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The recognition and cleavage of RNA in the antisense oligo–RNA hybrid duplexes by RNase H

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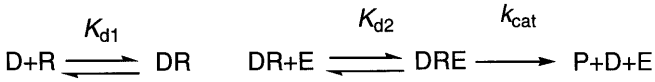
Abstract—The different extent of the target RNA cleavage at $t_{99.9\%}$ by RNase H in the AON–RNA duplexes, at the RNA saturation condition by antisense oligo, is due to different recognition and catalytic properties of RNase H toward the hybrids owing to different substituents at the 3′-end of the AONs, not owing to the different thermodynamic stabilities of the antisense oligonucleotide (AON)–RNA duplexes.

Conjugation of various chromophores and hydrophobic moieties to the antisense oligonucleotides (AON) has emerged as a challenging area of research because they form stable hybrid duplexes with the target mRNA, promoting its cleavage by RNase H. These AON conjugates have shown many important advantages over the native AON: (i) they form stable AON/RNA duplexes,[1,2] (ii) they promote cleavage of the target RNA effectively,[1,2] (iii) they improve bioavailability and the cellular delivery of AONs,[3] and (iv) they also improve the stability of AON towards various exonucleases.[4a,b] Recently, acridine conjugated AONs targeted to β-globin mRNA were shown to be more potent inhibitors of β-globin synthesis than the unmodified AONs.[5] Cholesterol conjugated AONs targeted to 27bp mRNA fragment of *Ha-ras* oncogene were able to promote a higher extent of the target RNA hydrolysis by RNase H compared to the non-conjugated counterpart.[6]

It has also been shown that chromophores tethered to either 3′- or 5′-end of the AONs increase the affinity of the AONs to the RNA target without altering the global helical structure of the corresponding AON/RNA duplexes, which has been thought to provide faster RNase H hydrolysis of the RNA moiety compared to that in the native duplex.[1] Recent studies have however shown that the 3′-conjugation results in the maximal RNase H potency.[2] We here show that 3'-tethering of chromophores to the AONs, as in **2** and **3**, not only improves $\Delta G°$ of the binding to the target RNA **4**, but also improves the kinetic properties of the RNase H promoted cleavage reaction because of favorable enzyme recognition and catalysis (Scheme 1).



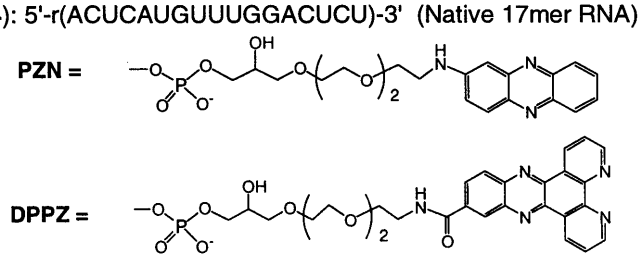
Scheme 1. Kinetic scheme of the RNase H hydrolysis; D: AON; R: target RNA; K_{d1}: equilibrium constant of dissociation of a duplex DR; K_{d2}: equilibrium constant of dissociation of the substrate-enzyme complex DRE.

The free energies of stabilization ($\Delta G°_{295}$) at 25°C were found to be -32.2 kJ mol^{-1} for the native AON/RNA (**1**+**4**, $T_m = 22.1$°C), -38.4 kJ mol^{-1} for Pzn-AON/RNA (**2**+**4**, $T_m = 28.6$°C) and -41.7 kJ mol^{-1} for Dppz-AON/RNA (**3**+**4**, $T_m = 30.6$°C) hybrid duplexes.

The PAGE (Fig. 1) and the kinetic plot (Fig. 2) show the effect of the 3′-chromophore conjugated AONs on the RNase H promoted cleavage of the target RNA in the hybrid, compared to the native counterpart.

(1): 5'-d(TCCAAACAT)-3' (Native 9mer AON)
(2): 5'-d(TCCAAACAT)-3'-**Pzn** (Pzn-AON)
(3): 5'-d(TCCAAACAT)-3'-**Dppz** (Dppz-AON)

(4): 5'-r(ACUCAUGUUUGGACUCU)-3' (Native 17mer RNA)



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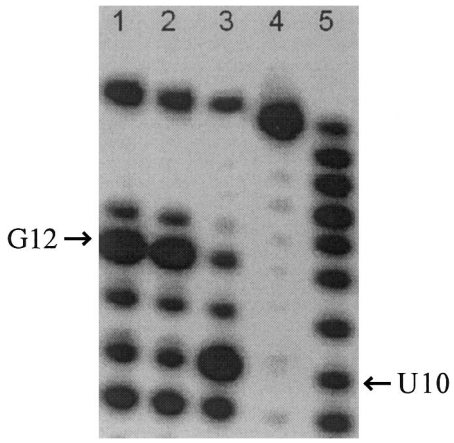

Figure 1. Autoradiogram of the 20% denaturing PAGE showing RNase H cleavage (after 2 h of incubation) of the 17*mer* target RNA **4** when hybridized with AONs (**1**–**3**). Lane 1: native 9 *mer* AON **1**; lane 2: Pzn-AON **2**; lane 3: Dppz-AON **3**; lane 4: ^{32}P-labelled target RNA **4** without AON; lane 5: snake venom PDE ladder. Conditions of cleavage reaction: AON (1 μM) and RNA (1 μM) in buffer, containing 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol (DTT), 21°C, 0.4 U of RNase H. Total reaction volume is 26 μl.

The extent of RNA hydrolysis after 1 min reaction time was chosen for comparison of the relative AON efficiencies of **2** and **3** with respect to the native **1**. It can be seen that after 1 min reaction time, all substrates were cleaved less than 30%, leaving the most of the

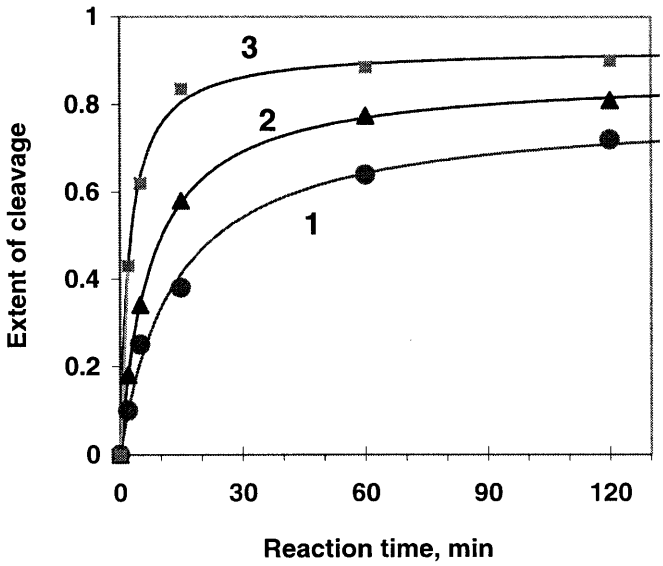

Figure 2. Extent of hydrolysis of the target RNA **4** in the AON–RNA hybrids by RNase H as a function of the reaction time. Curves 1, 2 and 3 correspond to the hybrid duplexes formed by native 9*mer* AON **1**, Pzn-AON **2** and Dppz-AON **3**. Conditions of cleavage reaction: AON (1 μM) and RNA **4** (1 μM) in buffer, containing 57 mMTris-HCl (pH 7.5), 57 mM KCl, 1 mM $MgCl_2$ and 2 mM DTT at 21°C, 0.3 U of RNase H. Total reaction volume is 26 μl.

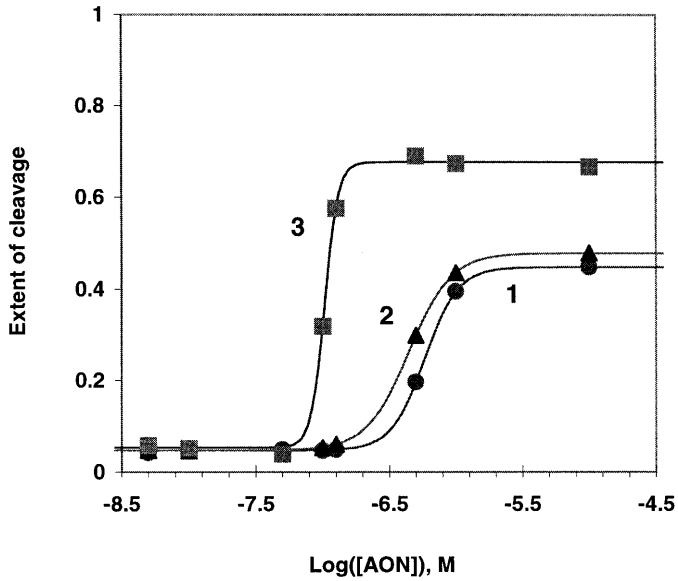

Figure 3. Extent of hydrolysis of the target RNA **4** (1 nM) in the AON–RNA hybrids by RNase H as a function of the logarithm of AON concentration (the concentrations of AONs range from 10^{-8}–10^{-5} M). Curves 1, 2 and 3 correspond to the hybrid duplexes formed by native 9*mer* AON **1**, Pzn-AON **2** and Dppz-AON **3**, respectively. Conditions of cleavage reaction are the same as in the legend of Fig. 1.

RNA intact. As evident from the kinetic data (Fig. 2), this time point lies within the linear region of the initial hydrolysis rate, and therefore the extent of hydrolysis after 1 min of reaction should directly correlate with the initial hydrolysis rate (V_0). This has led us to calculate the relative initial RNA hydrolysis rates in Pzn-AON/RNA and Dppz-AON/RNA hybrid duplexes compared to the native (**1**+**4**), showing that Dppz-AON **3** promotes 5.2 times and Azn-AON **2** 1.9 times faster hydrolysis of the target RNA compared to the native counterpart **1**.

To explore if the enhancement of the ΔG°_{295} of the chromophore-tethered AON–RNA hybrid duplexes, compared to the native, is exclusively responsible for the improved RNase H potency of the tethered AONs, the RNase H hydrolyses were performed under a condition where the target RNA is quantitatively converted to the corresponding AON/RNA hybrid with each AON (**1**–**3**) by using a large excess of AON (Fig. 3). Under such an RNA saturation condition (using 10-10000 times excess of AON relative to RNA), the starting initial concentration of the AON–RNA hybrid duplex in each reaction is the same. Hence the effect of the thermodynamics of the duplex formation in the RNase H cleavage reaction could be excluded. This gave us a unique opportunity to focus on the other factors controlling the relative affinity of the hybrid duplexes to RNase H and its catalytic properties, such as the subtle changes in the hybrid conformation and/or the change of aromatic properties of the 3′-tethered chromophore (Pzn in **2** compared to Dppz in **3**). Thus a fixed concentration of the target RNA **4**

was mixed with each AON (**1**–**3**) at different concentrations (from 10^{-8}–10^{-5} M), and the maximal extent of hydrolysis (after 2 h of incubation) of the resulting hybrid by RNase H was determined for each concentration. The results of these titrations (Fig. 3) clearly show that 100% duplex formation can be achieved with much lower Dppz-AON **3** concentration compared to Pzn-AON **2** or the native counterpart **1**. This means that as ΔG°_{295} of AON–RNA duplex stability increases, proportionally less amount of AON is required to saturate the target RNA. Inflection points of each titration curve in Fig. 3 reflect the thermodynamics of the AON/RNA duplex as well as substrate/enzyme complex formation, and are proportional to the corresponding dissociation constants K_{d1} and K_{d2} (Scheme 1), which is not possible to dissect in these specific experiments because of multiple cleavage sites in RNA (Fig. 1). It is however important to note that one should expect, despite different thermodynamic stabilities of the AON–RNA duplexes, the same degree of extent of RNA hydrolysis at $t_{99.9\%}$ for all the samples at the saturating AON concentrations provided all the hybrid duplexes were equally recognized by the enzyme. Clearly, this is not the case. The Fig. 3 shows that we have different extent of the RNA cleavage at $t_{99.9\%}$ (shown at the plateau, Fig. 3) for different AON–RNA duplex. This means that the catalytic activity of RNase H toward the RNA component in different AON/RNA hybrid duplexes are indeed different as a result of change of competing K_m and k_{cat} for each hybrid duplex substrate. This data shows that kinetic parameters of the RNase H promoted cleavage of the tethered AON/RNA hybrids are different from those of the unmodified counterpart. Clearly, these differences in the catalytic activity are either due to slight conformational changes of the helix of the hybrid substrates, or the preferential recognition of the tethered Dppz chromophore in **3** viz-a-viz Pzn in **2** compared to the native **1**. The relative global helical conformation of all three AON/RNA hybrids have remained identical as is evident from the fact that the CD spectra of all three AON–RNA hybrids generated from AON (**1**–**3**) and RNA **4**, despite that chemical nature of the 3′-terminal has been altered in all three cases, hence one can rule out the first alternative. This confirms that the change of the aromatic nature of the 3′-tethered chromophore changes the substrate recognition and catalysis by RNase H. This means that we should be able to steer the AON/RNA hybrid recognition and cleavage by RNase H more effectively in the future depending upon the engineering of the properties of the tethered chromophore.

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