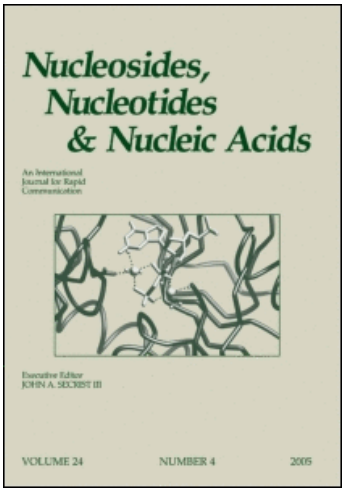

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Steven H. Krawczyk[a]; Norbert Bischofberger[a]; Linda C. Griffin[a]; Veronica S. Law[a]; Regan G. Shea[a]; S. Swaminathan[a]
[a] Gilead Sciences, 353 Lakeside Drive, Foster City, California

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STRUCTURE-ACTIVITY STUDY OF OLIGODEOXYNUCLEOTIDES WHICH INHIBIT THROMBIN

Steven H. Krawczyk; Norbert Bischofberger*; Linda C. Griffin; Veronica S. Law; Regan G. Shea; S. Swaminathan

Gilead Sciences, 353 Lakeside Drive, Foster City, California 94404

Abstract

The 15-mer oligodeoxynucleotide GGTTGGTGTGGTTGG is a potent inhibitor of thrombin and it forms a stable, highly compact structure in solution. Deletions and substitutions by abasic residues, 2'-deoxyinosine, 7-deaza-2'-deoxyguanosine and 8-methyl-2'-deoxyguanosine show that the structural features of the oligodeoxynucleotide are important for its biological activity.

Thrombin is a serine protease with multiple functions in hemostasis. Its role in coronary heart disease and other thrombotic disorders has prompted efforts towards the identification of specific inhibitors of thrombin,[1] and an understanding of their interaction with this enzyme.[2]

A report from our laboratory disclosed the selection of DNA aptamers by screening from a library of 10^{13} molecules of 96-mer single stranded oligodeoxynucleotides. The consensus sequences GGTTGGTGTGGTTGG (**1**) and GGTTGG (**10**) were found to be potent inhibitors of thrombin[3] with EC_{50} of 20 nM and 5 μM, respectively, in the purified fibrinogen clotting assay. Subsequent *in vivo* studies using cynomolgus monkeys demonstrated that **1** is a potent, rapid acting, anticoagulant with a half-life of approximately 2 min.[4] The short half-life of this inhibitor allowed the regional anticoagulation of an extra-corporeal hemofiltration circuit in sheep.[4] Moreover, in an *ex vivo* model of arterial thrombosis in a rabbit aorta, **1** was found to inhibit clot bound thrombin, whereas heparin was ineffective.[5] The rapid onset of action, the short half life *in vivo* and inhibitory activity of clot bound thrombin suggest that **1** may be useful in anticoagulation with extracorporeal circuits and may have distinct advantages in certain clinical settings.

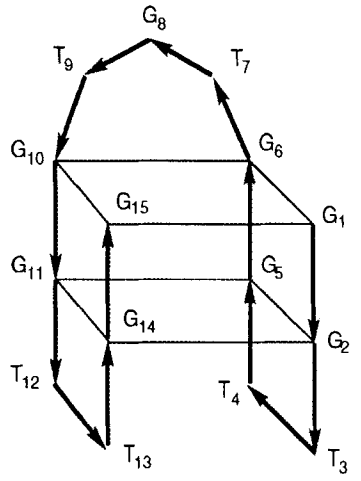


Figure 1

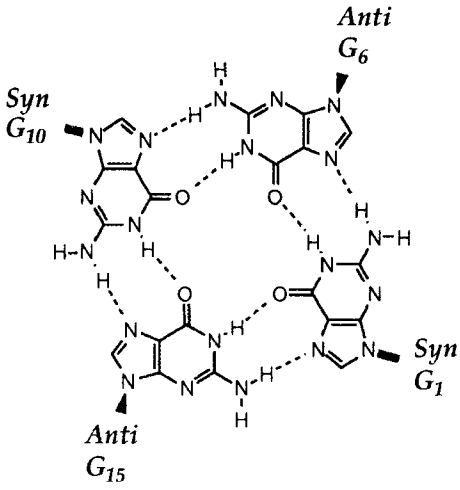


Figure 2

The three-dimensional solution structure of **1** has recently been solved using NMR techniques.[6-9] The structure adopted by this oligodeoxynucleotide consists of two stacked G-tetrads connected by two TT loops and a single TGT loop as shown in Figure 1.

A distinguishing feature of this structure is that within each of the stacked G-tetrads, two of the diagonally opposed deoxyguanosine residues adopt a *syn* conformation about their glycosidic bonds (i.e. G_1, G_{10} and G_5, G_{14} see Figure 1).

Lysine protection studies,[10] mutagenesis experiments,[11] NMR experiments[7] and X-ray crystallography[8] and the fact that **1** does not inhibit thrombin catalyzed chromogenic substrate cleavage[11] suggested that **1** was binding at the anion exosite of thrombin in a conformation little changed from that of the unbound species. To test this hypothesis and explore the SAR, we substituted individual residues of **1** with different deoxynucleoside analogs. As a first step, we investigated the influence of simple deletions from either end of **1** (oligomers **2** to **18**) (see Table 1).

The removal of a single deoxyguanosine residue from either terminus of oligomer **1** causes at least a 500-fold loss of activity. Upon making further residue deletions, some activity is regained when the 6-mer **10** is reached. To determine whether the 6-mer inhibits thrombin as a monomer or as a multimeric species, we measured the amount of thrombin inhibition as a function of the oligomer concentration. When the log thrombin inhibited was plotted against log of the 6-mer concentration a line with a slope of 1.95 was obtained (data not shown), indicating that the 6-mer acts as a dimer. The analogous experiment

TABLE 1

Thrombin time EC_{50} of truncated oligonucleotide analogs of **1**.

SEQUENCE		EC_{50} μM
GGTTGGTGTGGTTGG	**1**	0.020
GGTTGGTGTGGTTG	**2**	>10
GGTTGGTGTGGTT	**3**	>10
GGTTGGTGTGGT	**4**	>10
GGTTGGTGTGG	**5**	>10
GGTTGGTGTG	**6**	>10
GGTTGGTGT	**7**	>10
GGTTGGTG	**8**	>10
GGTTGGT	**9**	>10
GGTTGG	**10**	5
GTTGGTGTGGTTGG	**11**	>10
TTGGTGTGGTTGG	**12**	>10
TGGTGTGGTTGG	**13**	>10
GGTGTGGTTGG	**14**	>10
GTGTGGTTGG	**15**	>10
TGTGGTTGG	**16**	>10
GTGGTTGG	**17**	>10
TGGTTGG	**18**	>10

done with the 15-mer resulted in a line with the slope of 1.02, indicating that the 15-mer acts as a monomer. The fact that the 6-mer acts as a dimer indicates that the same G tetrad structural motif is responsible for its activity.

We then tested a series of fifteen analogs of oligomer **1** in which one of the fifteen residues has been sequentially replaced by an abasic site analog 1,4-anhydroribitol[12] (oligomers 19-33) or 2'-deoxyinosine (oligomers 34-42) or 7-deaza-2'-deoxyguanosine (oligomers 43-51) (see Table 2).

Replacement by an abasic residue of any of the guanine residues implicated in G-tetrad formation (G_1, 2, 5, 6 and G_{10}, 11, 14 and 15) leads to compounds devoid of substantial activity. Elimination of any of the three base residues of T_7, G_8, or T_9 affords oligomers which maintain submicromolar activity. The elimination of the thymine residue in position T_7 actually improves activity while the elimination of G_8 or T_9 lowers activity approximately by ten-fold as measured by the EC_{50}. This observation is consistent with the hypothesis that the deoxynucleosides in positions T_7, G_8 and T_9 serve as linkers which hold the rest of the residues in a configuration which is conducive to G-tetrad formation. Other base residues which can be removed without severely affecting activity are T_3 and T_{12} which result in approximately a ten-fold diminution of activity. The replacement of a deoxyguanosine residue by a deoxyinosine or a 7-deaza-2'-deoxyguanosine residue results

TABLE 2

Thrombin time EC50 of abasic, 2'-deoxyinosine, and 7-deaza-2'-deoxyguanosine substituted analogs of 1

SEQUENCE	X=	ABASIC		2'-Deoxy Inosine		7-Deaza-2'-Deoxy Guanosine		8-Methyl-2'-Deoxy Guanosine
				EC_{50} (μM)				
XGTTGGTGTGGTTGG	**19**	>2	**34**	>2	**43**	2	**52**	0.02
GXTTGGTGTGGTTGG	**20**	>2	**35**	>2	**44**	>2	**53**	0.16
GGXTGGTGTGGTTGG	**21**	0.08						
GGTXGGTGTGGTTGG	**22**	>2						
GGTTXGTGTGGTTGG	**23**	>2	**36**	>2	**45**	>2	**54**	0.02
GGTTGXTGTGGTTGG	**24**	>2	**37**	>2	**46**	>2	**55**	0.08
GGTTGGXGTGGTTGG	**25**	0.01						
GGTTGGTXTGGTTGG	**26**	0.12	**38**	0.04	**47**	0.05	**56**	0.02
GGTTGGTGXGGTTGG	**27**	0.15						
GGTTGGTGTXGTTGG	**28**	>2	**39**	>2	**48**	>2	**57**	0.02
GGTTGGTGTGXTTGG	**29**	>2	**40**	>2	**49**	>2	**58**	0.08
GGTTGGTGTGGXTGG	**30**	0.08						
GGTTGGTGTGGTXGG	**31**	>2						
GGTTGGTGTGGTTXG	**32**	>2	**41**	>2	**50**	>2	**59**	0.02
GGTTGGTGTGGTTGX	**33**	>2	**42**	>2	**51**	>2	**60**	0.06

in a more subtle perturbation than the removal of the entire base moiety, yet the absence of the 2-amino or 7-aza group should preclude the formation of a stable G-tetrad.[25] As the results in Table 2 indicate, the 2-amino group and the 7-aza functionality of G_1, G_2, G_5, G_6, G_{10}, G_{11}, G_{14}, and G_{15} are necessary for potent activity. The results of these experiments parallel those of the abasic substitution and support the notion that the loss of activity observed results from a disruption of the G-tetrad structure rather than the loss of a specific aptamer-thrombin interaction. The observation that deoxyinosine or deazadeoxyguanosine is tolerated in place of G_8 is consistent with this residue simply serving a linker function.

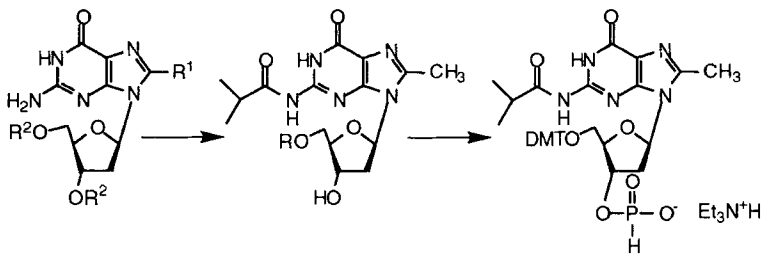
Finally, each dG position in oligomer **1** was substituted with an 8-methyl-2'-dG analog to test the hypothesis that G_1, G_5, G_{10} and G_{14} adopted the *syn* conformation as suggested by the structural model (oligomers **52-60**) (see Table 2). As can be seen, incorporation of 8-methyl-2'-deoxyguanosine into the anti G positions G_2, G_6, G_{11} and G_{15} leads to a slight decrease in activity, whereas incorporation into the *syn* G positions G_1, G_5, G_{10} and G_{14} and into the linker (G_8) has no effect. The observation that substitutions with 8-methyl-2'-dG at G_2, G_6, G_{11} or G_{15} are not very disruptive to activity can be understood by noting that the 8-methyl group does not preclude the anti confirmation, but disfavors it.[26]

Materials and Methods

Purified Fibrinogen Clotting Time. Since the oligodeoxynucleotides do not inhibit chromogenic substrate cleavage by thrombin, the inhibition of fibrinogen polymerization was determined in a fibrinogen clotting assay. ODN was incubated for ~1 min at 37°C in 200 μL buffer (20 mM tris acetate, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) containing 2 mg/mL purified human fibrinogen (Sigma). Thrombin (Haematologics) in the 100 μL buffer was pre-equilibrated for 1 min at 37°C and added to the DNA/fibrinogen solution (13 nM final thrombin concentration). Clotting times were measured using an automated fibrometer, which detects fibrin strand formation, and were the mean of three experiments. Clotting times in the absence of added oligonucleotide were in the range of 25-30 seconds. The concentration of oligonucleotide required to double the clotting time, corresponding to inhibition of approximately half of the thrombin, was determined and is reported as EC_{50}.

Dependence of thrombin inhibition on Oligomer (15-mer or 6-mer) concentration. Varying concentrations of oligomers (15-mer, 2.5-100 nM; 6-mer, 1-10 mM) were incubated for 1 min at 37°C in buffer (0.2 mL, 20 mM Tris-acetate, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) containing human fibrinogen (Sigma) at a final concentration of 2 mg/mL. Thrombin (0.1 mL in buffer, pre-equilibrated for 1 min at 37°C) was added to give a final concentration of 13 nM. Clotting times were measured using an automated fibrometer and the extent of thrombin inhibition was then calculated using a thrombin standard curve generated by measuring clotting time versus thrombin concentration. Log (Y/1-Y) (Y is the fraction of thrombin inhibited) versus log DNA concentration were plotted from two data sets. The slope of this plot for the 15-mer was 1.02 (R=0.99) and for the 6-mer it was 1.95 (R=0.99).

Synthesis of Monomers and Oligomers. 8-methyl-2'-deoxyguanosine[15] 62 was prepared from 61 by an adoption of the free radical methodology described for the ribonucleoside[16] (see Scheme 1) followed by deprotection to give 63. N^2 protection using the transient protection method[17] yielded 64. Protection of the 5'-hydroxyl with DMT-Cl and phosphitylation[18] gave 66. 7-deaza-2'-deoxyguanosine H-phosphonate was prepared as previously described.[13] 1,4-anhydro-2-deoxy-D-ribitol was prepared as described by Eritja *et al.*[12] and was converted to the cyanoethyl diisopropylphosphoramidite by the procedure described by Sinha *et al.*[14] Derivatized controlled pore glass (CPG) was prepared as described by Damha *et al.*[22] using the 5'-DMT derivatives of N^2- isobutyryl-7-deaza-2'-deoxyguanosine and 8-substituted N^2- isobutyryl-2'-deoxyguanosines. ODNs were synthesized by standard chemistry on CPG supports using MilliGen/Biosearch 8750 DNA synthesizers using H-phosphonate[23] or amidite[18] coupling methodology.



$R^1 = H$ $R_2 = Ac$ **61** R = H **64** **66**
$R^1 = CH_3$ $R_2 = Ac$ **62** R = DMT **65**
$R^1 = CH_3$ $R_2 = H$ **63**

Scheme 1

Purification of ODNs was accomplished by denaturing PAGE (10% acrylamide, 7M urea; 8-methyl-dG oligomers) or reversed-phase HPLC (abasic and 7-deaza-dG oligomers). ODNs were analyzed for purity and chemical integrity by anion-exchange HPLC, PAGE, and base composition analysis in the same equation.

3',5'-di-O-acetyl-8-methyl-2'-deoxyguanosine. A solution of 3',5'-di-O-acetyl-2'-deoxyguanosine (3.5 g, 10 mmol) in aqueous sulfuric acid (0.4 N, 50 mL) containing ferrous sulfate heptahydrate (11g, 39.6 mmol) was treated, dropwise, with a solution of 70% aqueous t-butyl hydroperoxide (5 mL) in aqueous sulfuric acid (25 mL, 0.4 N). After 0.5 h, the mixture was chilled to 15°C, the pH of the solution was adjusted to 6.5 with sodium bicarbonate, the brown precipitate was removed by filtration, and washed with methylene chloride/methanol (4:1, v:v). The filtrate was extracted with methylene chloride/methanol (4:1, v:v) and the extracts were combined with the precipitate washing. The combined extracts were filtered through a 7.5 cm wide by 1 cm deep silica gel pad and evaporated. The residue was crystallized from ethanol/water (19:1, v:v) to yield 1.52 g (41%) of the diacetate. ^{1}H-NMR (DMSO-d_6): 11.6 (bs, 1H, NH), 6.4 (bs, 2H, NH_2), 6.2 (t, 1H, H-1'), 5.6 (m, 1H, H-3'), 4.4-4.1 (3m, 3H, H-4', H-5'ab), 3.2, 2.3 (2m, 2H, H-2'ab), 2.4 (s, 3H, Me), 2.1, 1.9 (2s, 6H, Acetyl)

8-methyl-2'-deoxyguanosine. A solution of 3',5'-di-O-acetyl-8-methyl-2'-deoxyguanosine (2.0 g, 5.5 mmol) in methanolic ammonia (50 mL, saturated at 0°C) was kept at room temperature in a sealed flask for 24 h. The resulting suspension was chilled to -20°C and filtered to afford 1.2 g (78%) of product. ^{1}H-NMR (DMSO-d_6): 7.9 (bs, 1H, NH), 6.4 (bs, 2H, NH_2), 6.2 (t, 1H, H-1'), 5.4-4.6 (bs, 2H, 3',5'-OH), 4.3 (m, 1H, H-

3'), 3.7 (m, 1H, H-4'), 3.5 (m, 2H, H-5'ab), 2.8, 2.0 (2m, 2H, H-2'ab), 2.4 (s, 3H, Me) Anal. ($C_{11}H_{15}N_5O_4 \cdot 1.25\ H_2O$) C, H, N

N^2-isobutyryl-8-methyl-2'-deoxyguanosine. To a suspension of of 8-methyl-2'-deoxyguanosine (2.5 g, 8.9 mmol) in pyridine (100 mL) was added chlorotrimethylsilane (15 mL, 118.2 mmol). After 5 min, of isobutyric anhydride (10 mL, 60.3 mmol) was added. After 2 h, of ice (20 cc), followed by conc. aqueous ammonium hydroxide (20 mL) were added. The mixture was stirred for 0.5 h, evaporated, coevaporated with water, and the residue was triturated with ice cold water, the product was collected by filtration to yield 2.28 g (73%) of the title compound. ^{1}H-NMR (DMSO-d_6): 12.0, 11.5 (2bs, 2H, NH), 6.3 (t, 1H, H-1'), 5.2 (d, 1H, 3'-OH), 4.8 (bs, 1H, 5'-OH), 4.4 (m, 1H, H-3'), 3.7 (m, 1H, H-4'), 3.6(m, 2H, H-5'ab), 2.8 (2m, 2H, H-2'a, isobutyryl -CH), 2.5 (s, 3H, Me), 2.1 (m, 1H, H-2'b), 1.2 (m, 6H, isobutyryl-Me).

5'-O-(4,4'-dimethoxytrityl)-N^2-isobutyryl-8-methyl-2'-deoxyguanosine, 3'-H-phosphonate, triethylamine salt. A solution of N^2-isobutyryl-8-methyl-2'-deoxyguanosine (0.660 g, 1.88 mmol) in pyridine (50 mL) was treated with of 4,4'-dimethoxytrityl chloride (1.5 g, 4.43 mmol). After 2 h of stirring at room temperature, the mixture was partitioned between ethyl acetate and water, washed with water, then brine, and evaporated. The residue was chromatographed on a silica gel column using methylene chloride/methanol (9:1, v:v) to afford 0.74 g (60 %) of the 5'-dimethoxytrityl derivative. This material (0.5 g, 0.764 mmol) was dissolved in pyridine (25 mL), the resulting solution was chilled to 5°C and a solution of 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one in methylene chloride (1 M, 1.5 mL) was added. After 30 min, the reaction was quenched with ice cold triethylammonium bicarbonate buffer (1 M, 20 mL, pH 7.5) and the solution was extracted with methylene chloride. The organic extracts were evaporated, and the residue was chromatographed on a silica gel column using acetonitrile/water 9:1 v:v (containing 1% triethylamine). The residue left after evaporation of the product containing fraction was coevaporated with acetonitrile. Finally, the residue was dissolved in methylene chloride, and evaporated to afford 320 mg (51%) of the title compound as a crisp foam. ^{1}H-NMR (DMSO-d_6): 12.0, 11.7 (2bs, 2H, NH), 8.6-6.5 (m, 13H, DMT), 7.7, 5.7 (d, 1H, P-H), 6.2 (t, 1H, H-1'), 5.7 (m, 1H, H-3'), 4.0 (m, 1H, H-4'), 3.7 (s, 6H, OMe), 3.2 (m, 3H, H-5'ab, isobutyryl -CH), 2.9 (q, 6H, triethylammonium CH_2), 2.7, 2.3 (2m, 2H, H-2'ab), 2.5 (s, 3H, Me), 1.2 (m, 15H, isobutyryl-Me, triethylammonium-CH_3).

This material must be immediately converted to the DBU salt. The phosphonate was dissolved in methylene chloride (250 mL) the solution was shaken with DBU-bicarbonate buffer (0.1 N, 100 mL, pH=8.5). The organic layer was evaporated, and the residue was coevaporated with acetonitrile (5 x 50 mL) to yield material for DNA synthesis.

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