Metal Complex Conjugates of Antisense DNA Which Display Ribozyme-Like Activity

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One of the current challenges in supramolecular chemistry is to combine recognition with function so as to achieve systems that are capable of effecting enzyme-like catalytic modification of a chosen substrate. One area where this paradigm could be applied with particular benefit is in the field of RNA cleavage. Currently, catalytic site-specific RNA cleavage can be achieved using various enzyme- and ribozyme-based approaches.[1,2] However, no systems of completely artificial design are capable of achieving this particular supramolecular task.

Considerable progress has been made recently in terms of using oligonucleotide-appended, metal-based Lewis acidic functional groups to effect the site-directed cleavage of RNA.[3] We recently reported a synthetic approach in which a dysprosium(III) texaphyrin (DyTx) metal complex was attached to the 5′-end of oligodeoxynucleotides in the course of solid-phase synthesis (e.g., conjugate **1**, Figure 1).[4] Under conditions of conjugate excess, such 5′-derivatized constructs were shown to effect the site-specific cleavage of a cognate RNA sequence. This synthetic methodology has now been extended to the preparation of analogues in which the complex is attached at an internal position within the DNA oligomer (e.g., **2**, Figure 1).[5] This latter mode of complex attachment affords the possibility of RNA fragment release because the number of base pairs joining the RNA to the DNA conjugate is reduced by a factor of ca. 2 upon cleavage.[6]

The amount of cleavage of complementary RNA target **3** by DyTx−DNA conjugates **1** and **2** was evaluated (Figure 2). Under conditions of (>20-fold) conjugate excess, conjugates **1** and **2** displayed similar cleavage kinetics, with half-lives for RNA transesterification of ca. 2.4 and 2.2 h being recorded for these two species, respectively.[9,10] Addition of a 10-fold excess of substrate (to give 500 nM RNA), serves to illustrate an important difference between these two species, namely that one effects catalytic cleavage and the other does not. In

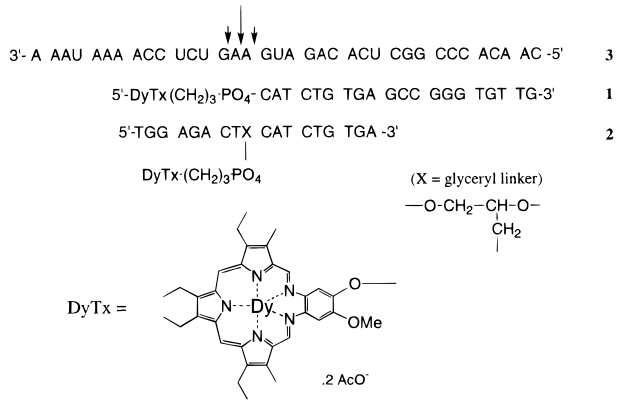

Figure 1. Structure and sequence of DyTx−DNA conjugates **1** and **2** analyzed in this study. Also shown is the sequence of complementary 5′-^{32}P-radiolabeled RNA target **3**. The larger arrow indicates the major site of cleavage produced upon incubation of this RNA target with conjugate **1** or **2** at 37 °C.

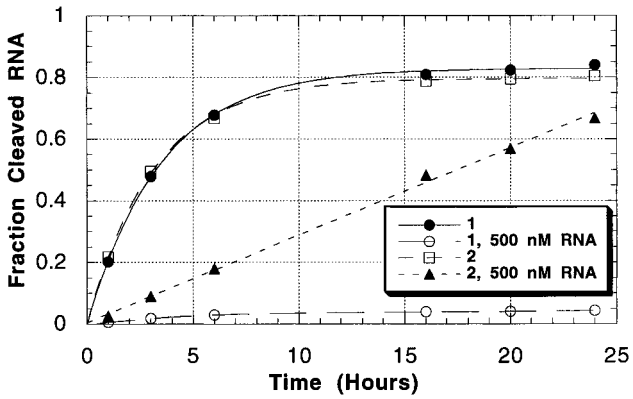

Figure 2. Comparison of RNA **3** cleavage by conjugates **1** and **2** under conditions of conjugate or substrate excess. Buffered solutions were prepared of each test species [50 nM DyTx−DNA conjugate, 50 mM HEPES, pH 7.5, 100 mM NaCl, 25 μM EDTA, 2 units/μL RNasin nuclease inhibitor (Promega Corp., Madison, WI), and 1 mM dithiothreitol, all concentrations final]. To assay under conditions of excess conjugate, the substrate, 5′- ^{32}P-radiolabeled RNA (ca. 2 nM), was incubated for 5 min at 60 °C and then added to the conjugate solutions. To assay under conditions of excess RNA, a mixture of 5′- ^{32}P-radiolabeled RNA (ca. 2 nM) and unlabeled RNA (500 nM) substrate were incubated together for 5 min at 60 °C, before adding to the conjugate solutions. The amount of site-specific RNA cleavage at 37 °C was determined as previously described.[4]

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(5) Internal asymmetric branching phosphoramidite (Clontech Laboratories, Palo Alto, CA) was deprotected using buffered hydrazine at the conclusion of DNA synthesis according to the manufacturer's protocol. The phosphoramidite derivative of DyTx was coupled to the resulting hydroxyl group, whereupon the DyTx−DNA conjugate was cleaved from the solid support and deprotected as previously described.[4] Compound **2** was purified by RP HPLC and preparative gel electrophoresis. Conjugates used in this study were characterized by positive ion MALDI mass spectral analysis (Charles Evans & Assoc., Redwood City, CA), cf. Supporting Information.
(6) The greater stability of RNA to transesterification within duplex regions[7] introduces a potential obstacle to cleavage by internally derivatized conjugates. Two groups have recently reported strategies to overcome this problem: creation of an unpaired residue at the site of cleavage through the use of an abasic linker[8] or the use of hybridization to form a two-base RNA bulge.[3e] For convenience of synthesis, we have chosen the former approach in this work.

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(9) Kinetic parameters were obtained following the method of Hendry et al.[10] by fitting the percentage of product observed to be formed (P_t) at any given time (t) to the equation $P_t = P_\infty - [\exp(-k_{obs}t)P_\Delta]$, where P_∞ is the amount of product at time $= \infty$, k_{obs} is the first-order rate constant for the reaction, and P_Δ is the difference between the percentage of products at $t = \infty$ and $t = 0$. Ca. 80% of the substrate was found to be cleaved by the end of the reaction. Remaining substrate appears to consist of nonhybrized material, as evidenced by its cleavage after a melting/reannealing protocol (cf., Supporting Information). k_{obs} was used to calculate the RNA half-life ($T_{1/2} = 0.693/k_{obs}$). Preincubation of conjugate **1** for 0, 12, or 24 h prior to addition of substrate **3** gave rise to identical cleavage profiles, confirming conjugate stability under the reaction conditions (cf., Supporting Information).
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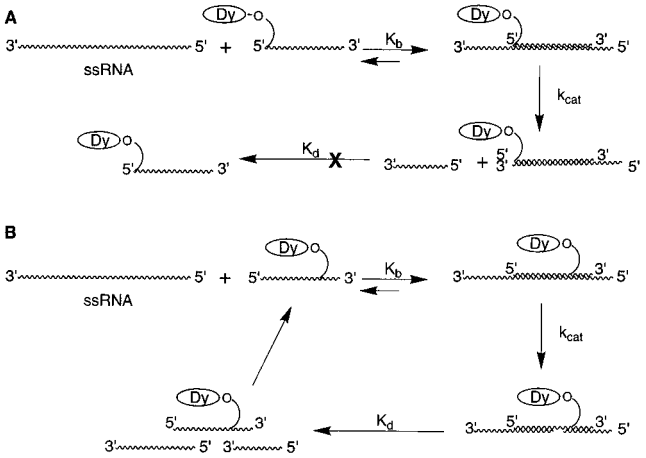

Figure 3. Schematic illustration of the differing interactions of DyTx−DNA conjugates **1** and **2** with RNA **3**. Part A describes interactions of 5′-derivatized conjugate **1** whereas part B pertains to internally derivatized conjugate **2**. DyTx complex is depicted by the circumscribed chemical symbol for dysprosium.

particular, using the 20-mer DNA conjugate **1**, ca. 5% of RNA is cleaved after 24 h, whereas in the reaction with internally derivatized conjugate **2**, cleavage of 67% of the total RNA is observed under identical conditions. This level of cleaved RNA, 335 nM after 24 h (67% of 500 nM total RNA), corresponds to a value that is 6.7 times the concentration of the DyTx−DNA conjugate **2** present in the reaction medium.[11]

The above data support the contention that the DyTx−DNA conjugate **2** is able to exhibit catalytic turnover, whereas the analogous 5′- coupled DyTx−DNA conjugate **1** is not. We explain this critical difference in function in terms of the schematic presented in Figure 3. Both agents bind (K_b) and cleave (k_{cat}) the RNA substrate in a similar fashion. However, RNA cleavage by conjugate **1** (Figure 3A) leaves the key RNA-cleaving agent bound as a 20-mer duplex comprised of the original conjugate **1** and the non-overhang portion of the RNA target. This structure is stable under the reaction conditions. As a consequence, dissociation of RNA-cleaving agent **1** is precluded and further reaction with other RNA substrate targets is prevented. In a similar way, product dissociation has been shown to inhibit turnover by hammerhead ribozymes containing long flanking arms.[12d] In the case of conjugate **2**, the observed RNA cleavage leads to strand breakage at a site internal to the duplex binding region (Figure 3B). The two resulting duplex domains (one a 9-mer and the other an 8-mer) are unstable under the reaction conditions. Conjugate **2** can dissociate from the cleaved, RNA-derived products. This frees up **2**, allowing it to bind and cleave additional RNA substrates.

Table 1. Initial Rate Data

conjugate	k_{cat} (h^{-1})[a]	K_M (nM)[a]
2	0.286 ± 0.057	20 ± 4
4	0.205 ± 0.004	69 ± 4
5	0.215 ± 0.007	6 ± 2

[a] Average values from three independent determinations (± standard deviation).

As would be predicted for an agent exhibiting turnover, there is an increase in the rate of RNA cleavage by **2** at higher substrate concentration. This observation suggested to us that internally modified DyTx−DNA conjugate **2** would exhibit saturation behavior upon titration with excess substrate. Indeed, we found that values of k_{cat} and K_M could be derived from initial rate plots for this agent, and also for congeners **4** and **5**, in which the length of the antisense portion was truncated or extended by two bases, respectively (cf., Table 1). The measured rates were found to vary between ca. 0.2 and 0.3 h^{-1}, while the values of K_M ranged from ca. 6 to 69 nM, depending on the length of the duplex formed upon hybridization with RNA **3**.

The first-order rate constant for DyTx−DNA conjugate **2** is ca. 10−1000-fold lower than those reported for hammerhead ribozymes.[10,12] Improving this rate presents a challenge for future development of this approach. However, there are aspects unique to this "ribozyme analogue" system that could prove advantageous under biological conditions. Specifically, a DyTx−DNA conjugate such as **2** is in itself sufficient for activity, in that the dysprosium(III) cation-derived "active site" is pre-programmed into the catalyst structure as a result of using a texaphyrin macrocycle. The rates of cleavage by hammerhead ribozymes, on the other hand, are cation-dependent and typically are measured in the presence of 10 mM free Mg(II). Such conditions may not be obtained in vivo.[13,14] Also, a DyTx−DNA conjugate such as **2** is in many ways structurally simpler than a ribozyme. In principle, it needs only to be of sufficient length to enable specific recognition of its cognate RNA substrate. This has potential benefits in that RNA-cleaving systems of shortened length would likely enable cellular uptake and should prove easier to prepare on large scale. Finally, in contrast to ribozyme-based approaches, there is no requirement to preserve ribonucleotide regions in the catalytic portion of the construct. As a result, the approach embodied in conjugate **2** should prove compatible with non-natural antisense backbones currently available or under development as potential therapeutics.

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Supporting Information Available: Initial rate plots for cleavage of RNA **3** by conjugates **2**, **4**, and **5**, description of the competition experiment, MALDI mass spectral data, description of the reannealing study, and conjugate stability data (8 pages). See any current masthead page for ordering and Internet access instructions.

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(11) To further evidence this difference in reactivity, cleavage of **3** by **2** and a 5′-derivatized DyTx−DNA conjugate which cleaves at an upstream site was monitored within the same reaction mixture. See Supporting Information for details.

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