Identification of efficient and sequence specific bimolecular artificial ribonucleases by a combinatorial approach†‡

Marcin Kalek,§[a] Peter Benediktson,[a] Birte Vester[b] and Jesper Wengel*[a]

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Chemically modified nucleotide monomers were incorporated into adjacent terminal positions of two separate oligonucleotides complementary to an RNA target; all possible combinations of the catalytic units were tested, resulting in an artificial nuclease that showed high activity and catalytic turnover.

There is a growing interest in artificial systems capable of sequence specific RNA cleavage as an alternative to RNAi, RNaseH, ribozyme and DNAzyme approaches for antisense therapy, and as general tools for RNA manipulation.[1–5] Artificial agents for DNA cleavage have also been developed.[6] In this paper, we present a novel combinatorial method that allows efficient identification and optimization of artificial ribonucleases, herein defined as oligonucleotides being conjugated with non-nucleotide moieties facilitating RNA cleavage.

The majority of sequence specific artificial ribonucleases discovered so far include an oligonucleotide unit to ensure target recognition, and an attached catalytic group able to mediate RNA phosphodiester backbone hydrolysis. Most attention has been devoted to metal complexes as catalytic groups which have also proven most efficient due to the fact that phosphodiester hydrolysis can be assisted by metal ions in several distinct catalytic pathways.[2,5] Although this versatility makes metal-dependent mononuclear artificial nucleases (Fig. 1 A) relatively efficient, its full potential is likely to be exploited only with dinuclear systems (Fig. 1 B–D). The cooperative action of two catalytic species has accordingly been reported to lead to a remarkable increase in phosphodiester cleavage activity.[7]

In the standard design of a dinuclear artificial nuclease (Fig. 1 B) two catalytic units are incorporated into a single oligonucleotide.[8] Recently, it was shown that introduction of a certain degree of flexibility into such system may result in increased cleavage efficiency[7] as demonstrated in an initial artificial nuclease design involving two oligonucleotides, each possessing a Cu(II) complex (Fig. 1 C). Upon interconnection of these two oligonucleotides by a flexible linker (Fig. 1 D) RNA cleavage capability increased, probably because a productive balance between nuclease flexibility, RNA target affinity and product release was reached.[7]

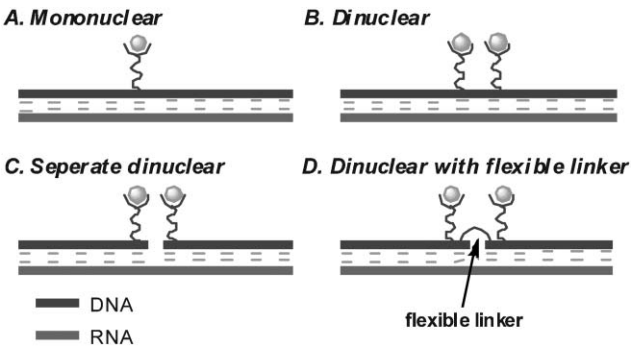

Fig. 1 Designs of artificial ribonucleases.

Rational design of artificial nucleases is inherently difficult and we therefore believe that a combinatorial approach would be the ultimate solution in the search for efficient systems. Inspired by the above mentioned work we adopted the separate dinuclear artificial nuclease setup as a base of our screening strategy. The approach relies on systematic testing of different constitutions of catalytic monomers incorporated into adjacent terminal positions of the two individual oligonucleotide strands (DNA1 and DNA2; Fig. 2)

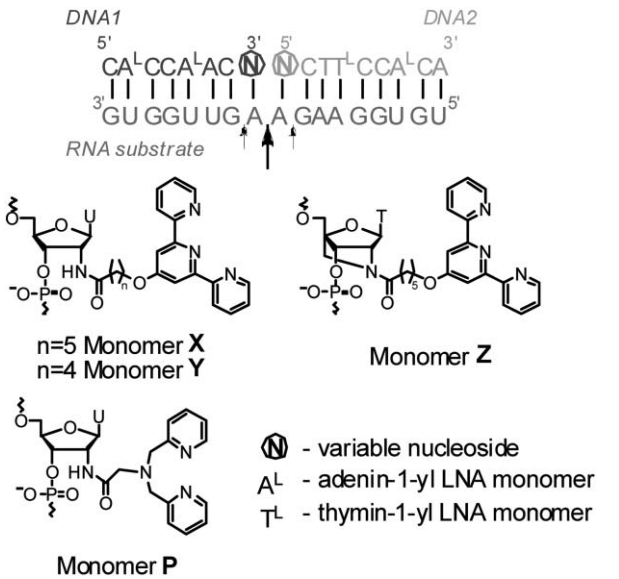

Fig. 2 Sequences of the bimolecular artificial nucleases and the RNA target. The arrows indicate cleavage sites. Also shown are structures of the monomers used in this study.

[a]*The Nucleic Acid Center, Department of Physics and Chemistry, University of Southern Denmark, DK-5230, Odense M, Denmark. E-mail: jwe@ifk.sdu.dk; Fax: +45 6550 4385; Tel: +45 6550 2510*
[b]*The Nucleic Acid Center, Department of Biochemistry and Molecular Biology, University of Southern Denmark, DK-5230, Odense M, Denmark*

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‡ The HTML version of this article has been enhanced with colour images.

§ Current affiliation: Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University.

by systematically combining these in all possible pairs. Such methodology is much more convenient than the preparation and screening of a standard dinuclear artificial nuclease library (Fig. 1 B), since it requires the synthesis of fewer oligonucleotides ($2n$ *vs.* n^2, possessing n different RNA cleaving units).

For this study four novel conjugates of nucleosides with metal complexing monomers were synthesized and incorporated into oligonucleotides DNA1 and DNA2 (Fig. 2).† Three of these conjugates contain terpyridine, a metal chelator widely used in artificial nucleases,[1,2] whereas the fourth contains a *N*,*N*-bis(2-pyridylmethyl)glycyl moiety.[5] The monomers differ in sugar puckering as the furanose ring in 2′-amino-DNA monomers (as in **X**, **Y** and **P**) is known to adopt an *S*-type conformation[9] and in 2′-amino-LNA monomers (as in **Z**) an *N*-type conformation.[10,11] DNA1 and DNA2 contain, in addition to the metal complexing monomers, two LNA monomers to ensure good RNA substrate affinity.[11] It should be noted that we have earlier demonstrated efficient RNA targeting for oligonucleotides composed of DNA, LNA and functionalized 2′-amino-DNA monomers.[12]

The cleavage efficiency of all sixteen oligonucleotide combinations (4 × 4) was assessed by incubating with complementary ^{32}P-labelled 17-mer RNA target. The RNA cleavage reactions were analyzed by gel electrophoresis (Fig. 3) and quantified by phosphor imager scanning. The screening was carried out under single turnover conditions with a DNA1 : DNA2 : RNA ratio of 4 : 4 : 1 in the presence of excess of Cu^{2+}. The tandem **YY** combination appeared to be the most efficient cleaving over 80% of the target RNA. Scission predominantly occurred between the nucleotides positioned opposite to the catalytic monomers, and to less extent in the adjacent positions. DNA1-**Y** and DNA2-**Y** when tested individually under similar conditions cleaved only 23% and 16% of the target, respectively, which proves a synergistic action of the two catalytic units in the experiment involving both DNA1-**Y** and DNA2-**Y**.†

Next, we investigated cleavage by the most effective combination with respect to the amount of Cu^{2+}. Surprisingly, maximum activity was observed in the presence of approximately one equivalent of Cu^{2+} (Fig. 4). Adding more Cu^{2+} decreased the level of cleavage and a plateau was reached when two or more Cu^{2+} equivalents were used. As virtually identical thermal stabilities were measured with one and five Cu^{2+} equivalents added,† the observed difference in cleavage efficiency is not related to target binding affinity. Instead the results suggest that two distinct mechanisms are operating in the presence of one and two Cu^{2+} ions, respectively, the former cleaving more efficiently than the latter.

To obtain deeper insight into the character of phosphodiester bond scission, MS analysis of the reaction mixture was carried out

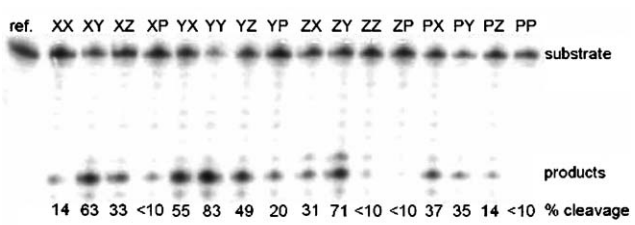

Fig. 3 Denaturating gel electrophoresis of the screening cleavage reactions with different combinations of monomers ($c_{DNA1\text{-}N}$ = $c_{DNA2\text{-}N}$ = 4 μM; c_{Cu2+} = 16 μM; c_{RNA} = 1 μM; 16 h at 37 °C).

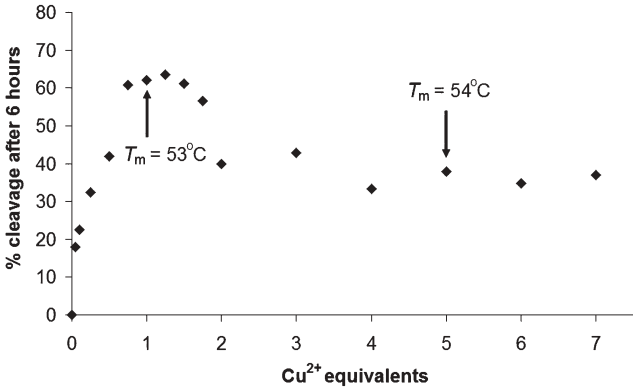

Fig. 4 Dependence of DNA1-**Y** : DNA2-**Y** activity on Cu^{2+} amount ($c_{DNA1\text{-}Y}$ = $c_{DNA2\text{-}Y}$ = c_{RNA} = 1 μM; 37 °C). During thermal denaturation experiments only a single transition was observed due to similar T_m values of the DNA1-**Y** : RNA and DNA2-**Y** : RNA duplexes.

after the cleavage of non-labeled RNA was completed. The MS spectra (Fig. 5) revealed, in addition to DNA1-**Y** and DNA2-**Y**, the presence of an RNA 5′-fragment (9-mer) bearing cyclic phosphate at its 3′ end, together with a 3′-fragment (8-mer possessing a free 5′-OH group) as the major species. These products directly indicate that RNA scission proceeds by a hydrolytic cleavage mechanism.[2] It means that the Cu^{2+} complex (or the two complexes) acts as a Lewis acid activating 2′-OH, the phosphate moiety, the leaving group and/or a water molecule.[5] The higher cleavage efficiency in the presence of only one Cu^{2+} equivalent indicates that a free terpyridine moiety is able to assist during the reaction, for instance by acting as a hydrogen bond acceptor that aids in forming the optimal catalytic center. In this context it should be noted that we do not anticipate the saturated $Cu(terpy)_2$ complex to be catalytically active, and if formed, that it will be in an equilibrium with the 'open' form.

Finally, we evaluated cleavage over time for the optimized artificial nuclease system at different ratios to target RNA (Fig. 6). Under single turnover conditions (4 : 4 : 1 and 2 : 2 : 1), the target

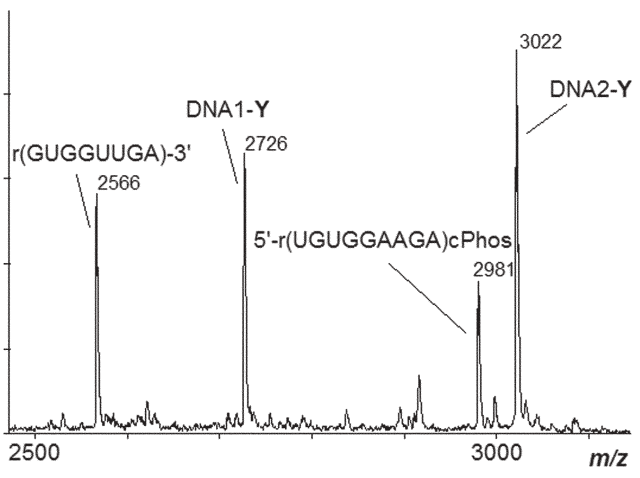

Fig. 5 MALDI mass spectra of the cleavage reaction of unlabelled RNA with DNA1-**Y** : DNA2-**Y**, in presence of Cu^{2+} ($c_{DNA1\text{-}Y}$ = $c_{DNA2\text{-}Y}$ = c_{RNA} = c_{Cu2+} = 1 μM; 37 °C, 20 h). Calcd. masses: DNA1-**Y** 2726, DNA2-**Y** 3022, 5′-r(UGUGGAAGA)cPhos 2981, r(GUGGUUGA)-3′ 2566.

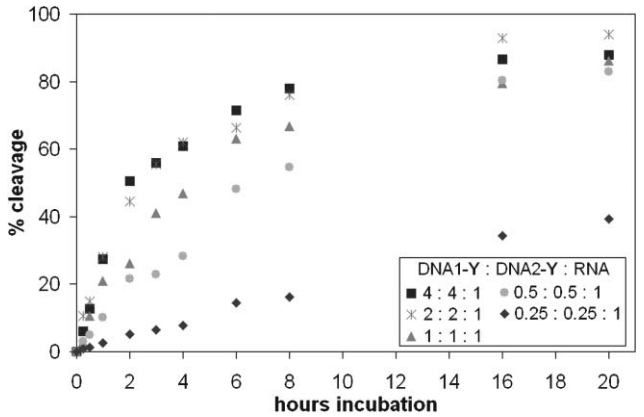

Fig. 6 Time dependent cleavage at different artificial nuclease : RNA ratios ($c_{DNA1\text{-}Y} = c_{DNA2\text{-}Y} = c_{Cu2+}$; c_{RNA} = 1 μM; 37 °C).

half-lifetime was found to be about two hours which corresponds to a relatively efficient cleavage compared to other reported artificial nucleases.[1,2,7] Additionally it is clear that even at 0.5 equivalent of the DNA strands relative to RNA, over 80% of the target was degraded after 20 h, demonstrating multiple turnover.

In conclusion, we have introduced a novel combinatorial principle for identifying efficient dinuclear artificial ribonuclease constructs by parallel screening. The principle offers the possibility of being further developed into a high-throughput approach by including larger libraries of nuclease constituents. In our opinion this would represent the ultimate strategy, since as we have also shown in this study, it is very difficult to predict the exact mechanism of action and efficiency of even well known systems. Additionally, despite the small library tested, we have identified a system that is comparable to the best artificial nuclease so far reported.

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