The RNase H affinity and cleavage of the target RNA in the antisense–RNA hybrid duplexes containing various 3′-tethered substituents in the antisense strand

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The kinetics of RNase H promoted cleavage of the RNA strand in the antisense oligonucleotide (AON)–RNA hybrid duplexes, with the 3′-end of the AON strand tethered with cholic acid (2), its triacetate (3), cholesterol (4) or dipyridophenazine (5), have been investigated by changing the concentration of both the AON and the RNA strands, while keeping the enzyme and the buffer concentration constant. It has been shown that the cleavage of the target RNA by RNase H in the conjugated AON (2–5)–RNA (6) hybrid duplexes, at saturation conditions for RNA in the presence of an excess of AON, is higher than in the native 9mer AON (1)–RNA (6) duplex. The RNA concentration-dependent kinetics of the RNase H promoted cleavage reaction gave values for $K_m$ and $V_{max}$ for RNA (6)–AON (1–5) duplexes. The $V_{max}$ values for all 3′-tethered AON–RNA duplexes were ~30% less efficient, and their $K_m$ were also 4–14 times less, than the native counterpart, which means that the 3′-tethered substituent decreases the catalytic activity of RNase H owing to the increased affinity toward the enzyme. Since the values of $V_{max}$ and $K_m$ change in a compensating manner at a fixed enzyme concentration, the $V_{max}/K_m$ ratio for all 3′-tethered AONs in the corresponding AON–RNA duplexes showed the unique nature of the 3′-tethered substituent, dictating the 3′-substituent-dependent enzyme affinity of the heteroduplex, in comparison with the native. Accordingly, 3′-tethered Dppz AON (5)–RNA duplex has a maximum affinity for RNase H, ~6-fold more compared to the native 9mer (1), and ~2-fold more compared to 3′-tethered cholic acid (2), its triacetate (3) or cholesterol (4) containing AON–RNA duplexes.

Introduction

RNase H occurs widely in various organisms, both in eukaryotes and prokaryotes. Various isotypes of RNase H seem to have similar nucleolytic activity and substrate requirements. We have however found one exception to this with our oxetane system: [1-(1-anhydro-β-D-psicofuranosyl)thymine]. We introduced up to 3 oxetane modifications in the AON strand (i.e. reduction of $T_m$ by ~18 °C) were nicely tolerated by RNase H, without any concomitant reduction of the rate of the RNase H cleavage reaction of the complementary RNA,
compared to the wild type heteroduplex.17c We are obviously intrigued by this new finding, in view of what is known about RNase recruitment of vis-à-vis conformational preorganization in the AON–RNA heteroduplexes by the AON.19d,e

In order to understand some of the underlying factors controlling the heteroduplex stability vis-à-vis catalytic RNA cleavage by RNase H in the AON–RNA heteroduplex 18c–e (Scheme 1), we here report the results of our kinetic studies on the RNase H binding affinity of AON–RNA heteroduplexes, as well as the catalytic cleavage rate of the complementary RNA strand in these heteroduplexes, containing AONs with the 3’-tethered aromatic or alicyclic substituents such as dipyridophenazine (Dppz), cholic acid (Chol), cholic acid triacetate (Chol–(Ac)₃), and cholesterol (Cholest).

Results

The stabilities of the heteroduplexes containing AONs tethered to different aromatic groups and the complementary RNA increases because of the stacking interaction of the tethered ligand with the AON–RNA helix, without compromising the RNase H promoted cleavage rate of the complementary RNA strand by RNase H.18f,g,21

- It was found that amongst all 3’-tethered AONs, the introduction of the 3’-Dppz moiety enhances the extent of RNase H cleavage of the target RNA in the RNA–AON duplexes most in comparison with the native 9mer AON.24 To our surprise, phenazine (Pzn) and positively charged phenazinium (Pznm)25 groups at the 3’-end of the conjugated AON in the AON–RNA hybrid have very similar effects on the RNase H promoted cleavage activity compared with the native 9mer AON, suggesting that RNase H does not have the ability to discriminate between a neutral and the positively charged chromophore.26 Earlier, it was also established by detailed NMR spectroscopy that the reason for dual stability in the presence of RNase H is owing to its stacking interaction with the neighboring nucleobases, which reduces the level of hydration in the minor and major grooves, compared to the native, thereby stabilizing the hydrogen bonds.18b,d

We here report on the influence of the biologically important 3’-hydrophobic groups tethered AONs (2–4) such as cholic acid 23c in 2, 3, 12-tri-O-acetylcholic acid 26b,c in 3 and cholesteryl 26d in 4 on the extent of complementary RNA cleavage in the AON–RNA duplexes by RNase H, and compared them with 3’-tethered AONs, the introduction of the 3’-Dppz moiety enhances the extent of RNase H cleavage of the target RNA in the RNA–AON duplexes in comparison with the native 9mer AON.24 To our surprise, phenazine (Pzn) and positively charged phenazinium (Pznm)25 groups at the 3’-end of the conjugated AON in the AON–RNA hybrid have very similar effects on the RNase H promoted cleavage activity compared with the native 9mer AON, suggesting that RNase H does not have the ability to discriminate between a neutral and the positively charged chromophore.26 Earlier, it was also established by detailed NMR spectroscopy that the reason for dual stability in the presence of RNase H is owing to its stacking interaction with the neighboring nucleobases, which reduces the level of hydration in the minor and major grooves, compared to the native, thereby stabilizing the hydrogen bonds.18b,d

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Thermodynamic stabilities of all AON (1–5)–RNA (6) hybrid duplexes are shown in Scheme 2. It was found that all 3’-tethered AONs (2–5)–RNA (6) duplexes are more stable (ΔTm ranges from +4 to +11°C) compared to the native 9mer AON (1), and that the 3’-Dppz group makes the corresponding AON (5)–RNA (6) duplex more stable (ΔTm = +11°C) than the other 3’-tethered AONs 2–4. The extent of RNA cleavage by RNase H in all AON (1–5)–RNA (6) duplexes was investigated both (i) by changing the AON concentration (Figs. 1–3) at constant concentration of RNA and RNase H in order to obtain saturation conditions for RNA (i.e. when RNA is completely bound to the AON in the duplex form) in the presence of an excess of AON, and also (ii) by changing the RNA concentration (Figs. 4–6) at a constant concentration of RNase H and AON, which is sufficient for complete saturation of RNA. The initial velocities (ν₀) have thus been obtained at four different RNA concentrations (ranging from 0.123 µM to 2.02 µM (Fig. 5 and Table 1)) at saturating conditions for RNA (6) by AON (1–5) (10 µM), which were subsequently plotted as a function of substrate, RNA–AON hybrid duplex, concentration (S₀) to give Kₙ and Vₘₘₜₙ from both the Michaelis equation and Lineweaver–Burk analysis.24

The summary of our investigation is as follows.

- The extent of the target RNA cleavage by RNase H in the hybrid RNA (6)–AON (2–5)–RNA duplexes at saturation conditions (when RNA is completely saturated by AON at low RNA concentration) is higher than in the native 9mer AON (1)–RNA (6) duplex (Figs. 1–3).

When the AON concentration for 1–5 is increased, we see an increase in the extent of cleavage along with the increase in the height of the saturation plateau (Fig. 3). The plot of this AON concentration-dependent study is hyperbolic in nature, which appears sigmoidal in shape in the logarithmic scale [AON] plot as shown in Fig. 3. This is contrary to what was reported26 earlier for cholesterol tethered AON (4), where it was shown to have a parabolic dependence of the extent of cleavage with increasing AON concentration, or to show a decrease of the RNase H response at a high concentration of AONs.26e

For AONs containing cholic acid (2), cholic acid triacetate (3) and cholesteryl (4), the cleavage sites have been found to be both at U9 and G12 positions in ca. 1 : 4 ratio (Fig. 1) of the complementary target RNA compared to that of the native 9mer AON (1), which had only a single cleavage site at the G12 position (Fig. 1). This shows that at least there are two binding sites as well as the cleavage sites in AONs (2–4)–RNA complexed to that of the native counterpart.

On the other hand, the 3’-Dppz tethered AON (5) promotes only a single cleavage site at the U10 position of the target RNA (Fig. 1) with a maximal extent of cleavage both at the saturating and non-saturating conditions of RNA, compared to all other AONs, specially at a low AON concentration (Fig. 2A and Fig. 3), showing that the 3’-Dppz group has not only promoted higher cleavage of RNA, but also forces the RNase H to bind and cleave at distinctly different sites from those of the native as well as for AONs (2–4)–RNA duplex.

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Since the Vₘₘₜₙ and Kₙ was performed by two methods: (i) from the 1/ν₀ versus 1/[S₀] plots, where [S₀] = [RNA], or (ii) directly from ν₀ versus [S₀] plots,24 which give the Michaelis dependency of ν₀ from [S₀] described by the equation: ν₀ = Vₘₘₜₙ[S₀]/(Kₙ + [S₀]). Observed values of Vₘₘₜₙ and Kₙ by these methods were found to be in good agreement (Figs. 7A and 7B).

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The Vₘₘₜₙ and Kₙ value for AON (5)–RNA is six-fold more than the native counterpart (Fig. 7C). On the other hand, for 3’-tethered cholic acid (2), its triacetate (3) or cholesteryl (4) complex, the RNase binds both to the 3’-tethered ligand as well as within the heteroduplex, hence giving two different cleavage sites, which can be seen in the Vₘₘₜₙ/Kₙ value for AON (2–4)–RNA.
Table 1 Dependence of the initial velocity ($v_0$) on the substrate concentration

<table>
<thead>
<tr>
<th>[RNA] = S₀/µM</th>
<th>$v_0$/µM min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9mer-AON</td>
</tr>
<tr>
<td>0.123</td>
<td>0.00166</td>
</tr>
<tr>
<td>0.523</td>
<td>0.00518</td>
</tr>
<tr>
<td>1.023</td>
<td>0.01180</td>
</tr>
<tr>
<td>2.023</td>
<td>0.01526</td>
</tr>
</tbody>
</table>

*a* Initial velocity has been calculated from the data taken up to 7.6 min into the reaction.

Table 2 Dependence of the extent of cleavage ($P/S₀$) of RNA on the substrate concentration

<table>
<thead>
<tr>
<th>[RNA] = S₀/µM</th>
<th>$α = P/S₀$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9mer-AON</td>
</tr>
<tr>
<td>0.123</td>
<td>0.0675</td>
</tr>
<tr>
<td>0.523</td>
<td>0.0495</td>
</tr>
<tr>
<td>1.023</td>
<td>0.0577</td>
</tr>
<tr>
<td>2.023</td>
<td>0.0377</td>
</tr>
</tbody>
</table>

*a* Extent of cleavage was calculated from the data taken during the first 5 min of the reaction.

RNA, which is three-fold more than the native counterpart (Fig. 7C). Such behavior of the Dppz-AON might be associated with the structure of the Dppz moiety itself, in which two pyridyl-nitrogens (N4 and N5) in the fused phenanthroline system can form a chelated complex with divalent metal ions such as Mg²⁺. Since Mg²⁺ ion is an important cofactor for RNase H¹³, it is likely that the Dppz group provides significant assistance by supplying Mg²⁺ ions to augment the RNase H affinity toward Dppz-AON in the corresponding hybrid duplex with RNA for subsequent RNase H cleavage.

Fig. 8 shows purities of all 3'-tethered AONs used in this work (Figs. 8A, B). It also shows (Fig. 8C) the relative retention times of a mixture of AONs 1-5 on a reversed-phase C18 column (solvent system: buffer A: 0.05 M LiClO₄, buffer B: 80% CH₃CN in 0.05 M LiClO₄, 0% → 100% B during 40 min, rate of elution 1.5 ml min⁻¹), showing the relative hydrophobicity of different 3'-tethered AONs, compared to the native RNA.

Scheme 2 Synthesis and structures of the antisense oligonucleotides, AONs, (1-5), target RNA (6) and the thermodynamic stability of the corresponding hybrid AON–RNA duplexes ($T_m$ values, see Experimental section for conditions). Reagents and conditions: (1): 3'-phosphate-9mer (1) (30 O.U.₂₆₀, 0.33 µmol), triphenylphosphine (200 µmol), 2,2'-dithiodiipyridine (200 µmol), 4-dimethylaminopyridine (400 µmol) and amine derivative $H_2N(CH_2)_2NHR$ (500 µmol) in dry dimethylformamide (300 µl) at RT (see ref. 22b,c). Yields of 2-5 vary from 50–70%.
Table 3  Kinetic characteristics* of RNA cleavage by RNase H from AON-RNA duplex

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{\text{max}}$/µM min$^{-1}$</th>
<th>$K_m$/µM</th>
<th>$k_{\text{cat}}$/min$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9mer-AON(1)-RNA(6)</td>
<td>(3.19 ± 0.45) × 10$^{-2}$</td>
<td>2.10 ± 0.29</td>
<td>16.6 ± 2.3</td>
</tr>
<tr>
<td>Chol-AON(2)-RNA(6)</td>
<td>(1.93 ± 0.71) × 10$^{-2}$</td>
<td>0.39 ± 0.14</td>
<td>10.1 ± 3.7</td>
</tr>
<tr>
<td>Chol(Ac)3-AON(3)-RNA(6)</td>
<td>(1.99 ± 0.20) × 10$^{-2}$</td>
<td>0.58 ± 0.06</td>
<td>10.4 ± 1.1</td>
</tr>
<tr>
<td>Cholest-AON(4)-RNA(6)</td>
<td>(1.74 ± 0.12) × 10$^{-2}$</td>
<td>0.38 ± 0.03</td>
<td>9.06 ± 0.6</td>
</tr>
<tr>
<td>Dppz-AON(5)-RNA(6)</td>
<td>(1.62 ± 0.35) × 10$^{-2}$</td>
<td>0.18 ± 0.04</td>
<td>8.4 ± 1.8</td>
</tr>
</tbody>
</table>

* $V_{\text{max}} = k_{\text{cat}}E_0$ ($E_0 = 2 \times 10^{-2}$ U µl$^{-1}$ = 1.92 × 10$^{-3}$ µM, specific activity = 420000 U mg$^{-1}$ = 1.134 × 10$^{-13}$ moles per unit, MW = 21000 g mol$^{-1}$).

native, which might give us an estimate of how easily these AONs may penetrate inside the cell. It can be seen that they can be classified on the basis of their relative hydrophobicities in the following manner, as judged by their relative retention times ($t_R$) in the reversed-phase column: 1 ($t_R$ = 9.9 min) < 2 ($t_R$ = 12.6 min) < 3 ($t_R$ = 15.4 min) < 4 ($t_R$ = 19.9 min) < 5 ($t_R$ = 35.6 min). We however have not found any correlation between the hydrophobicity of Chol-, Chol(Ac)$^3$- and Cholest-AONs (Fig. 8C) and theirs kinetic parameters ($K_m$, $V_{\text{max}}$ and $k_{\text{cat}}$) (Fig. 7, Table 3). It suggests that the recognition of AON–RNA duplex by RNase H is not associated with hydrophobic properties of the 3′-tethered moiety in the AONs, which is also consistent with the fact that 3′-tethered Pzn and Pznm promote comparable rates of cleavage of RNA.$^{18d}$

Discussion

The dependence of initial velocity, $v_0$, of cleavage reaction from substrate concentration ($S_0 = [\text{RNA}]$) has been found (Fig. 5, Table 1), which can be interpreted according to the Michaelis equation [eqn. (1)]:

$$v_0 = \frac{V_{\text{max}}S_0}{K_m + S_0}$$

where $v_0$, in unsaturated conditions (when $S_0 \leq K_m$), increases with the increase of $S_0$ and has a saturation plateau when $S_0 \rightarrow \infty$ (Fig. 5).

Fig. 1  (A) Autoradiogram of the 20% denaturing PAGE showing RNase H cleavage (after 30 min of incubation) of the 17mer target RNA (6) when hybridized with AONs (1–5). Lane 1: native 9mer AON (1); lane 2: Chol-AON (2); lane 3: Chol(Ac)$^3$-AON (3); lane 4: Cholest-AON (4); lane 5: Dppz-AON (5); lane R: $^{32}$P-labelled target RNA (6) without AON; lane L: snake venom PDE ladder. Conditions of cleavage reaction: AON (0.1 µM, 1 µM and 10 µM) and RNA (0.01 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, 1 U of RNase H. Total reaction volume is 30 µl. RNase H degradation pattern of the 17mer RNA target hybridized with native 9mer AON (B), Chol-, Chol(Ac)$^3$- and Cholest-AONs (C), Dppz-AON (D). The vertical arrows with nucleotide sequence number indicate the site of cleavage on the RNA strand.

In this case, different AONs are working in different manners in different substrate concentration (compare the area where \( S_0 < 1 \) µM with the area where \( S_0 > 2 \) µM in Fig. 5 and Table 1). Thus, Dppz-AON (5) has a maximal and the 9mer AON (1) has a minimal cleavage reaction in the area when \( S_0 < 0.2 \) µM and the relative activity switches in the opposite direction in the area when \( S_0 > 3 \) µM (Fig. 5B).

The Michaelis equation [eqn. (1)] can be written in different forms at different substrate concentrations. (A) Under low substrate concentrations, when \( S_0 \ll K_m \), eqn. (1) takes the form of eqn. (2).

\[
v_0 = \frac{V_{\text{max}} S_0}{K_m + S_0} = \frac{V_{\text{max}} S_0}{K_m}
\]

This means that \( v_0 \) is linearly dependent upon the substrate concentration with the linear coefficient of \( V_{\text{max}}/K_m \) which is maximal for the duplex formed with Dppz-AON (5) and minimal for the duplex formed with 9mer AON (1) (Fig. 7C).

(B) Under large substrate concentrations, when \( S_0 \gg K_m \), eqn. (1) can be rewritten as eqn. (3).

\[
v_0 = \frac{V_{\text{max}} S_0}{K_m + S_0} = V_{\text{max}} = k_{\text{cat}} E_0
\]

This means that the initial velocity of the reaction in this case is dependent only on \( V_{\text{max}} \) or, alternatively, on \( k_{\text{cat}} \) and \( E_0 \). The initial velocity or efficiency of the reaction in this case is dependent on \( V_{\text{max}} \), which for all 3'-tethered AON-RNA duplexes is \( \sim 30\% \) less efficient than the natural counterpart, which means that the 3'-tethered substituent decreases the catalytic activity of RNase H. The \( V_{\text{max}} \) is maximal for native 9mer AON and relatively less for Dppz-AON (Fig. 7A and Table 3).

It is known that RNase H concentration under the physiological conditions is \( \sim 10^{-7} \) micromoles per litre and mRNA concentration is \( \sim 10^{-5} \) micromoles per litre. It means that our conjugated AONs are working (Fig. 5) with improved efficiency under putative physiological conditions compared with the native 9mer AON, when \( [RNA] = 0.1 \) micromoles per litre. Under a constant RNase H concentration and time, but with a large substrate concentration and rich saturation conditions for the enzyme, \( v_0 \) should be maximal for the native 9mer AON, but the extent of cleavage, since that is not dictated by \( K_m \) or \( [S_0] \), will be low for all AONs because the amount of product formation will be the same. Clearly, the extent of the RNA cleavage, \( \alpha \), will be low if the initial substrate concentration \( [S_0] \) is high, and in this case, it is possible to cleave only a small part of the initial mRNA. So, \( v_0 \) of the reaction does not say very much about the extent of final cleavage, \( \alpha = [\Delta P]/[S_0] \) where \( [\Delta P] \) is product of cleavage in the reaction time \( \Delta t \).

For this reason we have investigated also the dependence of the extent of cleavage (during 5 min) \( \alpha = [\Delta P]/[S_0] \) on the substrate concentration \( [S_0] = [RNA] \) (Fig. 6, Table 2).

The extent of the cleavage in this case is decreased with increasing substrate concentration in accordance with a modified form, eqn. (6), of the Michaelis equation (1):

\[
v_0 = \frac{V_{\text{max}} S_0}{K_m + S_0}
\]

where:

\[
v_0 = \frac{\Delta P}{\Delta t}
\]

and we have for \( \Delta P \):

\[
In this case, the extent of cleavage is dependent only on the substrate concentration because, when $S_0 \ll K_m$, the extent of the cleavage is not dependent upon the substrate concentration because, when $S_0 \ll K_m$, eqn. (6) can be written as eqn. (7):

$$\alpha = \frac{V_{\text{max}} S_0}{(K_m + S_0)} \frac{\Delta t}{(K_m + S_0)} = \frac{V_{\text{max}} \Delta t}{K_m} \quad (7)$$

Eqn. (7) explains our experimental data, where Dppz-AON has maximal cleavage compared with all other AONs, in a situation when the substrate concentration is less than the $K_m$ value (Table 3, see the area around $S_0 = 0.1 \mu M$ in Fig. 6) because the Dppz-AON–RNA substrate has a maximal value of $V_{\text{max}}/K_m$ (Fig. 7C).

But, when substrate concentration is increasing, the extent of the cleavage should decrease and become close to zero according to eqn. (6), and this dependence has a hyperbolic shape (see area around $S_0$ more than $0.1 \mu M$ in Fig. 6A).

Thus, our conjugated AONs have better cleaving characteristics both in terms of initial velocity and the extent of the cleavage than the native 9mer AON under low RNA concentration conditions ($0.1 \mu M$ per litre), as under physiological conditions.

The turnover $(N_t)$ of RNase H can be calculated by using eqn. (8):

$$N_t = \frac{P_t}{E_0} \quad (8)$$

where $N_t$ indicates the number of RNA molecules ($P_t$) that the enzyme cleaves during the time, $t$. The values for $k_{\text{cat}}$ and $K_m$ allow us to define the $N_t$ at a substrate concentration, $S_0$, according to eqn. (9), which is derived from eqns. (4), (5) and (8):

$$N_t = \frac{\Delta P}{E_0} = \frac{V_{\text{max}} S_0}{(K_m + S_0) E_0} \Delta t = \frac{k_{\text{cat}} E_0 S_0}{(K_m + S_0) E_0} \Delta t = \frac{S_0}{K_m + S_0} k_{\text{cat}} \Delta t = F_s N_{\text{max}}$$

where $F_s$ is the extent of saturation of enzyme, $E$, as shown in eqn. (10), by the substrate, $S$, which is equal to zero when $[S] = 0$, and equal to 1 when $[S] \gg K_m$ or $[S] \to \infty$;

$$F_s = \frac{[\text{ES}]}{[E_0]} = \frac{S_0}{K_m + S_0} \quad (10)$$

ES is the enzyme–substrate complex, and $N_{\text{max}}$ is a maximal turn over of enzyme, when the extent of saturation of the enzyme by substrate is maximal, otherwise $F_s = 1$:

$$N_{\text{max}} = k_{\text{cat}} \frac{S_0}{(K_m + S_0)} \Delta t$$

Eqn. (9) means that the turnover of the enzyme is dependent on factors such as the time of reaction, the $k_{\text{cat}}$ and $F_s$ [eqn. (10)], which is dependent on both $K_m$ and $S_0$. It means that at different substrate concentrations $S_0$, turnover of enzyme, $N_t$, will be different. We have therefore calculated the values for $N_t$ for each AONs at different substrate concentration during 5 min of reaction (Table 4).

These data show that the turnover of RNase H for Dppz-AON–RNA $(N_t = 17.4)$ at low RNA concentration $([S] = 0.123 \mu M)$ is more compared with the native 9mer AON. This is because Dppz-AON–RNA substrate under these conditions saturates the RNase H more ($F_s = 0.41$ or $41\%$) compared to the native 9mer AON $(N_t = 4.6, F_s = 0.006$ or $0.6\%)$. But, at 2 $\mu M$ substrate concentration, RNase H has nearly the same turnover, $N_t$ (Table 4) because at these conditions values of $F_s$ increase for all AONs.

It should be added that all compared data for $V_{\text{max}}$, $\alpha$ and $N_t$ were obtained using large concentration of AONs (10 $\mu M$) (Fig. 4, 5). Under these conditions, whether Dppz-AON can work better or worse compared to the native 9mer AON, depends entirely upon the substrate (i.e. heteroduplex)
Fig. 4  Extent of hydrolysis of the target RNA (6) in the AON–RNA hybrids by RNase H as a function of the reaction time. Curves 1–5 in (A) to (D) correspond to the hybrid duplexes formed by native 9mer AON (1), Chol-AON (2), Chol(Ac)₃-AON (3), Cholest-AON (4) and Dppz-AON (5) respectively. Conditions of the cleavage reactions: AON concentration (10 µM) and RNA (6) were varied at 0.123 µM in (A), 0.523 µM in (B), 1.02 µM in (C), and 2.02 µM in (D) in buffer, containing 60 mM Tris-HCl (pH 7.5), 60 mM KCl, 10 mM MgCl₂ and 1 mM DTT at 21 °C, 0.5 units of RNase H. Total reaction volume is 30 µl.

concentration. When AON concentration is however low, [AON] = 0.1 µM (Fig. 2A and Fig. 3), all kinetic parameters ν₀, α and Nₐ additionally depend upon the extent of saturation RNA by AON, FRNA [eqn. (12)], which is equal to ca. 60% for Dppz-AON and less then 10% for all other AONs (Fig. 3). The extent of saturation of RNA by AON, FRNA, can be written according to eqn. (12):

\[
F_{RNA} = \frac{[DR]}{[R_0]} = \frac{D_0}{K_0 + D_0} 
\]

where DR is an AON–RNA hybrid duplex, D₀ is initial AON concentration, R₀ is initial RNA concentration and K₀ is the equilibrium constant of dissociation of the hybrid AON–RNA. In conditions when RNA is not saturated by AON and S₀ ≪ K₀, initial velocity, ν₀, or extent of cleavage, α, of reaction, can be rewritten as eqn. (13) or (14) by combining eqns. (2), (6), (12):

\[
ν₀ = \frac{V_{max}}{K_m} S₀ + \frac{V_{max}}{K_m} [DR] = \frac{V_{max}}{K_m} F_{RNA} R₀ 
\]

\[
α = \frac{ΔP}{R₀} = \frac{V_{max}[DR]}{(K_m + [DR])R₀} Δt = \frac{V_{max}}{K_m} \frac{[DR]}{R₀} Δt = \frac{V_{max}}{K_m} F_{RNA} Δt 
\]

According to eqn. (13) the initial velocity of reaction at constant RNA concentration depends upon two parameters: \(V_{max}/K_m\) ratio and the thermostability of the AON–RNA duplex, which is maximal for Dppz-AON–RNA substrate compared with other AON–RNA substrates. The same conclusion can be drawn in the case of the extent of cleavage in Fig. 3. According to eqn. (14) the extent of cleavage during the same time period of reaction depends upon two parameters: \(V_{max}/K_m\) ratio as well as on the thermostability of the AON–RNA duplex, and does not depend upon the RNA concentration. Both of these characteristics, value of \(V_{max}/K_m\) and FRNA, are much better for Dppz-AON compared with native 9mer AON. This explains why we have different saturation plateaux for different AONs in the AON concentration dependent plots, as shown in Fig. 3. Thus, a maximal extent of RNA cleavage is obtained for Dppz-AON, compared to 9mer AON or other AONs (Fig. 2A and Fig. 3) at 0.1 µM concentration of AON, which is an important characteristic fulfilled by Dppz-AON in order to be a potentially important antisense substance.

The relative retention times of a mixture of AONs 1–5 on a reversed-phase C18 column (Fig. 8) showing the relative hydrophobicity of different 3'-tethered AONs, compared to the native gives an insight, in a model system, into how easily these AONs may penetrate inside the cell. Since cholesterol-tethered AON 4 is retained most in the reversed-phase column, it can be assumed that it might be able to penetrate through the cellular membrane most easily. Since we do not find any correlation between the hydrophobicity of Chol-, Chol(Ac)₃- and Cholest-AONs (Fig. 8C) and their kinetic parameters (Kₐ, Vₐ, and k_cat) (Fig. 7, Table 3), it is likely that the recognition and kinetic degradation of the RNA in AON–RNA duplex by RNase H is not dictated by the hydrophobic properties of the 3'-tethered moiety in the AONs.

Materials and methods

Materials

T4 polynucleotide kinase (30 units per µL) and E. coli RNase H (5 units per µL, specific activity 420000 units mg⁻¹, molecular weight 21000 g mol⁻¹) and [γ⁻³²P]ATP were purchased from Amersham Pharmacia Biotech (Sweden), phosphodiesterase I from Crotalus adamanteus venom was from SIGMA. Oligonucleotide (1)²²⁴ and RNA (6) were synthesized using an Applied Biosystems 392 automated DNA/RNA synthesizer. Syntheses of the 3'-conjugated AONs (2-5) were carried out by post synthetic methods starting from the same 3'-phosphate tethered 9mer oligonucleotide (1), which was conjugated with amino derivatives of the corresponding cholic acid,²²⁵ 3,7,12-tri-O-acetylcholic acid,²²⁶ cholesterol²²⁷ or dipyridophenazine²²⁸ as previously described²² (Scheme 2). All AONs 1-5 were characterized by NMR, UV, electrophoresis and enzymatic digestions and compared with the physicochemical properties reported in the literature.²²⁵ ²²⁶ ²²⁷ ²²⁸ ²²² The column chromatographic separations were performed using Merck G 60 silica gel. A Biacore HPLC equipment with Pump Model 2250, Manometric Module Lambda 1010 connected to a DataApex CSW computer program for gradient control were used for analytical and semi-preparative RP-HPLC separations on Kromasil 100 C18 column (5 µm, 250 × 8 mm).

UV melting experiments

Determination of the Tₘ values of the AON–RNA hybrid duplexes was carried out in the same buffer as for RNase H degradation: 20 mM Tris-HCl (pH 8), 100 mM KCl and 10 mM MgCl₂. Absorbance was monitored at 260 nm in the temperature range from 3 °C to 70 °C using Lambda 40 UV spectrophotometer equipped with Peltier temperature programmer with the heating rate of 1 °C per minute. Prior to the measurements samples (1 : 1 mixture of 1 µl AON and 1 µl RNA) were preannealed by heating to 80 °C for 5 min followed by slow cooling to 3 °C and 30 min equilibration at this temperature.

³²P Labeling of oligonucleotides

The oligoribonucleotide and oligodeoxyribonucleotides were 5'-end labeled with ³²P using T4 polynucleotide kinase, [γ⁻³²P]-ATP and standard procedure. Labeled RNAs were purified by 20% 7 M urea denaturing polyacrylamide denaturing gel
Table 4: Turnovers ($N_t$ and $N_{\text{max}}$) and extent of saturation of enzyme by AON–RNA substrates, $F_e$, for RNase H cleavage reactions at different substrate concentrations, $S_0$.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>[RNA] = $S_0/\mu$M</th>
<th>$F_e = E/S_0 = S_0/(K_m + S_0)$</th>
<th>$N_{\text{max}} = V_{\text{max}}/t$</th>
<th>$N_t = F_e N_{\text{max}}$</th>
<th>$N_{\text{relative}} = N_t(AON)/N_t(9\text{mer})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9mer-AON(1)-RNA(6)</td>
<td>0.123</td>
<td>0.055</td>
<td>83.1</td>
<td>4.6</td>
<td>1</td>
</tr>
<tr>
<td>Chol-AON(2)-RNA(6)</td>
<td>0.123</td>
<td>0.239</td>
<td>50.3</td>
<td>12.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Chol(Ac1)-AON(3)-RNA(6)</td>
<td>0.123</td>
<td>0.176</td>
<td>51.8</td>
<td>9.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Cholest-AON(4)-RNA(6)</td>
<td>0.123</td>
<td>0.246</td>
<td>45.3</td>
<td>11.2</td>
<td>2.4</td>
</tr>
<tr>
<td>Dppz-AON(5)-RNA(6)</td>
<td>0.123</td>
<td>0.413</td>
<td>42.2</td>
<td>17.4</td>
<td>3.8</td>
</tr>
<tr>
<td>9mer-AON(1)-RNA(6)</td>
<td>0.523</td>
<td>0.199</td>
<td>83.1</td>
<td>16.6</td>
<td>1</td>
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<tr>
<td>Chol-AON(2)-RNA(6)</td>
<td>0.523</td>
<td>0.572</td>
<td>50.3</td>
<td>28.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Chol(Ac1)-AON(3)-RNA(6)</td>
<td>0.523</td>
<td>0.476</td>
<td>51.8</td>
<td>24.7</td>
<td>1.5</td>
</tr>
<tr>
<td>Cholest-AON(4)-RNA(6)</td>
<td>0.523</td>
<td>0.582</td>
<td>45.3</td>
<td>26.4</td>
<td>1.6</td>
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<tr>
<td>Dppz-AON(5)-RNA(6)</td>
<td>0.523</td>
<td>0.749</td>
<td>42.2</td>
<td>31.6</td>
<td>1.9</td>
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<tr>
<td>9mer-AON(1)-RNA(6)</td>
<td>1.023</td>
<td>0.328</td>
<td>83.1</td>
<td>27.2</td>
<td>1</td>
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<tr>
<td>Chol-AON(2)-RNA(6)</td>
<td>1.023</td>
<td>0.723</td>
<td>50.3</td>
<td>36.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Chol(Ac1)-AON(3)-RNA(6)</td>
<td>1.023</td>
<td>0.640</td>
<td>51.8</td>
<td>33.2</td>
<td>1.2</td>
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<tr>
<td>Cholest-AON(4)-RNA(6)</td>
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<td>0.731</td>
<td>45.3</td>
<td>31.1</td>
<td>1.2</td>
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<td>Dppz-AON(5)-RNA(6)</td>
<td>1.023</td>
<td>0.854</td>
<td>42.2</td>
<td>36.0</td>
<td>1.3</td>
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<td>9mer-AON(1)-RNA(6)</td>
<td>2.023</td>
<td>0.491</td>
<td>83.1</td>
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<td>1</td>
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<td>Chol-AON(2)-RNA(6)</td>
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<td>0.838</td>
<td>50.3</td>
<td>42.1</td>
<td>1.0</td>
</tr>
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<td>Chol(Ac1)-AON(3)-RNA(6)</td>
<td>2.023</td>
<td>0.778</td>
<td>51.8</td>
<td>40.4</td>
<td>0.99</td>
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<tr>
<td>Cholest-AON(4)-RNA(6)</td>
<td>2.023</td>
<td>0.843</td>
<td>45.3</td>
<td>38.2</td>
<td>0.94</td>
</tr>
<tr>
<td>Dppz-AON(5)-RNA(6)</td>
<td>2.023</td>
<td>0.920</td>
<td>42.2</td>
<td>38.8</td>
<td>0.95</td>
</tr>
<tr>
<td>9mer-AON(1)-RNA(6)</td>
<td>4</td>
<td>0.656</td>
<td>83.1</td>
<td>54.5</td>
<td>1</td>
</tr>
<tr>
<td>Chol-AON(2)-RNA(6)</td>
<td>4</td>
<td>0.911</td>
<td>50.3</td>
<td>45.8</td>
<td>0.84</td>
</tr>
<tr>
<td>Chol(Ac1)-AON(3)-RNA(6)</td>
<td>4</td>
<td>0.874</td>
<td>51.8</td>
<td>45.3</td>
<td>0.83</td>
</tr>
<tr>
<td>Cholest-AON(4)-RNA(6)</td>
<td>4</td>
<td>0.914</td>
<td>45.3</td>
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<td>0.76</td>
</tr>
<tr>
<td>Dppz-AON(5)-RNA(6)</td>
<td>4</td>
<td>0.958</td>
<td>42.2</td>
<td>40.4</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Fig. 7: (A) and (B) $V_{\text{max}}$ and $K_m$ values for the RNase H promoted hydrolysis of AON (1–5)–RNA (6) substrates were determined by a plot of $1/v_0$ versus $1/[S_0]$ (open bars) and Michaelis equation (filled bars). (C) Comparison of the $V_{\text{max}}/K_m$ values. The relative $V_{\text{max}}/K_m$ value for each tethered AON (2–5)–RNA duplex hybrid duplex was obtained by dividing the $V_{\text{max}}/K_m$ value for each tethered AON (2–5)–RNA duplex by the corresponding value for AON (1)–RNA duplex.
electrophoresis (PAGE) and specific activities were measured using a Beckman LS 3801 counter.

Snake venom PDE ladder

5′-[32P]P-labeled RNA (1.3 µM, specific activity 500 000 cpn) was incubated with 100 ng of the phosphodiesterase I in buffer, containing 50 mM Tris-HCl (pH 8), 5 mM MgCl2 at 21 °C. Total reaction volume was 50 µl. After 2, 10, 15, 30, 40 and 60 minutes aliquots (correspondingly 5, 6, 9, 9, 10 and 10 µl) were mixed with stop solution (correspondingly 10, 12, 18, 18 and 20 µl), containing 0.05 M disodium salt of ethylene-diaminetetraacetic acid (EDTA), 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol in 95% formamide. All aliquots were mixed and subjected to 20% 7 M urea denaturing gel electrophoresis and visualized by autoradiography.

Kinetics

AON concentration dependent experiments. AONs (0.1 µM, 1 µM, or 1 µM), with 32P-labeled RNA (0.01 µM, specific activity 50 000 cpn) were incubated with 0.5 or 1 units of RNase H in buffer, containing 20 mM Tris-HCl (pH 8), 100 mM KCl, 10 mM MgCl2 and 1 mM DTT at 21 °C. Total reaction volume was 30 µl. Prior to the addition of the enzyme reaction components were preannealed in the reaction buffer by heating at 80 °C for 5 min followed by 1.5 h equilibration at 21 °C. After 3–120 minutes aliquots (5 µl) were mixed with stop solution (5 µl), containing 0.05 M disodium salt of ethylenediaminetetraacetic acid (EDTA), 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol in 95% formamide. This samples were subjected to 20% 7 M urea polyacrylamide denaturing gel electrophoresis and visualized by autoradiography. Quantitation of cleavage products was performed using a Molecular Dynamics PhosphorImager.

RNA concentration dependent experiments. 32P-Labeled RNA (0.123 µM, 0.523 µM, 1.02 µM or 2.02 µM, specific activity 50 000 cpn) with AONs (10 µM) were incubated with 0.5 units of RNase H in buffer, containing 60 mM Tris-HCl (pH 7.5), 60 mM KCl, 10 mM MgCl2, and 1 mM DTT at 21 °C. Total reaction volume was 30 µl. Prior to the addition of the enzyme concentration components were preannealed in the reaction buffer by heating at 80 °C for 5 min followed by 1.5 h equilibration at 21 °C. After 3–10 minutes aliquots (5 µl) were mixed with stop solution (5 µl) and subjected to 20% 7 M urea denaturing gel electrophoresis. The kinetic parameters $K_m$ and $V_{max}$ were obtained by two methods: from the 1/$v_0$ versus 1/$[S]$ plots, where $[S]$ = [RNA], or directly from $v_0$ versus $[S]$ plots. Values of $K_m$ and $V_{max}$ at the last method were determined directly from $v_0$ versus $[S]$ plots by using of correlation SigmaPlot Program, where correlation equation was: $y = ax/(b + x)$.

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References
