Cleavage of yeast tRNA^{Phe} with complementary oligonucleotide conjugated to a small ribonuclease mimic

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Abstract An oligonucleotide conjugate bearing a chemical construct mimicking the catalytic center of ribonuclease A has been designed and studied. The conjugate efficiently cleaves yeast tRNA^{Phe} at a single site adjacent to the target complementary sequence.

Key words: Antisense oligonucleotide; Ribonuclease mimic; tRNA; RNA cleavage

1. Introduction

In the last years a great progress has been made in development of oligonucleotide-based therapeutics targeted to specific RNAs of infectious agents and cellular RNAs encoding disease-causing products [1]. The currently used phosphodiester oligonucleotides and phosphorothioate oligonucleotide analogs trigger cleavage of the target RNAs by cellular enzyme RNase H that attacks the RNA strands in duplexes formed by RNA and DNA. This catalytic reaction provides the basis of efficiency of these oligonucleotides in vivo. Binding of other known oligonucleotide analogs to RNA yields complexes, that are not substrates for RNase H and generally these analogs cannot efficiently arrest functions of target RNAs except when they interfere with specific protein–mRNA interactions [2]. Therefore some alternative approaches for improving antisense efficiency of oligonucleotide analogs are needed.

The efficacy of antisense oligonucleotides can be increased by conjugating them to reactive groups capable of irreversible damaging nucleic acids [3]. Of the reactive groups, particularly promising are those catalyzing hydrolytic cleavage of RNA, because they do not affect biopolymers other than RNA and should be highly efficient due to their catalytic nature [4–6]. Groups catalyzing RNA hydrolysis can be designed by mimicking active centers of ribonucleases. We have demonstrated a possibility to cleave RNA with small molecules containing imidazole residues conjugated by linkers of variable length and flexibility to intercalating dyes [7] and to cationic molecules [8,9]. Conjugation of imidazole-bearing constructs to oligonucleotides was shown to yield molecules cleaving RNA at specific target sequences [10–12]. However efficacy of the described RNA-cleaving oligonucleotide conjugates is rather poor. Reasonable cleavage can be achieved only after long incubation of target RNAs with conjugates in conditions inconsistent with biological experiments.

We have attempted to design improved RNase mimics and optimize the linker structure connecting them to oligonucleotide. We have designed an oligonucleotide conjugate that causes efficient cleavage of specific target sequence in RNA in physiological conditions.

2. Materials and methods

All buffer solutions used in the experiments were prepared using Milli-Q water, contained 1 mM EDTA and were filtered through membrane filters with 0.2 μm pores.

[γ-^{32}P]ATP was from Biosan Co., Russia. Enzymes and chemicals used for end-labeling of RNA and the electrophoretic analysis were as described in [13]. Yeast tRNA^{Phe} isolated by established procedures was a generous gift from Dr. G. Keith (Institute de Biologie Moleculaire et Cellulaire de CNRS, Strasbourg, France).

3′-[^{32}P]tRNA^{Phe} was obtained according to published protocols [14]. 15 μl reaction mixture contained 50 mM HEPES–KOH, pH 7.5; 10 mM $MgCl_2$; 10% DMSO; 0.1 mM ATP; 2 mM DTE; 100 μg/ml BSA; 160 pmol of tRNA^{Phe}; 200 μCi 5′-[^{32}P]pCp and 20 units of T4 RNA ligase. The reaction was performed at 4°C overnight. After labeling, the tRNA was purified by electrophoresis in 12% polyacrylamide–8 M urea gel. The labeled RNA was eluted from the gel by 0.5 M ammonium acetate containing 1.0 mM EDTA and 0.1% SDS. After ethanol precipitation, the tRNA was dissolved in water and stored at −20°C. The specific activity of the obtained [^{32}P]tRNA^{Phe} was 5×10^5 cpm/pmol.

2.1. Synthesis of the oligonucleotide conjugated to RNA-cleaving bis-imidazole construct (2B-R) (Scheme 1)

Oligonucleotide GATCGAACACAGGACCT (further in the text 2B) was synthesized by using the phosphotriester method [15]. The synthesis of RNA cleaving histamine-β-alanyl-histidyl-capromayl-ω-maleimide (compound **2** in Scheme 1) will be published elsewhere. The 9-aminomethylanthracene was attached to the terminal 5′-phosphate of oligonucleotide that had been activated with a mixture of triphenylphosphine (Ph_3P) and 2,2′-dipyridyl disulfide ($PyS)_2$ in the presence of 4-(*N*,*N*-dimethylamino)pyridine (DMAP) [16]. Shortly, the reaction mixture containing 0.5 μmol of the *N*-cetyl-*N*,*N*,*N*-trimethylammonium salt of the oligonucleotide, 15 μmol of Ph_3P, 15 μmol of $(PyS)_2$ and 30 μmol of DMAP in 0.1 ml dimethylformamide (DMFA) was incubated for 12 min at room temperature. Then 30 μmol of 9-(aminomethyl)anthracene in 20 μl of DMFA was added and the solution was incubated for 1 h at 25°C. The anthracene derivative of oligodeoxyribonucleotide (**1**) was precipitated with 2% $LiClO_4$ in acetone. Purification of the anthracene oligonucleotide derivative (**1**) was performed by reversed-phase HPLC using a LiChrosorb RP-18, 10 μm (Merck, FRG), 4.6×250 mm column, a Waters 600E chromatograph and a Waters 484 tunable absorbance detector (USA). A linear gradient (2 ml/min) from 0 to 30% of acetonitrile in 0.05 M $LiClO_4$ pH 7.5 was used. Lithium salt of anthracene oligonucleotide derivatives (**1**) (0.2–0.5 μmol) was dissolved in 30 μl of H_2O and 25 μmol of the compound **2** in 10 μl DMFA was added. The reaction mixture was incubated for 2 h at 50°C and oligonucleotide conjugate **3** (further in the text named as 2B-R) was precipitated with

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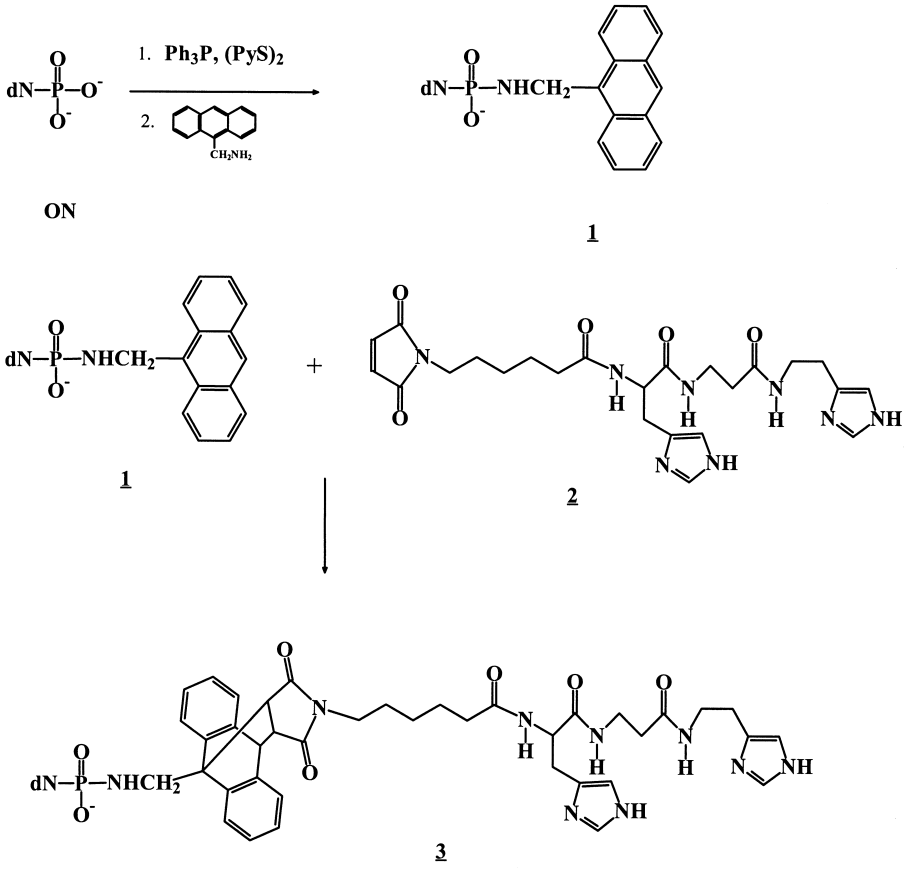


Scheme 1. Conjugation of the *bis*-imidazole construct to the oligonucleotide.

2% $LiClO_4$ in acetone and purified by reversed-phase HPLC in the same conditions.

2.2. Hydrolysis of tRNA^{Phe} by oligonucleotide conjugate 2B-R

Reaction mixtures (10 μl) contained 50 mM imidazole buffer, pH 7.0, 200 mM KCl, 1 mM EDTA, 100 μg/ml tRNA carrier (total tRNA from *Escherichia coli*), 5×10^{-7} M 3′-[^{32}P]tRNA^{Phe} and oligonucleotide conjugate 2B-R at concentrations ranging from 5×10^{-7} M to 7×10^{-4} M as indicated in the legends to the figures. The reaction mixtures were incubated at 37°C. The reactions were quenched by precipitation of tRNA with 150 ml of 2% lithium perchlorate solution in acetone. RNA was collected by centrifugation and dissolved in the loading buffer (6 M urea, 0.025% bromophenol blue, 0.025% xylene cyanol). The cleavage products were analyzed by electrophoresis in 12% polyacrylamide–8 M urea gel. To identify the cleavage sites, imidazole ladder and partial T1 RNase digest of the tRNA were run in parallel with the cleavage products. To get quantitative data, the gels were dried, radioactive bands were cut out of the gel and their radioactivity was determined by Cherenkov's counting.

2.3. Inhibition of the tRNA^{Phe} cleavage by oligonucleotides

The following oligonucleotides were used as inhibitors:

1A	GGTGCGAATTCTG	(complementary sequence is 63–76)
1B	TGGTGCGAATTCTG	(64–76)
1D	TGGTGCGAATTCTGT	(62–76)
2A	GGATCGAACACAGGACCT	(44–61)
2B	GATCGAACACAGGACCT	(44–60)

In the competition experiments, standard reaction mixture (volume 10 μl, containing 50 mM imidazole buffer, pH 7.0, 200 mM KCl, 1 mM EDTA, 100 μg/ml tRNA carrier, 5×10^{-7} M 3′-[^{32}P]tRNA^{Phe}) lacking 2B-R and supplemented with one of the oligonucleotide competitors at a concentration of 5×10^{-5} M was incubated for 10 min at 37°C. After the incubation, the oligonucleotide conjugate 2B-R was added up to a concentration of 5×10^{-6} M and the reaction was incubated at 37°C for different times (from 30 min to 2 h). The reaction was quenched and analyzed as described above.

3. Results and discussion

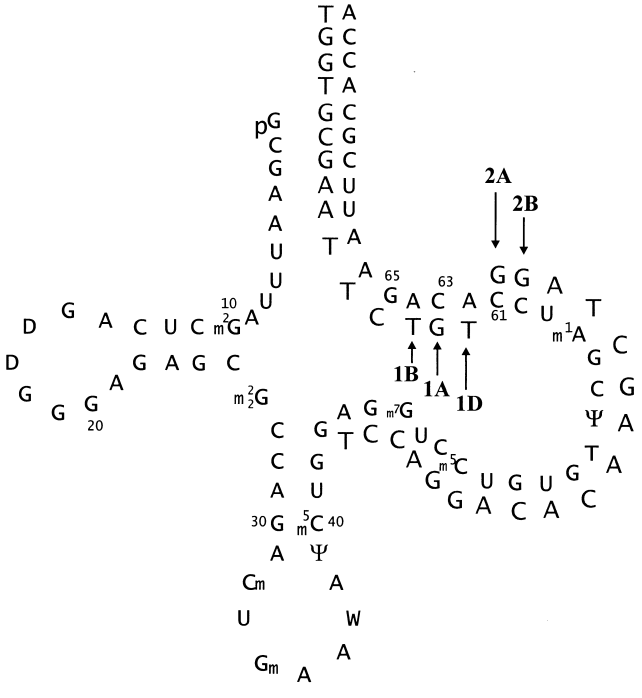
The synthesis of oligonucleotide conjugate is presented in Scheme 1. Anthracene derivative of oligonucleotide was synthesized by condensation of activated 5′-phosphate of oligonucleotide with 9-(aminomethyl)anthracene. Ph_3P and 2,2′-dipyridyl-disulfide in the presence of DMAP activate exclusively the terminal phosphate group of oligonucleotide without interacting with internucleotide phosphate groups and heterocyclic bases [16]. The derivatized oligonucleotide is eluted from the HPLC columns after the parent oligonucleotide (The conditions of HPLC isolation are indicated in Section 2). In the course of anion-exchange chromatography (Polisil-SA 15 μm, 2.5×30 mm column with a linear gradient of K_2HPO_4/KH_2PO_4, pH 7.0; 0–0.8 M, flow rate = 50 μl/min was employed) the oligonucleotide derivative is eluted as compound having one negative charge less than the parent oligonucleotide, in accordance with the expected behavior of the structure in which the oligonucleotide and anthracene residue are connected by a phosphoramide bond. The UV absorption spectra of the conjugate have a λ_{max} at 388 nm, characteristic of the connected anthracene residue [17]. Incubation of **1** with **2** yielded the conjugate, the cycloaddition product **3** (2B-R). The isolated compound was homogeneous according to the

data of anion-exchange and reversed-phase chromatography. The yield was 85–90%.

Earlier we have shown, that oligonucleotides conjugated to constructs with imidazole groups can bind to complementary sequences within the T-loop of $tRNA^{Phe}$ and cleave the molecule at the target sequence CA [12]. However detailed investigation of the process has shown that the conjugates were unstable in the experimental conditions. Hydrolysis of the phosphoramide bond connecting the catalytic group and the oligonucleotide occurred, apparently due to intramolecular catalysis by the imidazoles juxtaposed to the linkage. As a result, efficiency of the RNA cleavage by the conjugates was decreased and varied in different reaction conditions. In the conjugate described in the present paper, a bulky rigid spacer structure separates the phosphoramide bond and the catalytic group and interferes with the intramolecular reaction.

Oligonucleotide 2B is complementary to sequence 44–60 in the $tRNA^{Phe}$ including the variable loop and part of the T-arm of the tRNA (Fig. 1). Earlier it was found, that 2B can bind to the tRNA in physiological conditions [18]. The RNA cleaving group (compound **2**) conjugated to the 5′-terminal phosphate of the oligonucleotide via a linker can reach the sequence CACA that is particularly susceptible to cleavage by small ribonuclease mimics [8]. Fig. 2 displays an autoradiograph of the cleavage patterns of the 3′-end-labeled $tRNA^{Phe}$ by the oligonucleotide conjugate 2B-R. Spontaneous degradation of tRNA in these conditions in the absence of the conjugate, in the presence of the parent non-modified oligonucleotide and non-linked small ribonuclease mimic does not occur (Fig. 2). It is seen, that the major cleavage (~90%) occurs within the C63–A64 sequence (Figs. 1 and 2). Besides there appears a few faint cleavages within C61–A62, U8–A9 and C75–C76. The ratios between tRNA cleavage products at C63–A64 and C61–A62 sequences adjacent to each other are probably consistent with the sterical availability of A61–C62, A63–C64 phosphodiester bonds towards hydrolysis by 2B-R. Sequences U8–A9 and C75–A76 are highly sensitive to the cleavage by RNase A and imidazole containing constructs [5,9]. Therefore, the faint cleavage within these sites might result either from sequence specific cleavage (due to the length of the reactive part of 2B-R) or tRNA spontaneous cleavage in heteroduplex with the oligonucleotide conjugate. RNA is cleaved by conjugate 2B-R very rapidly. At conjugate concentration 10 μM, providing efficiency of the complex formation, the RNA is cleaved predominantly at the C63–A64 bond with a halflife time of approximately 30 min, and at [2B-R] = 50 μM the tRNA halflife time is measured by minutes (Fig. 2).

To prove the affinity character of the cleavage, the reaction was performed in the presence of oligonucleotide inhibitors (Fig. 3). As expected, the reaction was suppressed by the oligonucleotides complementary to the target sequence (2A



3' terminal tRNA sequence

3'-ACCACGCUUAAGACACCUAGCΨTGUGUCCUGGA-5'

1B TGGTGCGAATTCT
1A TGGTGCGAATTCTG
1D TGGTGCGAATTCTGT
2A GGATCGAACACAGGACCT
2B GATCGAACACAGGACCT

Fig. 1. Yeast $tRNA^{Phe}$: cloverleaf structure with indicated target sites for oligonucleotides 1B, 1A, 1D, 2A, 2B. Sequences of the oligonucleotides are presented at the bottom.

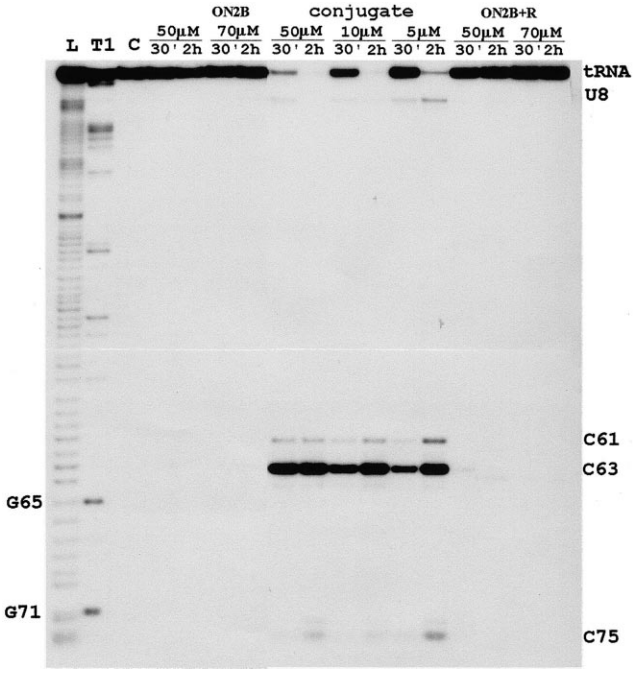


Fig. 2. Cleavage of 3′-end-labeled yeast $tRNA^{Phe}$ with oligonucleotide conjugate 2B-R. Autoradiograph of 12% polyacrylamide–8 M urea gel after analysis the products of $tRNA^{Phe}$ cleavage with 2B-R. Cleavage conditions: 37°C, 50 mM imidazole, pH 7.0, 200 mM KCl, 0.1 mM EDTA, 100 μg/ml of tRNA carrier, 5×10^{-7} M 3′-[^{32}P]$tRNA^{Phe}$. Lanes L and T1 represent the imidazole ladder and partial $tRNA^{Phe}$ digestion with RNase T1; lane C, incubation of $tRNA^{Phe}$ without oligonucleotides. Lanes ON2B, incubation of $tRNA^{Phe}$ in the presence of oligonucleotide 2B; lanes Conjugate, incubation of $tRNA^{Phe}$ in the presence of oligonucleotide conjugate 2B-R and lanes ON2B+R, $tRNA^{Phe}$ incubated in the presence of equimolar mixture of oligonucleotide 2B and compound **2** (see Scheme 1). Reaction time and oligonucleotide concentrations are indicated on the top of the gel. The cleavage positions are indicated on the right.

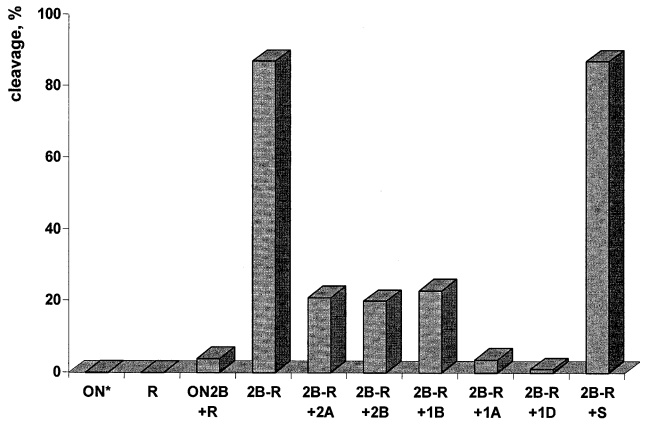


Fig. 3. Effect of oligonucleotide competitors on the cleavage of 3′-end-labeled yeast $tRNA^{Phe}$ with oligonucleotide conjugate 2B-R. Cleavage conditions were: 37°C, 2 h, 50 mM imidazole, pH 7.0, 200 mM KCl, 1 mM EDTA, 100 μg/ml tRNA carrier, 5×10^{-7} M 3′-[^{32}P]$tRNA^{Phe}$; the concentration of 2B-R was 10 μM, concentrations of oligonucleotides 2A, 2B, 1B, 1A, 1D and S (scrambled oligonucleotide) were 100 μM. R – incubation of tRNA in the presence of compound **2**, ON* – incubation of $tRNA^{Phe}$ in the presence of 100 μM of one of the oligonucleotides 2A, 2B, 1B, 1A, 1D or S.

and 2B) and by oligonucleotides forming duplex with the sequence to be cleaved (1A and 1D). Oligonucleotide 1B forms a duplex with the sequence adjacent to the linkage C63–A64 to be cleaved and partially suppress the reaction, apparently because the adjacent duplex reduced the flexibility of the internucleotide linkage, needed for the transesterification process.

Sequence specific hydrolysis of RNA was achieved using oligonucleotides conjugated to groups with chelated metal ions (for reviews see [4,5]). The most promising results have been obtained with terpyridyl–Cu(II) [19], the iminodiacetate–Lu(II) complex [20,21] and terpyridine-derived europium complexes [22,23]. In the last case cleavage efficiency compatible with potential biological applications was observed. However, possibilities of using the conjugates with chelated metal ions are limited by relative instability of the complexes in in vivo conditions. Attempts have been made to design RNA cleaving oligonucleotide conjugates with small organic RNA cleaving groups. Cleaving of RNA was shown to occur in complexes of RNA with complementary oligonucleotides bearing an ethylenediamine residue [24–27], alternating amino acids with basic and hydrophobic side chains [28] and constructs with imidazole groups [10–12,29,30].

The oligonucleotide conjugate described in the present paper demonstrates high cleavage efficiency. In optimal conditions, the halflife time of the RNA complexed to conjugate 2BR is measured by minutes. This efficiency is achieved by design of the structure of the RNA cleaving group and by choice of the target RNA sequence that is particularly sensitive to RNase mimics. Further investigation of factors affecting the susceptibility of phosphodiester bonds to hydrolyzing agents and optimization of structure of the catalytic group will lead to design of efficient RNA-cleaving oligonucleotide conjugates that will find applications in development of antisense therapeutics.

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