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Modified RNA sequence pools for *in vitro* selection

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ABSTRACT

We report the use of modified RNA, in which the 2′-OH group of pyrimidines is replaced by a 2′-amino (2′-NH_2) group to identify high affinity ligands specific for human neutrophil elastase (HNE) by *in vitro* selection. Compared to unmodified RNA the 2′-NH_2-modified RNA ligands show enhanced stability in human serum and urine. Use of RNase T1 cleavage data in the presence of K^+ and Li^+ ions suggests that the modified RNA ligands selected for HNE form an intermolecular G-quartet structure.

INTRODUCTION

Due to the propensity of oligonucleotides to assume a wide range of structures such as pseudoknots, stem loops, bent helices, bulges, triplexes and quadruplexes, a random sequence pool of oligonucleotides represents a library of molecules with different shapes. Systematic Evolution of Ligands by EXponential enrichment (SELEX) is an *in vitro* selection technique for isolating high affinity molecules (ligands) to a target from such a library of oligonucleotides (1,2). *In vitro* selection techniques have permitted the isolation of nucleic acid molecules with high-affinity binding for a wide array of targets, ranging from large proteins (1,3–8) to small molecules (9–12). In many cases, these high affinity ligands also inhibit certain biological properties of their target proteins (3, 6,7). In addition, selection–amplification techniques have also been used to alter the activities of existing ribozymes (13–15), and to select for RNA molecules with novel catalytic properties (16–18). Identification of pharmaceutically relavent nucleic acid molecules by *in vitro* selection will demand nucleic acids that are stable in biological fluids. Hence, making such ligands nuclease-resistant to increase their survival time in biological fluids is essential for nucleic acid-based therapeutic and diagnostic agents.

Modifications to a ligand after *in vitro* selection could alter the shape and/or the stereoelectronic properties which in turn will change high-affinity binding. One approach for obtaining modified oligonucleotide ligands with high-affinity binding is to initiate the SELEX process with a pool of modified oligonucleotides. Unnatural functional groups can be introduced to several positions in a nucleotide. To be successful in SELEX, however, such modifications must be compatible with the enzymes used in this procedure. In that regard the 2′ position of the sugar and the C-5 position of pyrimidines are attractive positions for modifications. Pyrimidines with different groups such as biotin or 1,10-phenanthroline attached to the C-5 position of pyrimidines have been shown to be enzymatically incorporated into RNA and DNA (19,20). Such substitutions are not limited to pyrimidines (21). Various modifications at the 2′ position of the sugar moiety have also been described (22–24). The substitution of 2′-OH group of pyrimidines by 2′-NH_2 group have been shown to render RNA resistant to pyrimidine-specific endonucleases (22,25).

In this study we describe the isolation of 2′-NH_2-modified RNA ligands (Fig. 1a) for human neutrophil elastase (HNE). Excess HNE activity is implicated in several disease states such as pulmonary emphysema, adult respiratory distress syndrome, chronic bronchitis and cystic fibrosis (26–30). Therefore, the development of specific inhibitors to HNE could have therapeutic value.

MATERIALS AND METHODS

HNE was obtained from Athens Research and Technology (Athens, Georgia). N-methoxysuccinyl Ala-Ala-Pro-Val-paranitroanilide, N-methoxy-succinyl Ala-Ala-Pro-Val-chloromethyl ketone and pooled human serum were obtained from Sigma. An improved protocol has been used for the synthesis of 2′-NH_2-UTP and 2′-NH_2-CTP (31).

SELEX

A detailed procedure of SELEX has been previously described (1). Five nanomoles of the synthetic DNA template [5′-GCCGG ATCCGGGCCTCATGTCGAA-$(N)_{40}$-TTGAGCGTTTATT-CTGAGCTCCC-3′] was amplified by the polymerase chain reaction (PCR) with 5′-CCGAAGCTTAATACGACTCAC-TATAGGGAGCTCAGAATAAACGCTCAA-3′ and 5′-G-CC-GGATCCGGGCCTCATGTCGAA-3′ as primers. The PCR products were used as a template for transcription *in vitro* by T7 RNA polymerase in a buffer containing 2 mM each of ATP, GTP, 2′-NH_2CTP and 2′-NH_2UTP, 40 mM Tris–HCl (pH 8.0), 12 mM $MgCl_2$, 1 mM spermidine, 5 mM DTT, 0.002% Triton X-100 and 4% polyethelene glycol (w/v) at room temperature for 4 h. Typically 100 pmoles of template was used in 1 mL reaction volume with 100 U of polymerase. The full-length transcripts were gel purified, resuspended in binding buffer [150 mM NaCl, 100 mM Tris–HCl (pH 7.0), 2 mM $MgCl_2$ and 6 mM KCl], heated to 70°C for 3 min, chilled on ice, and

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incubated with HNE at 37°C for 10 min. The protein–RNA mixture was then filtered through a prewet nitrocellulose filter followed by a 5 mL wash of binding buffer. The filter-retained RNAs were recovered, reverse transcribed by avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences) at 48°C for 45 min. The resulting cDNA was amplified by PCR and used as the template to obtain RNA for the next round of selection. To avoid the selection and amplification of undesired nitrocellulose binding RNA molecules, the newly transcribed RNA pool was passed through several layers of nitrocellulose filters (negative selection) before the next round of selection with HNE. To enrich high affinity RNA selective pressure was applied by decreasing the HNE concentration as the number of rounds advanced.

Determination of equilibrium dissociation constants

For these experiments internally radiolabeled RNAs were synthesized by *in vitro* transcription with ^{32}P-α-ATP. Gel-purified radiolabeled transcript obtained from a given clone (< 1 pM) were incubated with varying amounts of HNE in binding buffer containing 0.02% HSA (human serum albumin) for 10 min at 37°C. RNA-protein mixtures were filtered through prewet nitrocellulose filters (0.2 μ) followed by a 5 mL wash of binding buffer. The radioactivity retained on the filters was counted. Background binding of RNA to filters in the absence of HNE was also determined and was used for background correction. Assuming equimolar binding of RNA to HNE nonlinear least square method was used to fit the data by using the software package Kaleidagraph (Synergy Software, Reading, PA) to obtain equilibrium dissociation constant K_d (7, 32).

RNase T1 cleavage

Gel-purified RNAs were dephosphorylated with bacterial alkaline phosphatase, and treated with ^{32}P-γ-ATP and T4 polynucleotide kinase. The 5'-labeled RNA (~ 1 pmole) was resuspended in 20 μL of either KCl buffer [150 mM KCl and 100 mM Tris–HCl (pH 7.0)] or LiCl buffer [150 mM LiCl and 100 mM Tris–HCl (pH 7.0)], heated to 70°C, chilled on ice and transfered to ambient temperature before 0.2 U of RNase T1 was added. The cleavage reaction was proceeded for 10 or 20 min at ambient temperature and stopped by adding tRNA followed by ethanol precipitation. Ethanol precipitated RNA was run on 10% polyacrylamide sequencing gels, and gels were autoradiographed.

Native gel electrophoresis

Labeled RNA (~ 1 pmole) was resuspended either in KCl buffer or in LiCl buffer, heated to 70°C, chilled on ice and loaded onto a 6% native polyacrylamide gel polymerized in standard TBE buffer mixed with the appropriate monovalent cation (50 mM). Gels were run at room temperature for 10 hr at ~ 10 mA in standard TBE buffer in the presence of either 50 mM KCl or 50 mM LiCl.

Stability in biological fluids

One picomole of internally radiolabeled RNA (either 2'-NH_2-modified ligand or unmodified RNA of the same sequence) in 5 μL was incubated with 95 μL of either human serum or human urine at 37°C for indicated period of time. Samples were extracted with phenol and chloroform and RNA was precipitated with ethanol after adding tRNA as a carrier. RNA samples were then run on denaturing 8% polyacrylamide gels and the radioactivity of the full-length RNA was quantitated by using Fuji phosphorimager. The following relationship was used to fit the data by using Kaleidagraph software package.

$$signal = A\ e^{-kt} + C$$

where *signal* is the % full-length RNA recovered at a given time (t), $-k$ is first order rate constant, A is the intercept on y axis and C is a constant value at time infinity.

Enzyme activity assay

Enzyme activity assays were carried out in 96-well microtiter plates containing 200 μL of binding buffer [0.01% Triton X-100, 0.05% DMSO, 0.02% HSA, and 1 mM N-methoxy succinyl-Ala-Ala-Pro-Val-paranitroanilide as the substrate], and a given RNA ligand at various concentrations. At time zero, HNE was added with mixing to 10 nM final concentration, and the absorbance at 405 nm was determined at every 5 min in BioTek microtiter plate reader.

RESULTS

For each round the SELEX process requires transcription of RNA, isolation and reverse transcription of the target-bound RNA and PCR amplification of the cDNA. In order to introduce modifications to RNA, a modified nucleotide triphosphate must be a substrate for T7 RNA polymerase. The modified RNA, on the other hand, must be a substrate for reverse transcriptase for cDNA synthesis. We observed that the use of 2'-NH_2-UTP and 2'-NH_2-CTP in place of UTP and CTP in the transcription reaction gave full-length transcripts with ~50 % yield relative to normal 2'-OH transcripts. The observed decreased yield associated with the 2'-NH_2-modified transcripts was shown to be due to an increase in abortive initiation. In later experiments yields comparable to normal 2'-OH transcripts were obtained by excluding pyrimidines in the initial 12 nucleotides of the transcript. By using radiolabeled primers the yield of cDNA made from the 2'-NH_2-modified RNA was found to be comparable to that obtained from normal 2'-OH RNA (data not shown). These results indicated the compatibility of the 2'-NH_2-modified pyrimidines in the SELEX procedure.

The selection of high-affinity binding ligands for HNE was started from 2'-NH_2-modified random sequence pool of RNA

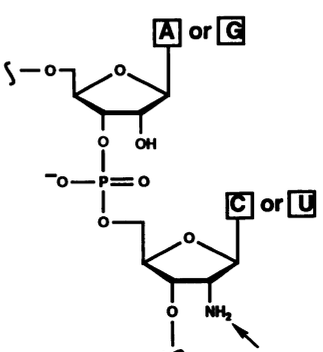

Figure 1. Schematic view of 2'-NH_2-modified RNA used in the study. In RNA molecules pyrimidines (C: cytosine and U: uracil) contain 2'-NH_2-modified ribose sugar whereas purines (A: adenine and G: guanine) contain natural ribose sugar.

with a complexity of $\sim 10^{14}$ different sequences. The selection procedure was based on the separation of HNE-bound RNA molecules on nitrocellulose filters, and subsequent amplification of bound RNAs by reverse transcription, PCR and *in vitro* transcription. After fifteen rounds, the K_d of the enriched pool toward HNE was about 70 nM compared to the unselected random pool which had an apparent K_d of 1 μM. At this point, PCR-amplified DNA was cloned and sequenced. Selected RNA sequences contained more or less equal proportions of ribopurine and 2'-NH_2-pyrimidine residues (Table 1), indicating the absence of a bias in favor of purine ribonucleotides.

The K_ds of several different representative RNA ligands from each class for HNE was determined by nitrocellulose filter binding (Table I). Overall, selected ligands showed high-affinity binding for HNE, with certain ligands having K_ds as low as 7 nM. RNA ligands selected for HNE did not show high-affinity binding to basic fibroblast growth factor and thrombin, (K_ds were > 1 μM) indicating that these ligands are specific for HNE. The lack of high-affinity binding to a closely related protease, porcine pancreatic elastase (Figure 2, squares), which is $\sim 43\%$ homologous to HNE with residues involved in peptide cleavage strictly conserved (33), further reinforces the specificity of SELEX-derived ligands.

The requirement of the 2'-NH_2 group for high-affinity binding was tested by synthesizing the 2'-OH transcripts and the ssDNA versions of several ligands. The binding of 2'-OH version of ligand 34 (triangles) in comparision with that of its 2'-NH_2 form (circles) is shown in Figure 2. Both ssDNA and 2'-OH RNA exhibited relatively low affinities toward HNE (K_ds were > 1 μM), suggesting that the presence of the 2'-NH_2 groups is critical for high-affinity binding to HNE.

The majority of selected sequences have a string of seven consecutive guanines. The presence of G-rich sequences in all three classes may allow RNA molecules to form G-quartet structures. In addition, some members of the Class I have the potential to form a stem-loop structure as illustrated in Figure 3a. Even though 2'-NH_2-modified RNA molecules are resistant to ribonuclease A (RNase A) (22), a purine-specific RNase such as RNase T1 (isolated from Aspergillus oryzae) can cleave modified RNAs at unmodified guanine residues.

The RNase T1 cleavage patterns of two ligands (34 and 19) are shown in Figure 3b. In LiCl buffer (lanes 2 and 3) the enzyme cleaves RNA virtually at all guanines, an expected cleavage pattern for RNase T1. The cleavage pattern in LiCl is very much similar to that observed in standard RNase T1 buffer (7 M urea, 20 mM sodium citrate, 1 mM EDTA, pH 5.0) (data not shown). However, in KCl buffer certain guanines in both sequences showed resistance toward RNase T1 (lanes 4 and 5). These guanines include the seven contiguous guanines (G_7) and the adjacent three guanines (G_3) toward the 5' end of the two sequences. The observed pattern of RNAse T1 cleavage data does not support either the stem-loop structure (Figure 3a-I) or the intramolecular G-quartet structure (Figure 3a-II) containing several unstructured guanines of the G_7 stretch which should be cleaved by RNase T1. The RNase T1 resistance of G_7 and G_3 in KCl could be due to their participation in a structure stabilized by K^+ ions. The G-quartet structure is known to be stabilized by K^+ ions, but not by Li^+ ions (34–37). It is therefore likely that the RNA ligands form an intermolecular G-quartet structure as illustrated in Figure 3a-III. In sequence 34, the single guanine residue that lies between G_7 and G_3 remained sensitive to RNase T1 in both LiCl and KCl buffers (Figure 3b-I), suggesting that it is not involved in a G-quartet, as expected. Thus G_7 and G_3 represent two G-quartet forming motifs. Formation of an U-quartet at the 3' end of a stable G-quartet has been reported recently (38). There are U residues at both ends of the G_7 stretch in sequence 34 and therefore the formation of a U-quartet is also a possibility.

Both RNA sequences showed multiple bands with retarded gel mobility when ran on native gels in the presence of KCl indicating intermolecular complex formation. The same RNA sequences ran as monomers in a native LiCl gel, suggesting that Li^+ ions do not favor intermolecular complex formation (data not shown). Both native gel electrophoresis and RNase T1 cleavage data therefore support the formation of intermolecular complexes

Table I. Sequences derived from HNE selection and K_d values of representative sequences

	Clone Number	Sequence (5'-3')	Kd (nM)
Class I	(10) 30	GAGUAGGCUUGACGCCUGGGGGGGUAUGGCUUCGACUGCG	10 ± 2
	44	GAGUAGGCUUGACGCUGGGGGGGUAUGGCUUCGACUUGCG	
	16	AGAGGUAGGCUUGACGCCUGGGGGGGUAUGGCUCCGACUGCG	
	2	GGCAGACAUACGCCUUGGGGGGGUGUCAGGGUAUAUGGCA	
	14	GGCAGACAUACGCCUUGGGGGGGUUUCAGGGUAUAUGGCA	17 ± 1
	(2) 21	GGUAGACAUACGCCUUGGGGGGGUGUCAGGGUAUAUGGUA	
	24	GGUAGACAUACGCCUUGGGGGGGUAUCAGGGUAUAUGGUA	
	34	GGUAGACAUACGCCUUGGGGGGGUGCCAGGGUAUAUGGUA	7 ± 2
	57	GGCAGACAUACGCCUUGGGGGGGUGUCAGGGUAUAUGGUA	
	(3) 19	UGCCCGGCAGUACUGCACGGCCUCGGGGGGGACCAGGGAG	7 ± 1
	28	AUCGGAUAGCGCCGCGAUGGCGUCUGGGGGGGACCAGGGA	
	46	UGGAUAGCGCUGCGUGACCGCGCUGGGGGGGUUGGCAGGG	12 ± 3
Class II	6	GAAUGAAUGUGAUGAACAGGUGUCGGGGUGGAUGGGUGG	
	9	GGAAUGAAUGUGAUGAACAGGUGUCGGGGUGGAUGGGUGG	9 ± 2
	(2) 45	AGAAUGAAUGUGAUGAACAGGUGUCGGGGUGGAUGGGUGG	
	26	CGCCUUAGUCGCCAUAAUGCUGUCGGGGUGGAUAGGGUGG	
	11	GACGCGUGCUGGCCUCGACCGUGUGGGGUGCGGAUGGGUGG	
Class III	4.	CGGAUCGGGCGGUCGUCUAGCGGGAUGGCGUGCGGGUGGA	
	35	GUACUACUGCAAGCCCGUGUGGCGCGGUCAGUGGGUGGCC	
	50	UGACCGGGUCGACAUGCCUGAGGUGUGGCCAGUGGGUGG	
	22	GGCCCUCUGAACCGUCUGGGCGUGGUUAGUGGGUGGC	24 ± 4
	25	UGACCGGGCCGACAUGCUUGAGGUGUGGCCCAGUGGGUGG	
	60	GUACUACUGCAAGCCCGUGUGGCGCGGUCAGUGGGUGGC	28 ± 8
		5'-GGGAGCUCAGAAUAAACGCUCAA- -UUGAGCGUUUAUUCUGAGCUCCCGGAUCCGGC-3'	

Sequences are aligned to show sequence homology. The number of clones carrying the same sequence is shown in parentheses. The bold sequence shown at the bottom indicates 5'- and 3'-fixed sequences.

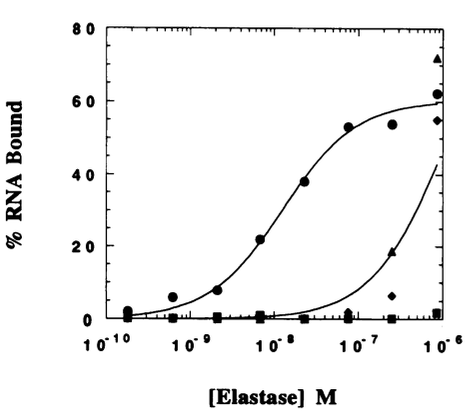

Figure 2. A representative set of binding curves for HNE binding. Data was obtained by nitrocellulose filter binding as described in Materials and Methods. Circles, binding of 2'-NH_2-modified RNA sequence 34 to HNE; squares, binding of 2'-NH_2-modified RNA sequence 34 to porcine pancreatic elastase; triangles, binding of 2'-OH version of the sequence 34 to HNE; diamonds, 2'-NH_2-modified random pool binding to HNE. For clarity, curve fitting is shown only for sequence 34 and random pool binding to HNE.

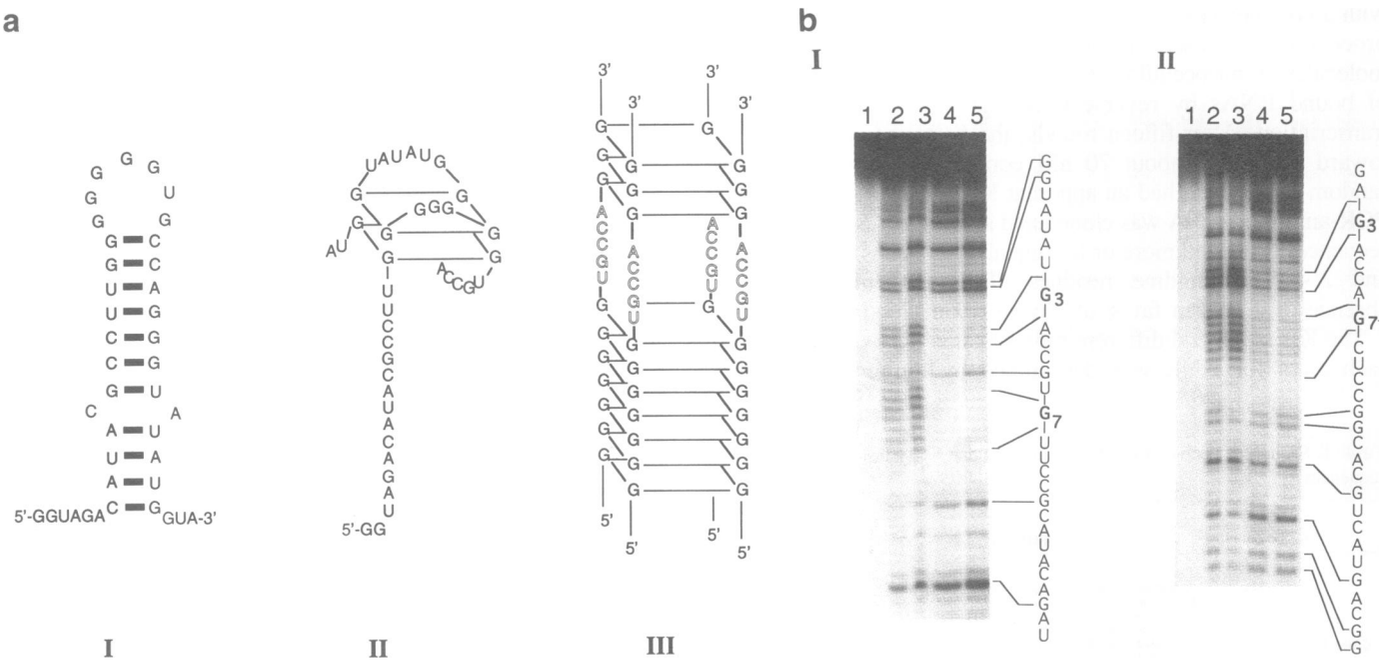

Figure 3. (a) Possible secondary structures (I. the stem-loop structure; II. an intramolecular G-quartet; III. intermolecular G-quartet) for sequence 34 belonging to class I. The intramolecular G-quartet structure illustrates one of several possible intramolecular G-quartets available for the sequence. Outlined letters indicate connecting nucleotides between the two possible G-quartet motifs. (b) RNase T1 cleavage patterns of the sequence 34 (I) and the sequence 19 (II). Untreated RNA (lane 1); cleavage in 150 mM LiCl buffer for 10 min (lane 2) and for 20 min (lane 3); cleavage in 150 mM KCl buffer for 10 min (lane 4) and for 20 min (lane 5).

stabilized by G-quartet structure as illustrated in Figure 3a-III in these 2′-NH_2-modified RNA ligands.

To determine whether the putative G-quartet structure is involved in high-affinity binding to HNE, filter binding was carried out in KCl and LiCl buffers [150 mM KCl or LiCl, 100 mM Tris−HCl (pH 7.0), 2 mM Mg Cl_2]. RNA ligands bound with high affinity in KCl buffer but not in LiCl buffer. For example, the K_ds of the sequence 34 were 6 ± 3 nM in KCl buffer and 670 ± 410 nM in LiCl buffer. The affinity in KCl buffer is more than 100-fold higher than that in LiCl buffer. The potassium-dependant ligand affinity suggests that the proposed G-quartet is the likely structure responsible for high-affinity binding to HNE.

To be useful for therapeutic and diagnostic agents SELEX-derived ligands must have sufficient stability in biological fluids. RNA modified with 2′-NH_2-UTP has been shown to be 10^6-fold resistant to RNAse A as compared to unmodified RNA (22). The stability is expected to be even higher when both pyrimidines were modified at the 2′-position as studied here. We used human urine and serum to test and compare the stability of 2′-NH_2-modified RNA with that of unmodified natural RNA. The results of this experiment are shown in Figure 4. The unmodified RNA is rapidly degraded in both serum and urine (panel a). The half life of unmodified RNA in urine was estimated as ≤ 8 min whereas that value in serum was ≤ 4 min. As compared to unmodified RNA, the 2′-NH_2-modified RNA is significantly stable in both fluids (panel b). The observed half lifes of modified RNA in serum and urine were 20.2 ± 3.3 and 9.3 ± 1.8 h, respectively.

The pK_a of the 2′-NH_2 group is 6.2 (39, 40) and therefore at physiological pH (7.35−7.45), an RNA molecule consisting of 2′-NH_2-modified pyrimidines contains both protonated and unprotonated 2′-NH_2 groups. Consequently, it is likely that small changes in pH can affect the degree of protonation of a ligand, which in turn might affect the binding to a target molecule. Indeed, the binding of 2′-NH_2-modified RNA ligands to HNE was found to be highly pH dependent. The binding affinity of ligand 19 was profoundly decreased when the pH was changed from 7 by one unit in either direction (K_d changed ~ 60 fold). The highest affinity of the selected ligand occurs at the pH of the binding buffer (pH = 7.0) that was used in the selection. The overall effect of pH on ligand binding is an interplay between the ionization state of the protein (HNE, in this case) and the RNA ligand. The pH-induced change in affinity for a DNA sequence also selected for HNE was not as pronounced as that was observed for 2′-NH_2-modified RNA (unpublished data). Thus it is likely that the observed pH effect of 2′-NH_2-modified RNA is due to the pK_a of the 2′-NH_2 groups.

The ability of *in vitro*-selected ligands to inhibit HNE was assayed by using a chromogenic tetrapeptide substrate specific for HNE. The degree of HNE inhibition is RNA concentration dependent; however, even at moderately high RNA concentrations (~1 μM) inhibition did not exceed more than 30% (data not shown). Ala-Ala-Pro-Val-chloromethyl ketone is an active site directed irreversible inhibitor of HNE (41). The selected 2′-NH_2-modified RNA ligands retain high-affinity binding to HNE that was irreversibly inactivated with the chloromethyl ketone inhibitor, suggesting that these ligands neither bind at the active site nor in the substrate binding pocket. Ligands from different classes compete with each other for binding to HNE, suggesting that they share a common or overlapping binding site on the enzyme. There are 19 arginines

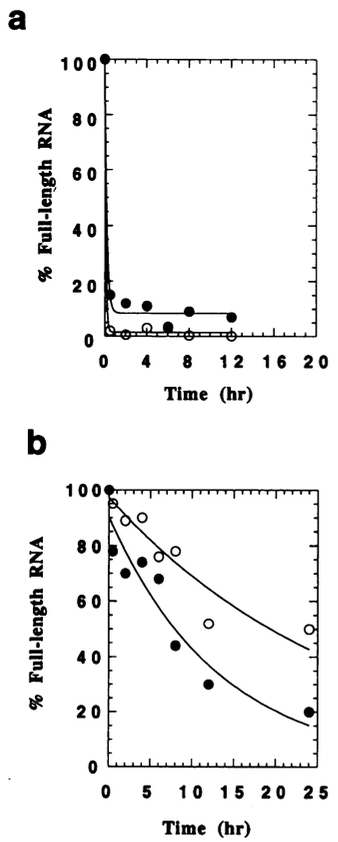

Figure 4. Stability of 2'-NH_2-modified and unmodified RNA in human serum and urine. Either unmodified RNA (**a**) or 2'-NH_2-modified RNA (**b**) was incubated either in 95% human serum (open circles) or in 95% human urine (closed circles) for indicated period of time at 37°C.

in HNE (33) forming an arginine belt on the surface (42). Based on elctrostatic interactions it is speculated that the arginine belt serves as a common binding site for *in vitro* selected RNA ligands.

DISCUSSION

RNA bearing the 2'-NH_2 modification is an example of the type of chemically altered oligonucleotides that can be used in SELEX. However, the available sites for modification in a triphosphate could be limited and not all possible substituents may work. In general, the use of modified oligonucleotides throughout the course of SELEX starting from the random pool is preferred to altering with RNA or DNA ligands after the selection process to make them nuclease resistant.

The presence of 2'-NH_2-substituted nucleotides significantly destabilizes duplexes of both RNA and DNA (39). Thus, during selection, 2'-NH_2-modified RNA may seek alternate structures with the best fit for the target. The selection of a G-quartet structure from a 2'-NH_2-modified RNA pool is therefore not surprising. Only the pyrimidines were 2'-NH_2-modified in the RNAs that we have used. Hence, the stability of a G-quartet motif in modified RNA in which the structured region consists of unmodified guanines may not be very different from that formed in regular RNA. However, experimental evidence such as melting temperatures are necessary to support this view. The selection of nucleic acid ligands with a G-quartet structure is not limited to 2'-NH_2-modified RNA. Nuclear magnetic resonance spectroscopy has revealed an intramolecular G-quartet in a single stranded DNA ligand selected for human thrombin (43).

The observation that binding of 2'-NH_2-modified RNA ligands is pH dependent has general ramifications. For example, by using two buffers with different pH values it would be possible to develop two ligands for a given target molecule that bind at two different pHs. Likewise, one can obtain ligands with high-affinity binding for a given target molecule at a specific pH, but that does not bind to the same protein at a different pH.

The modified ligands selected for HNE did not inhibit the enzyme. The selection scheme that we have chosen was based on affinity, a technically easy approach, rather than the inhibition of the enzyme, a technically challenging criterion. However, in the past affinity-based selections have yielded ligands that were inhibitory toward their target proteins (3, 6, 7), simply because the ligand binding sites were on or overlapping with the active site (or the business end) of the protein. Because of the presence of high-affinity nucleic acid binding sites that are remote from the active site of a target protein not all affinity-based selections generate ligands that are inhibitory toward the function of the target.

Even though the SELEX-derived modified RNA ligands are not efficient inhibitors for HNE they are specific high-affinity binding molecules that are resistant to nucleases. One approach for improving the inhibitory properties of such SELEX-derived tight binding RNA ligands would be the addition of a synthetic group (a substrate mimic) through a nonhydrolyzable linker of certain length to allow the synthetic moiety to reach and occupy the substrate binding pocket and/or the active site of the enzyme, blocking the occupancy of the natural substrate. Such experiments are currently in progress.

As demonstrated here, the use of pools containing unnatural or modified nucleic acids in SELEX expands the use and the potential of *in vitro* selection technology, especially in therapeutic and diagnostic areas where nuclease resistant oligonucleotides are desirable. The introduction of unnatural functional groups to nucleic acids may change the physical and chemical properties of the polymer. The use of such modified nucleic acid pools in SELEX may therefore produce novel nucleic acid ligands with different folding and binding properties previously unknown to nucleic acids.

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