Small Dumbbell Oligonucleotides Inhibitors of RNase H Activity of MOMULV Reverse Transcriptase

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Abstract: The small dumbbell oligonucleotides containing loops of phosphodiester (OL-1), two trimethylene, C_3 moieties in each loop (OL-2) and phosphorothioate (OL-3) linkages were synthesized. Incubation of OL-1 and OL-2 with S-1 nuclease generated break down products whereas incubation of OL-3 did not result in significant cleavage. Their binding to moloney murine leukemia virus reverse transcriptase was evaluated by PAGE band mobility shift assays. The OL-3 bound more strongly to the reverse transcriptase than OL-1 and OL-2. The dissociation constants evaluated using PAGE band mobility shift assays were of the order of 10^{-7} . Investigation of inhibition of RNase H activity of reverse transcriptase showed that the OL-3 is a better inhibitor of the retroviral RNase H activity than both OL-1 and OL-2. Thus OL-3 may be used as RNase H inhibitor. Our studies demonstrated that this particularly designed oligonucleotide (OL-3) displays an IC_{50} of 25 nM in its inhibition on the reverse transcriptase RNase H activity, a magnitude lower than that of first nucleotide reverse transcriptase of HIV-1, tenofovir, introduced by Gilead Science in the market.

Keywords: Small dumbbell oligonucleotides, MOMULV reverse transcriptase, Inhibition of RNase H activity of MMLV RT.

Introduction

Reverse transcription is a key enzyme for life cycle of retroviruses. It bears RNA and DNA dependent DNA polymerase activities. It also posses ribonuclease H activity. During reverse transcription of the viral genome, the RNase H domain of reverse transcriptase cleave the *t*-RNA Lys 3 primer required for minus strand synthesis, degrades the RNA templates during or after minus strand synthesis and removes the polypurine RNA primer during or after plus strand synthesis¹. RNase H activities are prerequisites to the integration of the viral genetic information into the host cell genome. Therefore any ligand able to block the

RNase H activity would prevent the development of retroviruses. However, up to now a very limited number of molecules have shown to display RNase H inhibitory properties²⁻³. There are several other published examples of HIV-RT inhibitors that block both DNA-polymerase and RNase H activities. Among these is *N*-(4-tert-butyl benzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH)⁴ illimaquinone, a natural product of marine origin, which inhibits the RNase H activity of HIV-1 RT, MLV-RT and *Escherichia coli*^{5,6}. Recently, a diketo acid, 4-{5-(benzoylamino)thien-2-yl}-2,4-dioxbutanoic acid has been shown to bind RNase H domain selectively⁷. Also it has recently been shown that 18 base pair RNA/RNA and 2',5'-RNA/RNA duplexes inhibited RNase H by competitively suppressing binding of the DNA/RNA substrate to HIV-1 RT⁸. These RNA/RNA and 2',5'-RNA duplexes also hinder *E.coli* RNase H activity. But the free ends make them susceptible to degradation by ubiquitous cellular nucleases predominantly by 3'-exonuclease type⁹. Recently, it has been shown that small unmodified RNA hairpins and dumbbells can selectively inhibit the RNase H activity of HIV-RT without affecting other cellular RNase H (*e.g.* *E. coli* and Human RNaseH) and do not disrupt the DNA polymerase activity¹⁰. The inhibitory potencies of several hairpins comprising DNA, RNA and 2'-5'-linked RNA segment have shown that hairpin structures having 3'-5'-linked rather than 2',5'-linked RNA loop are potent inhibitor¹¹. Recently we have shown that hairpin oligonucleotides containing phosphorothioate linkage at 3'-loop inhibit RNase H activity of MML-RT with IC₅₀ falling in the range of 140-150 nM.

The first oligonucleotide base drug entered into clinical trial in early 1990 was a 25 mer antisense PS-ODN¹² second antisense oligonucleotide drug¹³ which entered in clinical trial contained mixed phosphotriester and phosphorothioate backbone. Both were discontinued. The first oligonucleotide therapeutics, Vitravene¹⁴ approved by United State FDA is a 21-mer PS ODN. Tenofovir disoproxil fumarate (Gilead) is a nucleotide analogue reverse transcriptase inhibitor used as HIV-1 drug. *In vitro* tenofovir IC₅₀ was in the range of 0.04-8.5 μ M. The anti HIV-1 drugs used so far have shown various side effects. Patients who are taking anti-HIV drugs have suffered these drug toxicities for a long time. Dosages and combinations of drugs can be chosen so they don't kill the person, but they still can't be used at their most effective concentrations against HIV. Thus in order to develop more effective drug for retro virus infection we are investigating the binding and inhibitory properties of oligonucleotide with various chemistry to MOMULV Reverse Transcriptase. Although the catalytic activity of RNase H is restricted to RNA-DNA hybrids but enzymes can bind to different duplexes including DNA-DNA ones. As circular oligonucleotides display significant nuclease resistance¹⁵ they may be considered as good candidates to design RNase H inhibitors. Earlier we have reported binding of dumbbell oligonucleotides (containing 12 bp duplex in the centre) with MOMULV Reverse Transcriptase¹⁶. In order to optimize length of central duplex of the dumbbell oligonucleotides, we synthesized dumbbell oligonucleotides containing 8 bp duplex instead of 12 bp duplex. We report here, data on the design of dumbbell oligonucleotides (containing 8 bp duplex in the center) that aimed at inhibiting RNase H activity. The small dumbbell oligonucleotide containing phosphorothioate bonds in the loops displays an IC₅₀ of 25 nM in its inhibition on the reverse transcriptase RNase H activity. This magnitude is lower than that of first nucleotide reverse transcriptase of HIV-1, tenofovir, introduced by Gilead Science in the market.

Experimental

Moloney murine leukemia virus (MMLV) reverse transcriptase (RT) was purchased from Gibco-BRL. The concentration of protein, determined from its specific activity provided by

the supplier, was 7.6 pmole / μ L. The oligonucleotides *P*-CCTGTTTTTCAGGGA TTTTTGTC and *P*-CCTGsTsTsTsTsTsCAGGGAsTsTsTsTsTsGTC were synthesized on Millipore Expedite synthesizer by standard phosphoramidite chemistry, s denotes phosphorothioate linkage. In oligonucleotide OL-2 two trimethylene, C₃ moieties were added in each loop respectively as described earlier¹⁶. A phosphate group was introduced at the 5'-end by 2 (2'(4,4'-dimethoxytrityloxy) ethylsulfonyl) ethyl-(2-cyanoethyl)-*N,N'*-isopropyl phosphoramidite. The phosphorothioate part of the chimeric oligonucleotide OL-3 was synthesized by oxidizing phosphite diester bond to phosphorothioate by Beaucage reagent¹⁷. The oligonucleotides were purified by electrophoresis on 20% polyacrylamide gel containing 7 M urea. The upper bands corresponding to the longest oligomer, visualized by UV-shadowing were cut out. The gel pieces were soaked in water : methanol (80:20 v/v) overnight and volume was reduced to 100 μ L. Finally, the oligonucleotides were desalted on a Sephadex G 50 Column. The synthesis of small dumbbell oligonucleotides was carried out as described earlier¹⁶. S1 nuclease treatment was also carried out as described earlier¹⁶. Polyacrylamide gel electrophoresis band mobility shift assays were carried out as described elsewhere¹⁸. The binding constant K_a was evaluated from the MMLV-RT concentration at which 50% of the oligonucleotide was converted into oligonucleotide/RT complex: $K_a = \{ \text{MMLV RT} \} 50^{-1/2} (\text{OND})_0^{-1}$. Where $(\text{OND})_0$ is initial oligonucleotide concentration and $(\text{MMLV RT}) 50$ is the enzyme concentration inducing 50% band shift (Figure 2). For the Inhibition of RNase H activity of MMLV Reverse Transcriptase the synthesis of ³H-UTP-labeled RNA: RNA-DNA hybrid required for RNase H activity inhibition assay was prepared as described elsewhere¹⁹. Finally percentage RNase H inhibitions were plotted against oligonucleotides concentration (Figure 3).

Results and Discussion

The small dumbbell, OL-1 was prepared with two loops made up of 5 Ts containing phosphodiester bonds. The OL-2 was prepared with two loops made up of two C3 units and OL-3 was prepared with two loops made up of 5 Ts containing phosphorothioate linkages. The 3'-exonucleases are the most abundant DNases in biological media²⁰ but OL-1 will still be degraded by endonucleases. Indeed, incubation of OL-1 and OL-2 with S1 nuclease generated breakdown products as seen by electrophoretic analysis on a denaturing polyacrylamide gel. Incubation of OL-3 with S1 nuclease did not result in significant cleavage under conditions which led to degradation of OL-1 and OL-2 (Figure 1).

Figure 1. S-1 Nuclease digestion of OL-1 to OL-3

The addition of MMLV- RT to ³²P labeled dumbbell oligomers resulted in the appearance of slow migrating species due to protein -oligonucleotide complexes formation. The oligonucleotide amount in free and bound form was determined by scintillation counting. Figure 2 shows that OL-3 bound more strongly than OL-1 and OL-2. The binding in all the three cases reached to saturation suggesting specific binding of small dumbbell oligonucleotides to a particular site of the enzyme.

We next compared the inhibitory properties of dumbbell oligonucleotides on RNase H activity. As shown in Figure 3, substantial difference was observed. OL-3 was far better inhibitor than OL-1 and OL-2. The sequence, design of the oligonucleotides, K_d and % RNase H activity inhibition are tabulated in Table 1.

Figure 2. Binding curves of MMLV RT to small dumbbell oligonucleotides

Figure 3. Inhibition of RNase H activity of MMLV RT by small dumbbell oligonucleotides

The dissociation constant of OL-3 with RT evaluated from the titration curve fall in the micro molar range (Table 1). The dissociation constant of OL-1 and OL-2 could not be determined as saturation reached before 50% binding.

Table 1. Sequences of small dumbbell oligonucleotides and Percentage RNase H activity inhibition

S. No	Sequences	% RNase H Inhibition at		K _d (M ⁻¹) (K _d = 1/K _a)
		200 nM	50 nM	
OL-1	T T C A G G G A C A T T			
	T T T G T C C C T G T T T	17	10	
OL-2	C ₃ C A G G G A C A C ₃			
	P C ₃ G T C C C T G T C ₃ P	25	2	
OL-3	s T s T s C A G G G A C A s T s T			
	T s T s G T C C C T G T s T s T s	80	80	5.3X10 ⁻⁷
s denotes phosphorothioate linkage in the loops				

From the above studies it appears that the properties of these dumbbell oligonucleotides were mainly related to loop chemistry. The oligonucleotide's loop might interact with the catalytic site of the enzyme, preferably with the loops of the RNase H domain of the reverse transcriptase. The OL-3 bound more strongly with reverse transcriptase and was also a far better inhibitor of RNase H activity than OL-1 and OL-2. The phosphorothioate oligonucleotides have been shown to inhibit RNase H activity²¹. Therefore this interesting observation suggests that small dumbbell oligonucleotides containing phosphorothioate linkage (six on both sides) in the loops portion, such as OL-3, could be used as RNase H inhibitor at as low as 60 nM concentration and might be useful to interfere with retroviral cDNA synthesis. Our studies showed that small dumbbell oligonucleotide (containing 8 bp duplex in the center and phosphorothioate linkages in both the loops) displays an IC₅₀ of 25 nM in its inhibition on the reverse transcriptase RNase H activity, equal to that of dumbbell oligonucleotide (containing 12 bp duplex in the center and phosphorothioate linkages in both the loop). From this it can be concluded that dumbbell oligonucleotides containing 8 bp duplex in the center of the dumbbell can bind to the reverse transcriptase as effectively as 12 bp containing dumbbell oligonucleotides¹⁶. Our studies clearly showed that small dumbbell oligonucleotide containing phosphorothioate bonds in the loops displays an IC₅₀ of 25 nM in its inhibition on the reverse transcriptase RNase H activity, a magnitude lower than that of first nucleotide reverse transcriptase of HIV-1, tenofovir, introduced by Gilead Science in the market. Therefore, this investigation suggests that dumbbell oligonucleotide containing phosphorothioate linkages can be used as RNase H inhibitor and might be useful to interfere with retroviral cDNA synthesis.

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