Nuclease Resistance of Oligonucleotides Containing the Tricyclic Cytosine Analogues Phenoxazine and 9-(2-Aminoethoxy)-Phenoxazine ("G-clamp") and Origins of Their Nuclease Resistance Properties

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ABSTRACT: The tricyclic cytosine analogues phenoxazine and 9-(2-aminoethoxy)-phenoxazine ("G-clamp") are known to significantly enhance the binding affinity of oligonucleotides to their complementary target DNA or RNA strands. To investigate their effect on the nuclease resistance, they were incorporated into model oligomers with a natural phosphodiester backbone, and enzymatic degradation was monitored in an in vitro assay with snake venom phosphodiesterase as the hydrolytic enzyme. In both cases, a single incorporation at the 3′-terminus completely protected the oligonucleotides against 3′-exonuclease attack. Further investigations indicate that the observed high nuclease resistance is not due to the lack of binding affinity to the enzyme's active site, since these modified oligonucleotides were able to inhibit degradation of a natural DNA fragment by bovine intestinal mucosal phosphodiesterase in a dose-dependent manner.

In vivo potency and sequence-specific antisense activity of therapeutic ONs[1] in biological systems depend on many factors including their resistance to enzymatic degradation. For the design of potent antisense drugs, it is therefore essential to combine features, such as high binding affinity and mismatch selectivity with nuclease resistance. Unmodified phosphodiester ONs are degraded rapidly in biological fluids containing hydrolytic enzymes (*1*, *2*), and even the first generation antisense oligonucleotide drugs such as 2′-deoxyphosphorothioate oligonucleotides are subject to enzymatic degradation (*3*, *4*). Since 3′-exonuclease activity is predominantly responsible for enzymatic degradation in serum-containing medium and in various eukaryotic cell lines, modifications located at the 3′-terminus can significantly contribute to the nuclease resistance of an oligonucleotide (*1*, *3*). Such nuclease-resistance 3′-modifications include 3′-conjugates (*5*, *6*), cationic modifications (*7*−*9*), zwitterionic modifications in the 2′-position (*10*, *11*), geometrically altered linkages such as 2′-5′ linkages (*12*), L-nucleotides and analogues (*13*, *14*), α-anomeric oligonucleotides (*15*), 3′-3′-terminal linkages (*1*, *16*), 3′-3′-linked oligonucleotides (*17*), and 3′-loop oligonucleotides. (*4*, *18*). In many cases, these nuclease-resistant modifications and conjugates require numerous synthetic steps and modifications to the oligonucleotide synthesis protocol. In addition, there may be a loss in binding affinity to the target RNA due to these changes with geometrically altered linkages. 2′-Carbohydrate modifications (*19*, *20*) such as 2′-*O*-alkyl and 2′-*O*-alkoxyalkyl modifications provide both binding affinity and nuclease-resistance advantages. However, a minimum of three to five modified nucleosides are required at the 3′-end to provide sufficient nuclease resistance (*21*, *22*). On the other hand, some of the 2′-modifications such as 2′-F provide high binding affinity but fail to increase the nuclease resistance (*23*).

In addition to the conjugates and carbohydrate modifications mentioned above, numerous nucleobase modifications, designed to enhance the binding affinity of antisense ONs to their complementary target strands, have been synthesized (*20*, *24*, *25*). More recently, several tricyclic cytosine analogues, such as phenoxazine[2] (*26*), phenothiazine (*27*), and tetrafluorophenoxazine (*28*) have been developed. Incorporated into oligonucleotides, these base modifications were shown to hybridize with guanine and to enhance helical thermal stability by extended stacking interactions. Tetrafluorophenoxazine also hybridizes with adenine.

These helix-stabilizing properties could further be improved with 9-(2-aminoethoxy)-phenoxazine,[3] another cytosine analogue, which carries an additional aminoethoxy tether on its rigid phenoxazine scaffold and is termed "G-clamp" due to its high binding affinity for a complementary guanine nucleobase (*29*). Binding studies demonstrated that a single incorporation of 9-(2-aminoethoxy)-phenoxazine enhanced the binding affinity of a model oligonucleotide to its complementary target DNA or RNA with a ΔT_m of up to 18 °C relative to $dC5^{Me}$. This is the highest affinity enhancement attained so far with a single modification. Importantly, the

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[1] Abbreviations: BIPD, bovine intestinal mucosal phosphodiesterase; CGE, capillary gel electrophoresis; $dC5^{Me}$, 5-methylcytosine; DIEA, *N*,*N*-diisopropylethylamine; DMAP, 4-(dimethylamino)pyridine; DMT, 4, 4′-dimethoxytrityl; ΔT_m, difference in melting temperatures; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; LCAA-CPG, long-chain alkylamine controlled pore glass; ON, oligonucleotide; SVPD, snake venom phosphodiesterase; TEA, triethylamine; TLC, thin-layer chromatography; Tris-HCl tris(hydroxymethyl)-aminomethane hydrochloride.

[2] Chem. Abstr.: 3-(2-deoxy-β-D-erythro-pentofuranosyl)-1H-Pyrimido-[5,4-b][1,4]benzoxazin-2(3H)-one.

[3] Chem. Abstr.: 9-(2-aminoethoxy)-3-(2-deoxy-β-D-erythro-pentofuranosyl)-1H-Pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one.

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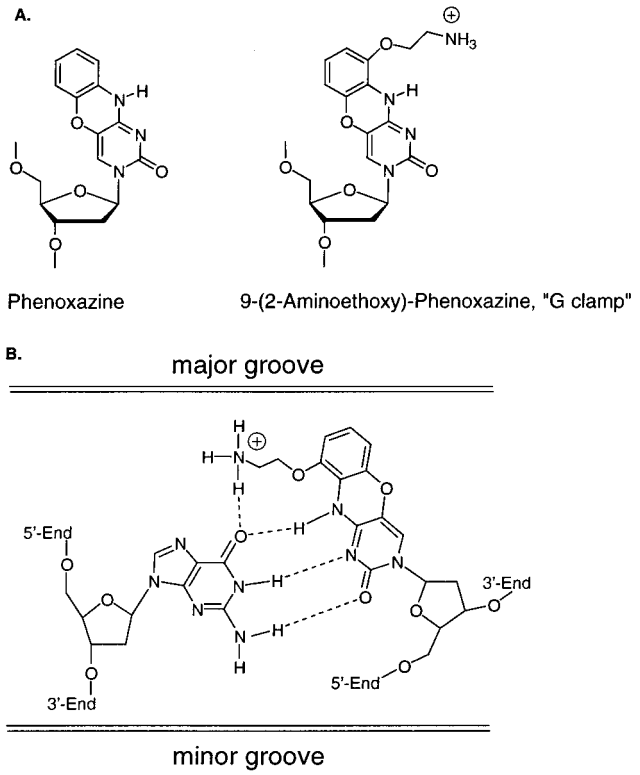
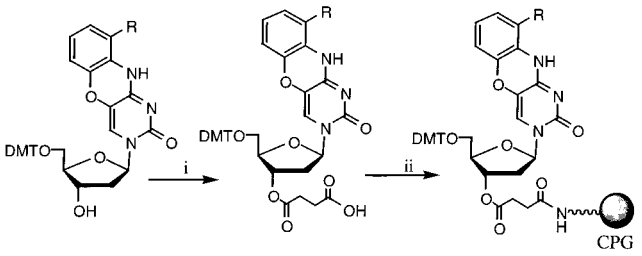


FIGURE 1: (A) Structure of phenoxazine and 9-(2-aminoethoxy)-phenoxazine modifications. (B) G−C* pair with 9-(2-aminoethoxy)-phenoxazine as the C mimic showing the proposed four hydrogen bonds.

gain in helical stability does not compromise the specificity of the oligonucleotide for complementary RNA. The T_m data indicate that G-clamp provides an even higher discrimination between the perfect match and mismatched sequences compared to $dC5^{Me}$ (*29*). The tethered amino group may act as a hydrogen bond donor and interact with the O6 of guanine on the Hoogsteen face of the base pair (Figure 1). Thus, the increased affinity of 9-(2-aminoethoxy)-phenoxazine is presumably due to the combination of extended base stacking and an additional specific hydrogen bond.

The enhanced binding affinity of the phenoxazine derivatives together with their uncompromised sequence specificity makes them valuable nucleobase analogues for the development of potent antisense-based drugs. In fact, promising data have been derived from in vitro experiments demonstrating that heptanucleotides containing phenoxazine substitutions are able to activate RNase H, enhance cellular uptake, and exhibit an increased antisense activity relative to 2′-deoxyphosphorothioate control ONs (*29*). The activity enhancement was even more pronounced in case of 9-(2-aminoethoxy)-phenoxazine, as a single substitution was shown to significantly improve the in vitro potency of 20-mer 2′-deoxyphosphorothioate oligonucleotides targeted against c-*raf* (*30*). To optimize oligonucleotide design and to better understand the impact of these heterocyclic modifications on the biological activity, it is important to evaluate their effect on the nuclease stability of the oligomers. In the present work, we investigated the influence of a single modification at the 3′-terminus on the enzymatic stability of ONs with a natural phosphodiester backbone.

Scheme 1[a]



1, **3**, **5**: R = H; **2**, **4**, **6**: R = $OCH_2CH_2NHCOCF_3$

[a] (i) Nucleoside **1** or **2**, succinic anhydride (1.5 equiv), DMAP [4-(dimethylamino)pyridine, 0.5 equiv], CH_2Cl_2/pyridine (5:1), r.t., 16 h; (ii) LCAA-CPG (long-chain alkylamine controlled pore glass, initial loading: 115 μmol/g), succinate **3** or **4** (1.5 equiv), HATU [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronuim hexafluorophosphate, 1.5 equiv], DIEA (*N*,*N*-diisopropylethylamine, 4 equiv), DMF, r.t., 16 h; final loading as determined by DMT-assay: **5**, 82 μmol/g; **6**, 65 μmol/g.

MATERIALS AND METHODS

9-(2-aminoethoxy)-Phenoxazine Succinate **4**. After drying at 50 °C in vacuo overnight, the 9-(2-aminoethoxy)-phenoxazine 2′-deoxynucleoside (*29*) (**2**, 0.51 g, 0684 mmol) was dissolved in anhydrous DCM/Pyr (5:1), and 0.103 g (1.03 mmol) succinic anhydride was added to the solution. Subsequently, 41.5 mg (0.34 mmol) of DMAP in 1 mL of DMF was added, and the mixture was stirred overnight. After completion of the reaction (as indicated by TLC), the solvent was evaporated in vacuo and the remaining yellow oil was dissolved in DCM, washed twice with 10% $NAHCO_3$(aq), 10% aqueous citrate, and brine. After the solvent was dried over Na_2SO_4, the organic phase was evaporated in vacuo to yield a yellow solid (0.45 g, 75%). MS (HR−FAB) *m*/*z* 897.256 $(M + Na)^+$. The phenoxazine succinate **3** was prepared by an analogous procedure in an isolated yield of 89% starting from nucleoside **1** (Scheme 1).

9-(2-aminoethoxy)-Phenoxazine Succinyl-LCAA-CPG ***6***. A total of 131 mg (0.15 mmol) of 9-(2-aminoethoxy)-phenoxazine succinate was dissolved in DMF, and 68 μL (0.4 mmol) DIEA was added. A solution of 57 mg (0.15 mmol) HATU in DMF was added to the mixture while stirring. Stirring was continued for about 1 min in order to allow preactivation before the mixture was added to 1 g of LCAA-CPG (initial loading: 115 μmol/g), and the suspension was shaken overnight. Subsequently, the resin was washed three times each with DMF, DCM, and CH_3CN and the unreacted amino groups of the resin were capped by shaking the resin with 0.24 mL (2 mmol) of ethyl trifluoroacetate and 0.28 mL (2 mmol) of TEA in 5 mL of MeOH. Finally, the resin was washed with MeOH, CH_3CN, and DCM and dried in vacuo. The loading with 9-(2-aminoethoxy)-phenoxazine succinate was determined by DMT assay (final loading = 65 μmol/g). The 2′-deoxy-phenoxazine CPG **5** was synthesized from the phenoxazine nucleoside succinate **3** using a similar procedure, and a final loading of 82 μmol/g was obtained (Scheme 1).

Oligonucleotide Synthesis. Solid-phase synthesis of oligonucleotides was carried out using standard phosphoramidite chemistry and an Applied Biosystems (Perkin-Elmer Corp.) DNA/RNA Synthesizer 380B. Cleavage and deprotection of the oligonucleotides containing phenoxazine and 9-(2-aminoethoxy)-phenoxazine units was performed using 28−30%

NH_3(aq) at 55 °C for 6 h and a solution of 40% $MeNH_2$(aq) and (1:1) for 4 h at r.t., respectively. The oligonucleotides were purified by reversed-phase HPLC using a 306 Piston Pump System, an 811C Dynamic Mixer, a 170 Diode Array Detector, and a 215 Liquid Handler together with the Unipoint Software from Gilson (Middleton, WI). The HPLC conditions were as follows: column, Waters Deltapak C_{18} reversed-phase (300 × 3.9 mm, 15 μm, 300 Å); solvent A, 0.1 M NH_4OAc in H_2O; solvent B, 0.1 M NH_4OAc in CH_3CN/H_2O (80:20); gradient, 0−40 min 0 to 50% B. After chromatographic purification, the oligonucleotides were desalted by RP-HPLC, lyophilized, and stored at −20 °C.

Nuclease Stability Determination. Oligonucleotides, at a final concentration of 2 μM, were incubated with snake venom phosphodiesterase (0.005 units/mL) in 50 mM Tris-HCl, pH 7.5, and 8 mM $MgCl_2$ at 37 °C. The total reaction volume was 100 μL. At each time point, 10 μL aliquots of each reaction mixture were placed in a 500 μL microfuge tube and put in a boiling water bath for 2 min. The sample was then cooled on ice, spun quickly to bring the entire volume to the bottom of the tube, and desalted on a Millipore 0.025 micron filter disk (Bedford, MA) that was floating in water in a 60 mm Petrie dish. After 30−60 min on the membrane, the sample was diluted with 200 μL of distilled H_2O and analyzed by gel-filled capillary electrophoresis. The oligonucleotide and metabolites were separated and analyzed using the Beckman P/ACE MDQ capillary electrophoresis instrument using a 100 μm ID 30 cm coated capillary (Beckman no. 477477) with eCAP ssDNA 100-R gel (Beckman no. 477621) and Tris-borate urea buffer (Beckman no. 338481). The samples were injected electrokinetically using a field strength of between 5 and 10 kV for a duration of between 5 and 10 s. Separation was achieved at 40 °C with an applied voltage of 15 kV. The percentage of full-length oligonucleotide was calculated by integration using Caesar ver. 6 software (Senetec Software, New Jersey) followed by correction for differences in extinction coefficient for oligonucleotides of different length.

RESULTS AND DISCUSSIONS

Phenoxazine **1** and 9-(2-aminoethoxy)-phenoxazine **2** nucleosides were prepared by modifying previously published procedures (*27*, *29*). The succinates **3** and **4** and the corresponding substituted solid supports **5** and **6** were prepared as outlined in Scheme 1. Using the CPG supports, the two cytidine analogues **1** and **2** were incorporated at the 3′ terminus of two model ONs **7** and **8**, respectively, with the sequence T_{18}dC* (dC* = phenoxazine or 9-(2-aminoethoxy)-phenoxazine deoxyribonucleoside). Solid-phase oligonucleotide synthesis was carried out using standard phosphoramidite chemistry. To prevent the addition of acrylonitrile, which is formed by cleaving the β-cyanoethyl phosphate protection, to the primary amino group of 9-(2-aminoethoxy)-phenoxazine, deprotection of ON **8** was performed with a 1:1 solution of $MeNH_2$ (40%, aq) and NH_3 (28−30%, aq) at r.t. for 4 h. The oligonucleotides were purified and desalted by reversed-phase HPLC.

SVPD and BIPD were utilized as the hydrolytic enzymes for in vitro nuclease resistance studies. Both enzymes predominantly exhibit 3′-exonuclease activity. An unmodified 19-mer oligothymidylate (ON **9**) was used as a control.

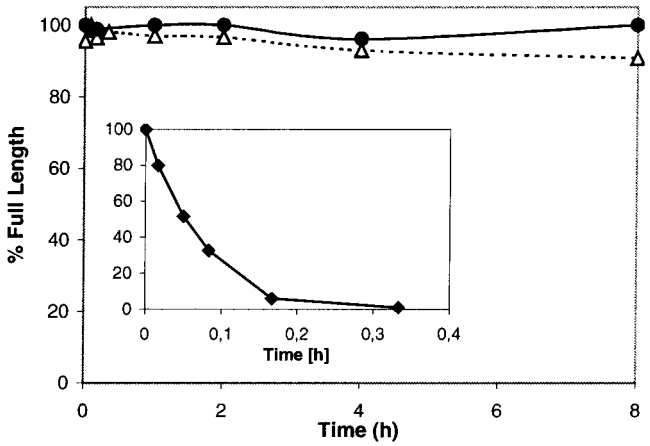


FIGURE 2: Degradation of ONs **7** (open triangles) and **8** (closed circles) as a function of incubation time and compared to an unmodified control ON **9** (closed diamonds, inset).

The progress of enzymatic degradation was monitored by CGE analysis.

The results of the nuclease resistance study with SVPD as the hydrolytic enzyme are shown in Figure 2. As expected, the unmodified control ON **9** (inset) was degraded rapidly by sequential removal of the terminal nucleotides. The half-life $t_{1/2}$ of the full-length oligonucleotide was reached at about 3 min. After 20 min of incubation, the full-length oligomer was almost completely degraded to a series of shorter fragments. In contrast, the modified ONs **7** and **8**, bearing a single nucleobase modification at their 3′-end, were not significantly degraded under these conditions even after an incubation time of 8 h. According to the degradation rates and the CGE profiles, there is no significant difference in the 3′-exonuclease resistance of oligomers modified with either phenoxazine or 9-(2-aminoethoxy)-phenoxazine. Using BIPD as the hydrolytic enzyme, very similar results for the nuclease resistance of both modified oligonucleotides **7** and **8** were obtained (data not shown).

In a second set of experiments, the inhibitory effects of phenoxazine and 9-(2-aminoethoxy)-phenoxazine oligonucleotides on the nuclease activity was investigated. The 19-mer oligothymidylate ON **9**, which was labeled with fluorescein at the 5′-terminus, was incubated with BIPD, and the enzymatic degradation was followed in the presence of ON **7** and **8**, respectively. The influence of the modified oligonucleotides on the nucleolytic activity was determined by looking at the overall velocity of the enzymatic reaction. All products of degradation were quantified at each time point, weighted considering their stage of degradation (n−x), and summarized to obtain the number of hydrolyzed linkages. The velocity of the enzymatic reaction was determined graphically from the number of hydrolyzed phosphodiester linkages as a function of the incubation time.

The velocity of the enzymatic degradation of unmodified ON **9** is plotted as a function of the concentration of ON **7** and **8** (Figure 3). The results demonstrate that both modified oligonucleotides have a distinct inhibitory effect on the enzymatic reaction. Again, no significant difference is detectable between phenoxazine and 9-(2-aminoethoxy)-phenoxazine. Both inhibit degradation of ON **9** at concentrations above 0.2 μM. The IC_{50} values are reached at about 0.5 μM, and at concentrations of 5 μM and higher the enzymatic reaction is almost completely inhibited.

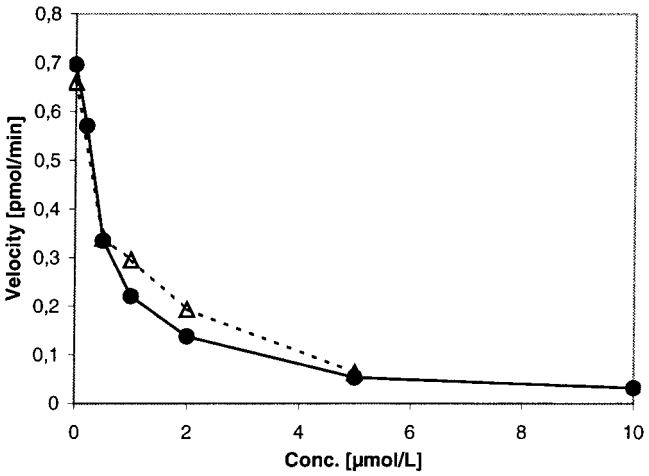


FIGURE 3: Velocity of the enzymatic reaction: hydrolysis of ON **9** with BIPD as a function of the concentration of co-incubated ON **7** (open triangles) and ON **8** (closed circles).

This experiment was driven by the question of why oligonucleotides bearing these tricyclic base modifications at their 3′-termini exhibit such extraordinary nuclease resistance. The nuclease resistance data demonstrate that, despite their natural phosphodiester backbones, both heterocyclic modifications provide an almost complete protection against 3′-exonuclease attack. One explanation could be that the bulky heterocycle moieties simply prevent the enzyme from binding to the 3′-terminus by steric hindrance, meaning that the oligonucleotides are *not* recognized as substrates. Alternatively, the modified oligonucleotides may bind to the active site of the enzyme without being hydrolyzed, which would directly affect the enzyme's activity. The observed decrease in the velocity of the enzymatic degradation of a natural DNA fragment indicates that oligonucleotides containing either phenoxazine or 9-(2-aminoethoxy)-phenoxazine residues are able to bind to the enzyme's active site. However, as it has been shown in the previous experiment, hydrolysis of the 3′-terminal nucleotide phosphodiester linkage is prevented due to the presence of the unnatural tricyclic base moieties. The dose dependence of the inhibitory effects with IC_{50} values of about 0.5 μmol suggests that the binding of the modified oligonucleotides is competitive and reversible.

The fact that there is no detectable difference between the nuclease resistance of ON **7** and ON **8** indicates that the observed stabilizing effect is mainly due to presence of the bulky unnatural heterocycles. With the present data, however, it remains unclear to what extent the positively charged amino tether of the 9-(2-aminoethoxy)-phenoxazine moiety contributes to the nuclease resistance of ON **8**. In previous studies, it has been shown that cationic modifications of the sugar moieties, such as 2′-*O*-aminoalkyl, can efficiently protect phosphodiester oligonucleotides from enzymatic degradation (*9*, *10*). Crystal structure studies of a complex formed between a 2′-aminopropyl-modified ON and an exonuclease (DNA polymerase I Klenow fragment) demonstrate that the aminopropyl residue prevents binding of a metal ion, which is needed to catalyze hydrolysis of the 3′-phosphodiester linkage (*31*). The amino tether of a 9-(2-aminoethoxy)-phenoxazine residue, however, protrudes into the major groove, while the 2′-modification points into the shallow groove of a duplex. Whether or not the positive charge of the latter can interfere with the metal binding of an exonuclease remains to be investigated.

CONCLUSIONS

In summary, the present work demonstrates that a single incorporation of phenoxazine or 9-(2-aminoethoxy)-phenoxazine at the 3′-end of an oligonucleotide efficiently protects against nucleolytic degradation by 3′-exonucleases. No significant difference has been observed between the two cytosine analogues as far as their effects on the nuclease resistance and their capability to inhibit nuclease activity are concerned. Their enhanced binding affinity, together with their uncompromised specificity, ability to support RNase H activity, and the observed nuclease resistance, makes these nucleobase analogues promising tools for the antisense strategy. As these modifications protect natural phosphodiester linkages against enzymatic hydrolysis, optimized placement of phenoxazine or the 9-(2-aminoethoxy)-phenoxazine may lead to the design of more potent antisense-based drugs. Enhanced stability of antisense oligonucleotides may reduce the frequency of dosing for chronic treatment and may allow oral administration of the drug. Both phenoxazine and 9-(2-aminoethoxy)-phenoxazine modifications have attributes that make them attractive candidates for further evaluation in vivo as potent antisense drugs.

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