target RNA's are varied to form differently sized bulges (0–5 nucleotides (nt)) and the different OBANs have different preferences for which sizes are preferentially cleaved. The OBAN with the centrally positioned C-5 linked zinc chelate preferentially cleaves 3 and 4-nt bulges, the OBAN with the 2′-linked chelate has a preference for slightly smaller bulges and the OBAN with a 5′-end chelate is more efficient the larger the bulge is. In addition the OBAN with the centrally positioned C-5 linked zinc chelate is shown to be a real enzyme, capable of turnover of substrate and displaying Michaelis–Menten behaviour. The main differences in efficiency of cleavage between the different OBAN–RNA substrate combinations are likely to be due to proximity factors *i.e.* the positioning of a catalytic group relative to cleaved phosphodiester functions. The model systems investigated partially display the importance of catalytic group positioning and should be useful in future development of more efficient OBANs.

Introduction

A number of approaches to synthetic artificial ribonucleases have been developed over the last decade and have been the topic of several recent reviews.[1] Apart from the scientific challenge in designing and synthesizing these artificial enzymes, the simultaneous development of antisense therapy[2] leads to the obvious possibility of combining these two concepts and thus using the artificial nuclease for medical applications. A huge number of oligonucleotide modifications have been developed particularly for the purpose of achieving stability towards degrading enzymes and for more favourable hybridisation to the target RNA.[2] The successful blocking of gene expression by antisense oligonucleotides is usually dependent on the host cell's RNase H cleaving the target mRNA and thereby allowing reuse of the antisense oligonucleotide in a catalytic cycle.[2] However, most modifications of oligonucleotides are not recognised by RNase H.[2] If the antisense oligonucleotide could carry a group that causes cleavage of the target RNA upon hybridisation this problem would be avoided. Recognition of the target substrate is reliably achieved through Watson–Crick base pairing and the concept should work in theory. In reality, the difficulty lies in developing a sufficiently efficient artificial enzyme.

We have taken on the task of developing an oligonucleotide based artificial nuclease (OBAN) that is efficient enough for antisense therapy (and possibly other applications in biotechnology and biomedical research). An OBAN system consists of an oligonucleotide (preferably modified in order to be stable to degrading enzymes and to enhance binding to the substrate) with a sequence complementary to the target RNA to which a cleaving agent is attached *via* a linker. For RNA the cleavage can be achieved most effectively through intramolecular transesterification of the phosphodiester linkages with the 2′-hydroxyl function. One obvious task is to develop catalytic groups to be able to achieve efficient cleavage. However, if the catalyst is too active, cleavage of non-hybridised non-complementary RNA could become a problem. Both for this reason and for high catalytic activity, the proximity factor is crucial *i.e.* a high intramolecularity between the cleavage agent and the target RNA is desirable. For a given system this is largely determined by the nature of the linker and its attachment position. Furthermore, the choice of target sequence may also be crucial in this respect. The nature of the substrate is also crucial for overall reactivity as it is well known that cleavage of RNA has a high dependence on sequence[3] and is considerably more reactive in the single stranded form (also as bulges or loops) than as a duplex.[4,5] Thus, the vulnerability of the substrate is also a key issue. Finally, it is recognised that in order to achieve catalytic turnover it is preferable to cleave the target RNA in a central part of the sequence so that the number of base pairs for each remaining fragment is greatly reduced and the product complex can readily dissociate.[6]

Our strategy is to work on a number of parallel developments. These are related to the choice of catalytic group, linker, linker position and selection of the target sequence. Although successful cleavage on biological targets has been obtained in systems where there is nearly full complementarity (except for the metal chelate bearing site) between OBAN and target[7,8] we mainly wished to explore systems that contain more vulnerable bulged out regions. We are using an RNA bulge with a known structure[9] as our initial model system to get some insights into the molecular requirements of the linker. As a first choice of catalytic group we have chosen the Zn(II) complex of a 2,9,-dimethylphenanthroline derivative. The parent compound (as the Cu(II) complex) was reported by Linkletter and Chin[10] to promote efficient cleavage of dinucleotides and was later shown to act as an RNA cleaver (both as the Zn(II) and Cu(II) complexes) when conjugated to a complementary

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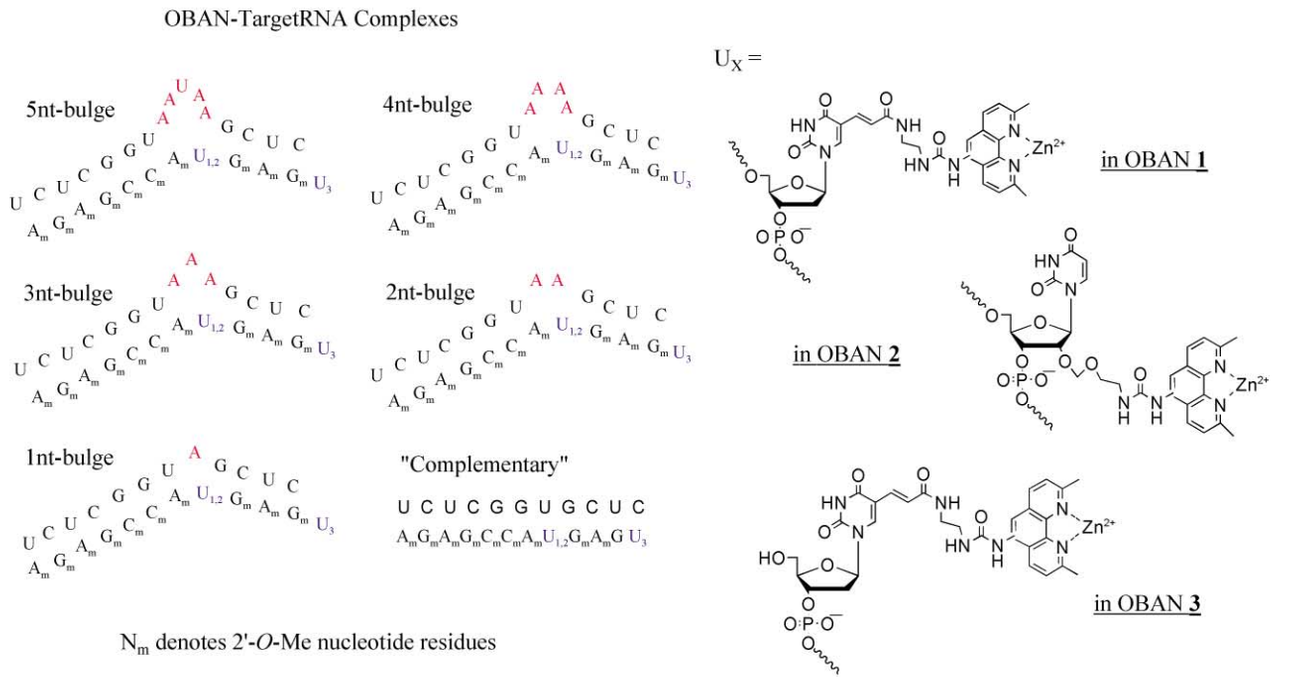

Fig. 1 Illustration of the different OBAN–RNA substrate complexes having different bulge sizes. Substrate RNA's are written from the 5′-end (from left to right) and OBAN RNA's from the 3′-end. N_m denotes 2′-*O*-Me nucleosides, $U_{1,2}$ denotes the position of building blocks carrying the modified nucleoside units of OBAN 1 or OBAN 2 and U_3 denotes the position of the modified building block of OBAN 3. U_3 is only present in OBAN 3.

oligodeoxyribonucleotide, although not in a catalytic fashion.[7,8,11] We have also reported the conjugation of this ligand to an 2′-*O*-methyloligoribonucleotide.[12] We have selected to work mainly with the Zn(II) complex, since this was reported to be nearly as effective as the Cu(II) complex[8] and use of Cu(II) at neutral pH in reasonable concentration is not possible due to severe precipitation of copper hydroxide.

In the present study we have investigated whether we can achieve cleavage of a chosen bulge system in a catalytic fashion and if the phosphodiester linkages within the bulge region can be reached from different linking positions of the OBAN. Furthermore, to explore the proximity issue, we have investigated the bulge size-dependence of the cleavage reactions.

Results and discussion

The designed OBAN systems consist of an 11- (or 12-) mer oligoribonucleotide with all but one ribose carrying a 2′-*O*-methyl substitution and either a centrally placed linker equipped nucleoside residue or an additional modified residue at the 5′-end (Fig. 1). The linking of a metal chelate from the C-5 position of a pyrimidine has been used before[5,13] but with a slightly different linker. Here we chose the commercially available linker shown in Fig. 1. The 2′-position has also been chosen for conjugation before,[5] but with another linker arm and in a different system. The 2′-*O*-methylsubstitution was chosen to achieve OBAN's that are more stable to degradation and with a higher binding affinity to the target oligoribonucleotide. The target structure is varied with respect to the bulge size starting with the bulge from the reported structure down to the non-bulge containing duplex (Fig. 1).

The modified oligonucleotides were conjugated by virtually quantitative formation of a urea link to the 2,9-dimethylphenanthroline derivative by reacting the oligonucleotide carrying a free amino linker with phenyloxycarbonyl-5-amino-2,9-dimethylphenanthroline, as previously reported.[12] UV melting points of the complexes between OBAN (without Zn(II) present) and different targets were determined. These did of course vary but were in all cases above 44 °C ensuring a minimum of 80–90% hybridisation at 37 °C, and thus an approximate maximum variation of 10–15% in the extent of hybridisation between the different complexes.

At first, the cleavage of the RNA target in the presence of OBAN **1** was investigated using equimolar amounts of OBAN and RNA target. Cleavage of the target was observed and variation of the substrate showed a strong dependence on the size of the bulge (Fig. 2). After 12 h the 3- and 4-nt bulges are cleaved to a substantially higher degree than the 5- and 2-nt bulges but cleavage of the 1-nt bulge and the non-bulge containing substrate cleavage can barely be detected. The bulge-size preferences are likely to be largely due to the proximity factor. Some influence from specific interactions between the 2,9-dimethylphenanthroline moiety and the adenine bases in the bulge is plausible, however, control experiments with external non-conjugated neocuproine complexes with the RNA substrates where the OBAN was replaced by the corresponding all 2′-*O*-methyloligoribonucleotides displayed a monotonic increase in cleavage rate with the number of phosphates in the bulge.[14]

To gather more detailed information on the preferred cleavage site(s) we performed LC-MS analysis of the reaction with the 3- and 4-nt bulges. For the 3-nt bulge there is a clear preference for cleavage between the first two (in the 5′ to 3′ direction) adenosine moieties in the bulge (Fig. 3). Almost 70% of the cleavage takes place at this site. For the 4-nt bulge the same site is preferred although in a less dominating way giving nearly half of the cleavage at this position (Fig. 3). OBAN **1** is clearly site-selective and the most likely cause is that the positions that are most readily cleaved are those that are most readily reached by the chosen linker. It would therefore be expected to find different preferences with other linkers and positions.

Our first preference was to explore OBAN **1** further. Could we consider it to be a real enzyme? Experiments with excess substrate were subsequently carried out and the substrate is indeed consumed (obviously at lower rate) at ratios of 1 : 2, 1 : 4 and 1 : 10 of OBAN *vs.* target RNA. The observed turnover (Fig. 4a) is actually not limited by the reaction itself nor by the stability of the OBAN (which can be purified and reused or more substrate can be added to the same mixture). A limitation to what can be observed is that as the amount of OBAN is

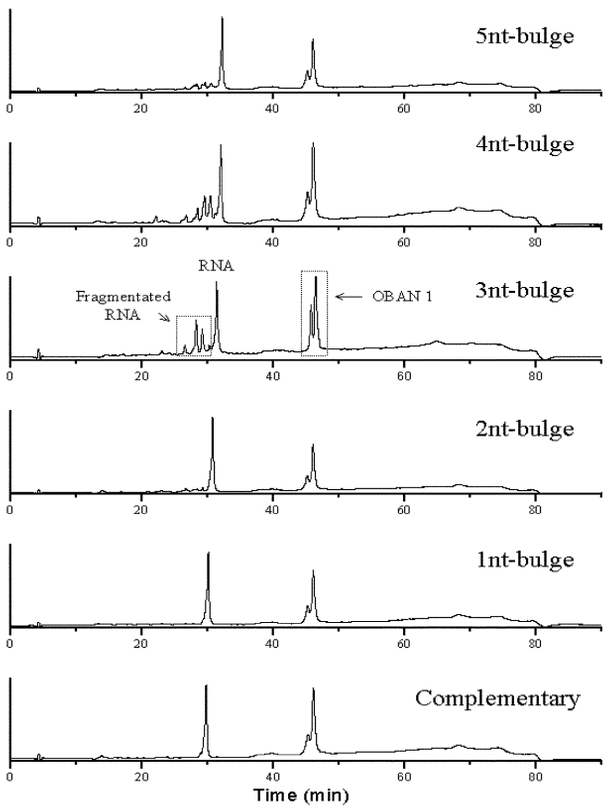

Fig. 2 HPLC chromatograms from the analysis of reaction mixtures of OBAN **1** and RNA substrates after 12 h, from the 5-nt bulge forming complex (top) to the complementary duplex (bottom). All reactions were carried out at 37 °C with a 4 μM conc. of both OBAN **1** and substrate RNA, in 10 mM HEPES buffer (pH 7.4) containing 0.1 M NaCl and 100 μM $Zn(NO_3)_2$. (The additional shoulder on the peak arising from OBAN **1** is because the HPLC analysis (see Experimental section) is carried out at pH 6.5, which is close to the pK_a of the chelate. At higher pH this is not present but the target RNA and fragments from cleavage are then less well separated).

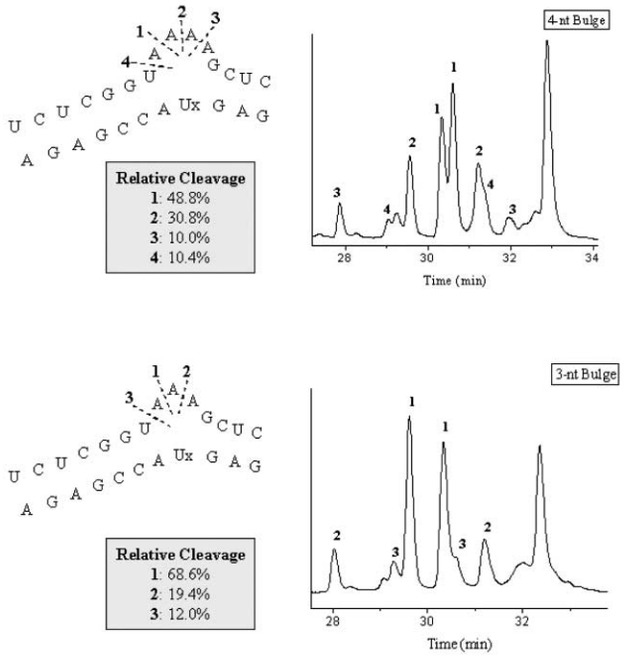

Fig. 3 LC-MS analysis of the fragments formed in the cleavage of the 4-nt and 3-nt bulge systems using OBAN **1** and substrate RNA's. The reactions were incubated for 17 h at 37 °C with a 4 μM conc. of both OBAN **1** and substrate RNA, in a 10 mM HEPES buffer (pH 7.4) containing 0.1 M NaCl and 100 μM $Zn(NO_3)_2$. The peak at the far right of the two chromatograms is the intact RNA substrate and the numbered peaks are fragments formed in the reaction. The numbers and the relative cleavage refer to cleavage at different positions of the bulges. M(−1) for fragments were detected using ESI-TOF MS. For 4-nt bulge: **1**; 2568 + 2189, **2**; 1859 + 2898, **3**; 1531 + 3209, **4**, 2222 + 2518. For 3-nt bulge: **1**; 1859 + 2549, **2**; 1530 + 2877, **3**; 2220 + 2189.

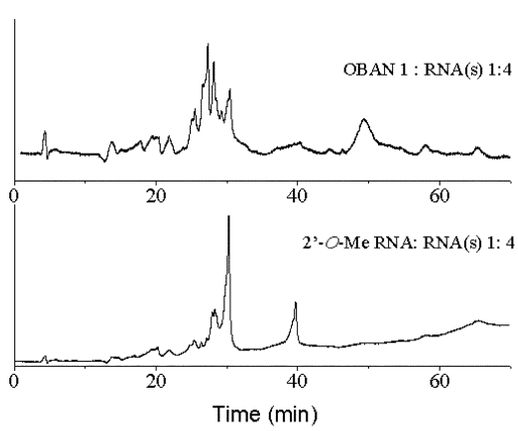

Fig. 4 HPLC chromatograms from the analysis of reactions with 4 times excess of RNA substrate. Reactions were carried out at 37 °C in a 10 mM HEPES buffer (pH 7.4) containing 0.1 M NaCl and 100 μM $Zn(NO_3)$ with a) a 1 μM conc. of OBAN **1** and a 4 μM conc. of substrate RNA or with b) a 1 μM conc. of all 2′-*O*-methyl-oligoribonucleotide (with same sequence as the OBAN's) and a 4 μM conc. of substrate RNA.

decreased, the excess of RNA in its single stranded form reacts sufficiently fast with free zinc aqua ions in a background reaction (Fig. 4b) that becomes predominant if the OBAN is present at too low a concentration (which also indicates that cleavage of the single stranded RNA is somewhat faster than that of the bulge with zinc aqua ions). This is a limitation of this particular metal chelate as the stability constant for the complex with Zn(II)[15] is only about 10^4, which means that an excess of zinc ion is necessary to keep the chelate nearly saturated.

Nevertheless, OBAN **1** gives turnover and we decided to explore the dependence of rate on the amount of enzyme and on the concentration of the Zn(II) cofactor. The rate of RNA cleavage displays Michaelis–Menten behaviour on the concentration of OBAN **1** (Fig. 5a) and as expected from the stability constant of the chelate there is also a dependence on Zn(II) concentration (Fig. 5b). Clearly OBAN **1** behaves in all respects as a real enzyme and is a starting point upon which more efficient systems can be built. One interesting question is just how much more efficient OBAN **1** is when bound to the target as compared to an equimolar concentration of an externally added catalyst, *i.e.* what is the intramolecularity of the system? Curve fitting to the data in Fig. 5a leads to a V_{max} of 20–22 × 10^{-6} s^{-1} and a K_M of about 3–3.5 × 10^{-6} M. The rate of cleavage of the 3-nt bulge formed in the RNA complexed with the all-2′-*O*-methyloligoribonucleotide (with a sequence corresponding to that of OBAN **1** but without linker and chelate) in a reaction mixture containing 10 μM 2,9-dimethylphenanthroline and 100 μM zinc ion was about 1 × 10^{-6} s^{-1}.[14] However, this is a maximum rate since that rate is, within error, almost the same as with 100 μM zinc ion alone. As the 2,9-dimethylphenanthroline is present at a 2.5 times higher concentration the intramolecularity can be estimated to have a minimum value of about 50. Thus, apart from giving the sequence selectivity through Watson–Crick base pairing, OBAN **1** also gives a higher rate of cleavage by bringing the metal chelate closer to the phosphodiester functions of the target RNA.

From this point we also wished to compare other linking positions for the present chelate. Two other OBANs, one with a centrally positioned linker but extending from the 2′-position (OBAN **2**) and one with a linker from the 5′-end of the modified oligonucleotide (OBAN **3**), were synthesized (Fig. 1).

Table 1 Rate constants for cleavage of the different bulges with OBAN **1** to **3**

Bulge size	k_{obs} (10^6 s^{-1}) OBAN **1**	OBAN **2**	OBAN **3**
5	9.1 ± 0.2	7.5 ± 0.7	5.8 ± 0.1
4	17.1 ± 0.4	4.8 ± 0.3	5.1 ± 0.1
3	14.1 ± 0.2	6.2 ± 0.4	4.0 ± 0.1
2	3.3 ± 0.2	7.5 ± 0.4	2.4 ± 0.1
1	n.d.	1.3 ± 0.1	n.d.
0	n.d.	n.d.	n.d.

Experiments carried out in 10 mM HEPES (pH 7.4), 0.1 M NaCl, substrate RNA's and OBAN's were present at 4 μM, all reactions were incubated at 37 °C. Due to some variation in melting points of the complexes the extent of bound RNA varies from 82 to 95%. Thus differences in observed rate constants smaller than 10–15% are insignificant.

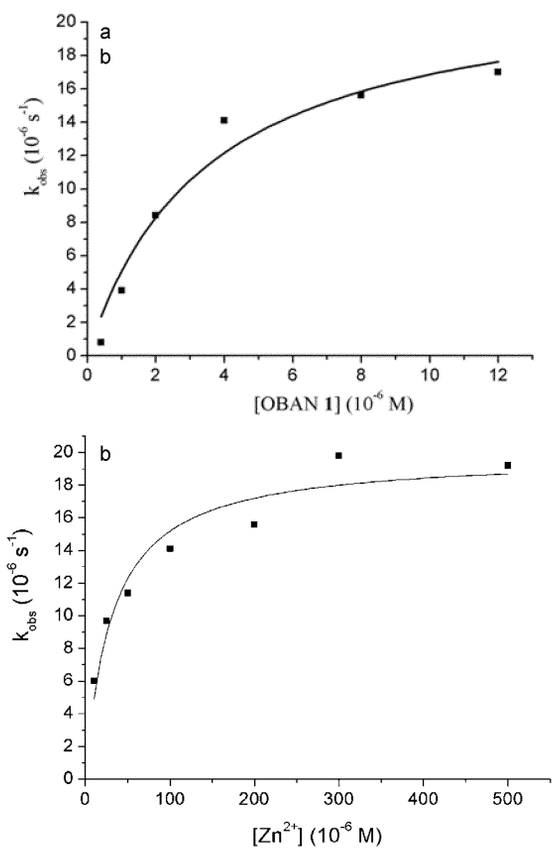

Fig. 5 a) Dependence of rate of cleavage of the 3-nt bulge on concentration of OBAN **1**. Reactions were performed with 4 μM substrate RNA, 0.1 M NaCl, 10 mM HEPES and 100 μM $Zn(NO_3)_2$ at 37 °C and pH 7.4. b) Dependence of rate of cleavage of the 3-nt bulge on concentration of zinc ion: 4 μM substrate RNA, 0.1 M NaCl, 10 mM HEPES and 4 μM OBAN **1** at 37 °C and pH 7.4.

These OBANs were then incubated with the different RNA substrates using the same conditions as with OBAN **1**. Both alternative OBAN systems cleave target RNAs but are in general less efficient than OBAN **1** (Table 1).

What is quite clear is that the bulge size preference is different for each system. As mentioned above, OBAN **1** preferentially cleaves the 3- and 4-nt bulges. OBAN **2**, which carries the chelate on a 2′-linker, displays a general shifting of the preference towards smaller bulges (Fig. 6). This is perhaps to be expected since in the three dimensional structure of the parent 5-nt bulge system[9] the 2′-position seems to point more away from the bulge. Although this system is heavily modified we suggest that this is why we see less efficient cleavage in the larger bulges with OBAN **2** than with OBAN **1** (the relative rates remain similar

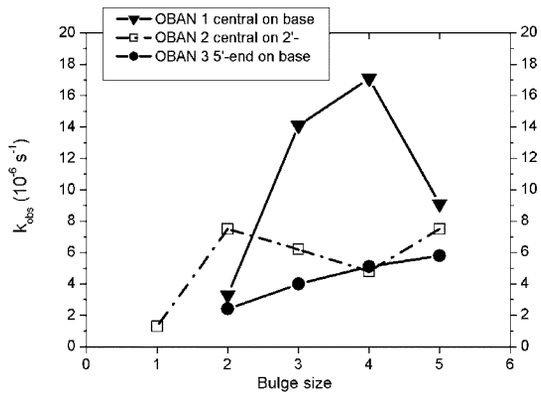

Fig. 6 Observed rate constants (k_{obs}) *vs* bulge sizes (Fig. 1) in cleavage of the RNA targets by OBAN **1**, **2** or **3** at 100 μM Zn^{2+}, pH 7.4 and 37 °C.

also at 10 mM Zn ion concentration, *i.e.*, for the 3-nt bulge the rates are for OBAN **1**: 6.0×10^{-6} s^{-1} and for OBAN **2**: 2.3×10^{-6} s^{-1}). In OBAN **1** the smaller bulges would be quite close to the linker position and presumably the linker then has to extend beyond the bulge giving a less favourable distance between the phosphodiesters and the chelate. Our results are also in agreement with those of Hall *et. al.*[5] that in cleavage of a 2-nt bulge found that a 2′-linked europium complex gave more efficient cleavage than a C-5 linked complex. In that study larger bulges were not explored and it is possible that the same tendency would be found in that system. Before data is available on a sufficient number of systems it is, however, not possible to generalise since each new system is likely to have a different structure, especially in the bulge region.

The 5′-end linked OBAN **3**, on the other hand, displays a shifting of the bulge size cleavage preference towards larger bulges giving the highest rate with the largest bulge (Fig. 6). This can again be explained by the proximity of the chelate to the cleaved phosphodiesters. The current model system has been chosen because the 5′-end of the OBAN strand is close in space to the bulge (in particular when this is a 5-nt bulge) thus giving us the possibility to cleave the target RNA centrally, thereby promoting release, even though the cleaver is attached at the end of the OBAN. As the linker is quite long (including one extra nucleotide unit) in OBAN **3**, it is hardly surprising to find that this system is the least efficient and it would also be expected that the largest bulge would interact most readily with the linked chelate. Although it is quite likely that the efficiency could be improved by the use of a shorter linker at the 5′-end, we have shown that it is possible to reach the bulge and obtain cleavage even though the linker is probably not ideal.

The reported OBANs can be desribed as artificial enzymes as they display real catalysis in the cleavage of RNA. Variation of the OBAN concentration gives a saturation curve, displaying Michaelis–Menten behaviour. The efficiencies of the present systems are in the same range as most other reported oligonucleotide conjugates, which is quite insufficient for most applications. The present study is most useful in the further development of more efficient OBAN's and our model system has proven to be versatile in accepting several linkage positions which interestingly also give different preferences for different substrate RNAs *i.e.* bulge sizes. Further studies in this model system where more combinations of different linkers, linker positions and catalytic group are presently being pursued.

Experimental

Oligonucleotides

All RNA substrates were bought from Dharmacon and were purified on HPLC first on an ion exchange column. Dionex NucleoPac PA-100 (4 × 250 mm) was used for analytical runs

and Dionex NucleoPac PA-100 (9 × 250 mm) for preparative runs, using a linear gradient of 0–90 mM $LiClO_4$ in 20 mM sodium acetate (pH 6.5), 30% CH_3CN. The collected fractions were lyophilized and then purified on reversed phase HPLC Hypersil ODS (250 × 4.6 mm) for analytical runs and Hypersil ODS (250 × 10 mm) for preparative runs. A linear gradient of 0–25% CH_3CN in 50 mM triethylammonium acetate (pH 6.5). The oligonucleotides were collected, lyophilized, dissolved in water and lyophilized again. The precursors to OBAN **1** and **2**, *i.e.*, the oligonucleotides carrying the deoxyuridine with a C-5 linker were purchased from Trilink Biotechnologies Inc. (San Diego, CA, USA) and were purified as above. The precursor to OBAN **2**, *i.e.*, the oligonucleotide carrying the 2′-aminolinker arm was synthesized as reported.[12] Conjugation to the chelate was performed in aqueous sodium tetraborate buffer (pH 8.5) by reaction of the precursor oligonucleotide with phenyloxycarbonyl-5-amino-2,9-dimethylphenanthroline. All oligonucleotides were characterised by ESI-TOF MS.

Cleavage reactions

Chemicals used were all of molecular biology grade. All kinetic runs were performed in 10 mM HEPES (Sigma) buffer at an ionic strength of 0.1 M (NaCl), pH 7.4, t = 37 °C. Reactions with equimolar concentrations of substrate and OBAN were performed with 4 μM OBAN and 4 μM substrate oligomers. Turnover experiments were done with 0.4, 1 or 2 μM OBAN while keeping the concentration of the substrate at 4 μM. The Michaelis–Menten Plot was derived from rate constants from reaction of the 4 mM 3-nt bulge RNA substrate with 0.4, 1, 2, 4, 8 and 12 mM OBAN **1** respectively. These rate constants were corrected by substracting the background rate caused by the Zn aqua ion on the single stranded RNA substrate (significant only when RNA substrate was in excess). Stock solutions of Zn^{2+}–HEPES–NaCl containing different concentrations of metal ions were mixed and the pH was set to 7.4. Typically for 100 μM Zn^{2+} experiments: 160 μL buffer containing the individual components at a concentration of 125 μM–0.0125 M–0.125 M was added to Wheaton vials after being filtered through a Millipore Ultrafree-MC 5K centrifugal filter device. Appropriate amounts of substrate RNA, OBAN and water were added to achieve the final concentrations 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 30 μL aliquots were withdrawn from the reaction and quenched on 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 50 μL with RP buffer A before HPLC analysis. The HPLC buffers used were buffer A: 0.1 M triethylammonium acetate at pH 6.5 and buffer B: buffer A containing 50% MeCN. The gradient was 0–5 min: 100% A; 5–40 min 0–30% B; 30–60 min 30–100% B; flow: 0.2 mL min^{-1}. A Jones Genesis C18 4 μm (250 × 2.1 mm) column was used.

LC-MS analyses were performed with the same buffer and gradient but at a buffer concentration of 50 mM. A capillary was mounted from the outlet of the UV detector of the HPLC to the ion source of a Micromass ES-TOF instrument. After calibration with polyalanine in negative mode, masses corresponding to the cleaved fragments could be detected. Cleaved fragments were detected as the 5′-OH containing fragment from the 3′-end and either the monophosphate or the 2′-, 3′-, cyclic phosphate or both from the 5′ end. 50 μL Of the reaction mixture corresponding to a total amount of 2×10^{-10} mol substrate and fragments was injected for each LC-MS analysis.

All raw chromatograms were imported into the Origin® software. Baseline correction was performed and the peaks were integrated. First-order rate constants for the OBAN induced cleavage of RNA substrates were obtained by quantifying the areas of the disappearing substrate RNA peaks S with that of the intact OBAN peak N. First-order rate constants was obtained by linear regression analysis of the plot of the normalised quote: ln (S/N) as a function of reaction time. As a control also the total area of fragments F were used to plot ln $F/(F + S)$ against reaction time. The differently obtained rate constants agreed within reasonable error, and in some cases, *e.g.* in turnover experiments when the OBAN peak was too small or too broad to be integrated accurately, only the latter method was employed.

Acknowledgements

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