



Synthesis and properties of hammerhead ribozymes stabilized against nucleases by different 2'-modifications: methoxyethoxy-, fluoro- and amino groups

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Abstract: 2'-modified ribonucleosides (2'-methoxyethoxy- \bigcirc , 2'-fluoro-2'-deoxypyrimidines \bigcirc and 2'-amino-2'-deoxyuridines \square) were introduced into hammerhead ribozymes. Kinetics and stability assays were performed. The lipophilicity characteristics of these 2'-modifications were investigated measuring partition coefficients and HPLC retention times. © 1997 Elsevier Science Ltd.

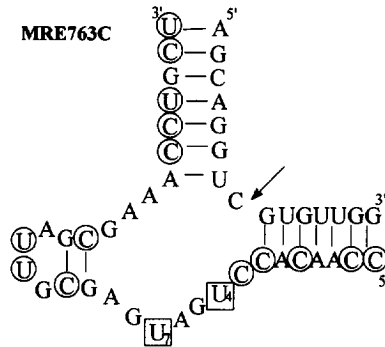
Ribozymes have potential as therapeutic entities for the treatment of e.g. cancer¹⁻³ or HIV⁴. For exogenous delivery into cells they have to be stabilized against nucleolytic degradation without loss of their catalytic efficiency.

Hammerhead ribozymes are catalytic RNA molecules which recognize substrates containing NUH base triplets (N can be any base; H can be A, C, or U) and cleave a phosphodiester bond on the 3'-side of H in *trans* position specifically in the presence of divalent metal ions (e.g. Mg^{2+})⁵⁻⁷. The 2'-position has to be modified as the 2'-hydroxyl group is involved in the degradation of the ribozyme. We examined the influence of chemical modifications such as 2'-methoxyethoxy- and 2'-fluoro-2'-deoxypyrimidine combined with 2'-amino-2'-deoxyuridines in U₄ and U₇ positions on the catalytic activity and stability of hammerhead ribozymes targeted against N-ras transcripts containing point mutations in codon 13. Previous studies^{8,9} showed that replacement of all pyrimidine nucleotides by their 2'-fluoro derivatives in the hammerhead domain except U₄ and U₇ positions demonstrated high cleavage activity. But due to the remaining unmodified ribose units they were degraded in cell culture supernatants containing 10 % FCS in less than 30 minutes. The substitution of the U₄ and U₇ positions in the catalytic core with 2'-amino-2'-deoxyuridine led only to a slight loss of catalytic activity compared to the unmodified one. But this structural change resulted in a remarkable stability against RNases present in the cell culture containing 10% FCS as described by Heidenreich et al.¹⁰. Beigelman et al. obtained also improved cellular stability with 2'-modifications such as 2'-C-allyl-, 2'-O-methyl- and 2'-amino groups¹¹. When we replaced the 2'-hydroxy group by methoxyethoxy in all pyrimidine nucleotides \bigcirc and in the U₄ and U₇ positions \square by 2'-amino-2'-deoxy groups in the ribozyme M3 (Figure 1), half of the cleavage efficiency

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toward the short synthetic oligonucleotides under 'steady-state' conditions was retained compared to the unmodified ribozyme (Table 1).

Figure 1. Design of the modified hammerhead ribozyme



For the 2'-fluoro-2'-deoxypyrimidines \bigcirc and the 2'-amino-2'-deoxyuridines \square modified ribozyme M1 (4- and 7-positions) only 40% of the wildtype activity was preserved. Total substitution, including U₄- and U₇-positions, of the 2'-hydroxy group by 2'-methoxyethoxy pyrimidine in the ribozyme M5 resulted in a complete loss of activity ($k_{\text{cat}}/K_M = 5.2 \cdot 10^{-2} \text{ min}^{-1} \cdot \text{nM}^{-1}$ for the unmodified ribozyme to $2.6 \cdot 10^{-4}$ for the modified ribozyme M5).

Table 1. K_M and k_{cat} values of ribozymes with short synthetic substrates

Ribozyme MRE763C	modification	k_{cat} [min^{-1}]	K_M [nM]	k_{cat}/K_M [$\text{min}^{-1} \cdot \text{nM}^{-1}$]	k_{cat}/K_M (relative)
	unmodified	3.760	72	0.05100	1
M1	FU, FC, U₄U₇-NH₂	0.300	16	0.01900	0.380
M3	MOE-T, MOE-C, U₄U₇-NH₂	0.530	22	0.02400	0.470
M5	MOE-T, MOE-C, U₄U₇-MOE	0.027	105	0.00026	0.005
M7	S, MOE-T, MOE-C, U₄U₇-NH₂	0.220	18	0.01200	0.230

The k_{cat} - and K_M -values were determined under 'steady-state' conditions. Terminal phosphorothioate groups are indicated by an S. The abbreviations MOE-T and MOE-C are 2'-methoxyethoxy-2'-deoxythymidine and 2'-methoxyethoxy-5-methylcytidine.

For further investigations, in order to optimize mRNA-cleavage, the *in vitro* transcribed N-ras RNA, a 849 nucleotide long transcript⁹, served as substrate. The catalytic efficiency was examined under 'single-turnover' conditions (Table 2). Modifications such as 2'-methoxyethoxy-2'-deoxypyrimidine and 2'-amino-2'-deoxyuridines in the ribozyme M3 (U₄- and U₇-positions) led only to a slight loss of activity. The k_{react}/K_M -value is $2370 \text{ s}^{-1} \cdot \text{M}^{-1}$ compared to $3752 \text{ s}^{-1} \cdot \text{M}^{-1}$ of the unmodified ribozyme. The ribozyme M1 containing 2'-amino-2'-deoxyuridines at positions U₄ and U₇ and 2'-fluoro-2'-deoxy groups possesses nearly equal k_{react}/K_M -

values compared to the above mentioned 2'-methoxyethoxy-2'-deoxypyrimidine and 2'-amino-2'-deoxyuridine containing ribozyme M3.

Table 2. k_{react}/K_M -values of different modified ribozymes with *in vitro* transcribed N-ras RNA

Ribozyme MRE763C	modification	k_{react} [10^{-6} s^{-1}]	K_M [nM]	k_{react}/K_M [$\text{s}^{-1} \text{ M}^{-1}$]	k_{react}/K_M (relative)
	unmodified	266	71	3752	1
M1	FU, FC, U ₄ U ₇ -NH ₂	173	71	2437	0.650
M2	MOE-T, MOE-C, U ₄ U ₇ -OH	147	51	2882	0.770
M3	MOE-T, MOE-C, U ₄ U ₇ -NH ₂	173	73	2370	0.630
M4	MOE-T, MOE-C, U ₄ U ₇ -F	39	135	288	0.077
M5	MOE-T, MOE-C, U ₄ U ₇ -MOE	nd	nd	nd	nd
M6	S, MOE-T, MOE-C, U ₄ U ₇ -F	nd	nd	nd	nd
M7	S, MOE-T, MOE-C, U ₄ U ₇ -NH ₂	27	190	142	0.038

The k_{react} - and K_M -values were determined under 'single-turnover' conditions [nd = not detectable].

In Table 3 the stability data of such modified ribozymes are listed. It is remarkable that the 2'-methoxyethoxy modified ribozymes are much more stable in cell culture supernatants supplemented with 10% FCS than the 2'-fluoro-2'-deoxy compound M1. This could have important implications for cell experiments.

Table 3. Stability of chemically modified ribozymes in cell culture supernatant supplemented with 10% FCS

Ribozyme MRE763C	modification	cell supernatants (half-life times)
	unmodified	0.5 min
M1	FU, FC, U ₄ U ₇ -NH ₂	50 h
M2	MOE-T, MOE-C, U ₄ U ₇ -OH	30.0 min
M3	MOE-T, MOE-C, U ₄ U ₇ -NH ₂	80 h
M4	MOE-T, MOE-C, U ₄ U ₇ -F	80 h
M5	MOE-T, MOE-C, U ₄ U ₇ -MOE	80 h
M6	S, MOE-T, MOE-C, U ₄ U ₇ -F	80 h
M7	S, MOE-T, MOE-C, U ₄ U ₇ -NH ₂	80 h

Beside improving the stability, the 2'-methoxyethoxy modifications may have an influence on the lipophilicity of the ribozyme. Increased lipophilic character should facilitate the membrane permeability. The partition coefficients between 1-octanol and water and the retention times of the nucleoside analogues reflect the change in lipophilicity. The data of the 2'-modified nucleosides in Table 4 were determined using 2'-deoxythymidine and uridine as references. Both, the partition coefficients and the retention times, indicate that the modified nucleosides are as lipophilic as 2'-deoxythymidine. The retention times obtained from reversed phase HPLC for

2'-methoxyethoxy nucleoside analogues are the highest of all analogues (Table 4). Altogether, we synthesized highly stable ribozymes with very good catalytic activities and with a higher lipophilicity of the monomers.

Table 4. Partition coefficient and retention time for nucleosides analogues

Nucleoside	partition coefficient P	retention time t_R [min]
2'-Deoxythymidine (dT)	0.060 ^a	n.d.
Uridine (U)	0.016	5.6
2'-Fluoro-2'-deoxyuridine (FU)	0.050	6.6
2'-O-Methyluridine (OMeU)	0.045	6.8
2'-O-Methoxyethoxythymidine (MOE-T)	0.053	7.5

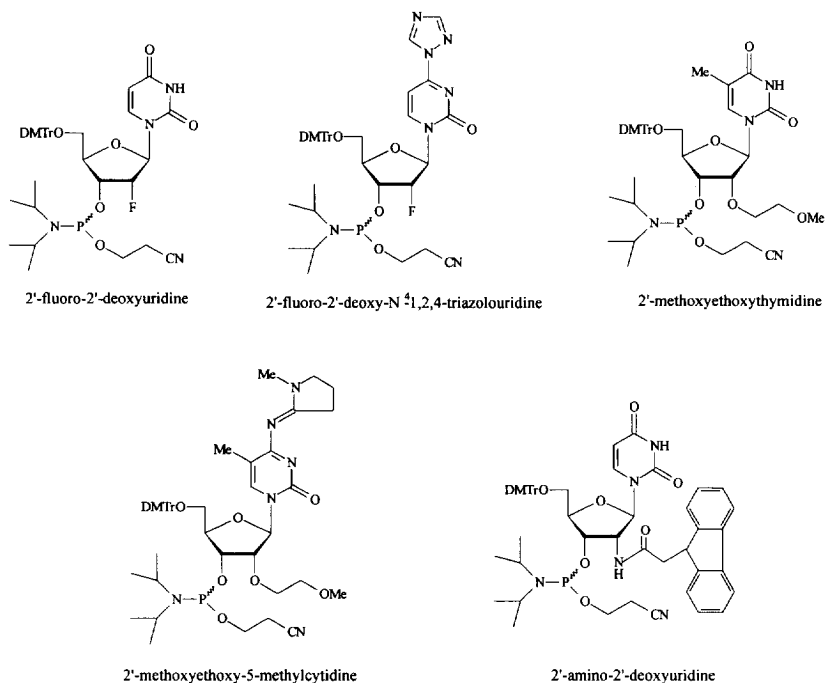
^a The partition coefficient was taken from the paper of Lien¹²; [n.d. = not determined].

Synthesis of the modified nucleotides and oligonucleotides

The 2'-methoxyethoxy modified ribonucleosides¹³ were a kind gift of Dr. P. Martin, Novartis, and the 2'-amino-2'-deoxyuridine was a kind gift of K. Jahn-Hoffmann.

Beside 2'-methoxyethoxy modifications, 2'-fluoro groups were incorporated into the ribozymes. The synthesis of 2'-fluoro-2'-deoxypyrimidine nucleosides and their incorporation into the hammerhead ribozymes together with 2'-methoxyethoxy and 2'-amino-2'-deoxy compounds was much more efficient and gave higher yields than described in Shah¹⁴ (Figure 2). The procedure is described below.

Figure 2. Different 2'-modified ribonucleosides incorporated in oligoribonucleotides



Modified synthesis of 2'-fluoro-2'-deoxyphosphoramidite

Uridine was transformed into 2,2'-anhydro-uridine according to the procedure of Verheyden et al.¹⁵ with HMPT. This was the starting material of the synthesis of 2'-fluoro-2'-deoxyuridine using HF-pyridine as fluorinating agent¹⁶. The 2'-fluoro-2'-deoxyuridine-3'-phosphoramidite was prepared by the method of Sinha¹⁷. The transformation of the 2'-fluoro-2'-deoxyuridine-3'-phosphoramidite to the 2'-fluoro-2'-deoxy-N⁴1,2,4-triazolouridine-3'-phosphoramidite is given below.

5'-O-dimethoxytrityl-2'-fluoro-2'-deoxy-N⁴1,2,4-triazolouridine-3'-O-(O-cyanoethyl-N,N-diisopropylamino)-phosphite

To an ice-cooled stirred suspension of 1,2,4-triazole (1.393 g, 20.2 mmol) in dry acetonitrile (26 ml) ($H_2O < 10$ ppm) was added slowly $POCl_3$ (0.67 g, 4.38 mmol) followed by dry Et_3N (3 ml). After 30 min a solution of 2'-fluoro-2'-deoxyuridine-3'-phosphoramidite (250 mg, 0.33 mmol) in dry acetonitrile (6.6 ml) was added over a period of 15 min. Ice-cooled stirring was continued for 2 h followed by 1.5 h at room temperature. The reaction solution was diluted with methylenechloride (60 ml) and extracted with a saturated $NaHCO_3$ solution (60 ml) and brine (30 ml). The organic layer was dried with Na_2SO_4 and evaporated to dryness. The crude compound was purified by column chromatography (ethyl acetate:hexane 8:2) and can be directly used as cytidine analogue in the oligonucleotide synthesis. Yield: 163 mg, 62%. 1H NMR ($CDCl_3$) (ppm): 9.25 (s, 1H, C-H, 1,2,4-triazole); 8.82 (d, 1H, H-6); 8.08 (s, 1H, C-H 1,2,4-triazole); 7.39-6.82 (m, 13H, arom. H); 6.54 (d, 1H, H-5); 6.25 (d, 1H, H-1'); 5.2 (dd, 1H, H-2'); 4.83-4.71 (m, 1H, H-3'); 4.36 (d, 1H, H-4'); 3.81 (d, 6H, $-OCH_3$); 3.79-3.52 (m, 6H, 2CH, H-5', H-5'', OCH_2); 2.46 (m, 2H, CH_2CN); 1.29-1.13 (m, 12H, $-CH_3$); ^{31}P NMR ($CDCl_3$) (ppm): 152.7 (d, J_{P-F} 5.7 Hz); 151.7 (d, J_{P-F} 9.6 Hz); ESI-MS (+) 799, 32 g/mol (800.9 g/mol); TLC (toluene:ethyl acetate 1:5); RF 0.52 and 0.43 (two diastereoisomers).

The 2'-modified phosphoramidites were introduced into the ribozyme with the aid of an Applied Biosystems 380B DNA Synthesizer on a 1 μ mol scale with final detritylation on the synthesizer. Oligoribonucleotides were synthesized and deprotected using standard protocols with 600 sec. coupling time for the modified phosphoramidites except for the oxidation procedure that was performed using tert.-butylhydroperoxide. The ribozymes were purified by polyacrylamide gel electrophoresis as described previously⁹. RNA concentration was measured by assuming an extinction coefficient at 260 nm of $6.6 \times 10^3 M^{-1} cm^{-1}$ ¹⁸.

The partition coefficients were determined in 1-octanol and water. Retention times were measured on a LiChrospher RP 18 column (3 mm x 125 mm) with acetonitrile/water as eluent. Nucleosides were eluted with a 20 min gradient from 0 to 100 % acetonitrile.

The cleavage kinetics with short synthetic oligoribonucleotide substrate or with the long N-ras transcript were performed as described previously⁹. The single-turnover k_{react}/K_M -values were determined as described previously⁸.

The stability of ribozymes in cell culture supernatants supplemented with 10% FCS was performed as described previously⁹. The degree of RNA digestion was visualized by silver staining and quantified by laser scanning densitometry. Half-life times were determined by the ratio between full-length oligoribonucleotides/degradation products and full-length oligoribonucleotides.

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