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SITE OF CHEMICAL MODIFICATIONS IN CpG CONTAINING PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDE MODULATES ITS IMMUNOSTIMULATORY ACTIVITY

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Abstract: Phosphorothioate oligodeoxynucleotides containing CpG motifs have immunostimulatory activity. Appropriate substitution of deoxynucleosides in the flanking region of CpG-containing phosphorothioate oligodeoxynucleotides with 2'-*O*-methylribonucleosides results in significant decreases or increases in their immunostimulatory activities. The results provide insights in how to chemically modify phosphorothioate oligodeoxynucleotides containing CpG motifs to suppress or enhance their immunostimulatory activity for different therapeutic uses.

Bacterial DNA or synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides (CpG motif) have been found to be immunostimulatory.[1,2] These sequences are known to enhance the natural killer cell activity of murine lymphocytes,[3] induce interferon-α and -γ secretion[4,5] and have shown antitumor activities.[6] Detailed studies have shown that the presence of a CpG motif and the nucleotide composition of its flanking sequence are key factors in the immunostimulatory activity.[3]

Phosphorothioate oligodeoxynucleotides (PS-oligos) are analogs of phosphodiester oligonucleotides in which one of the non-bridged oxygens of the internucleotide linkage is substituted by a sulfur. PS-oligos have been shown to be immunostimulatory.[7,8] A detailed study of PS-oligos demonstrated that the presence of CpG motif is primarily responsible for its immunostimulatory activity and that the immunostimulatory activity of PS-oligos is greater than that of their phosphodiester counterparts. The immunostimulatory activity of a PS-oligo containing a CpG motif also depends on the nucleotide in its flanking sequence.[9] Methylation of the cytosine of the CpG[9] and other modifications involving the internucleotide linkages or deoxyribose sugar of the CpG motif abolish the immunostimulatory activity.[10] PS-oligo containing CpG motif induce various cytokines including interferon-γ, IL-6, IL-12, TNF-α[11-14] and also chemokines.[13] Based on the therapeutic effects of some of these cytokines, PS-oligo containing CpG motifs have recently been used as anti-viral,[15,16] and anti-bacterial agents[17] and also as adjuvants[18] in various model studies.

PS-oligos of varying lengths and base compositions continue to be extensively studied as antisense agents.[19] Our earlier studies have shown that the side effects observed with PS-oligos are primarily due to their immunostimulatory activities, the severity of which is increased by the presence of a CpG motif.[19] Appropriate modifications of the CpG motif in PS-oligos can minimize these side effects.[19,20] We have found that substitution of certain deoxynucleosides in the flanking region of PS-oligos containing a CpG motif with 2'-*O*-methylribonucleosides (2'-OMe), has no effect or may actually increase the immunostimulatory activity. Thus, certain modifications may lead to oligonucleotides with increased side effects due to their increased immunostimulatory activity and could affect the mechanism of action. In addition, PS-oligos are being used as immune stimulators[15-18] and increased immunostimulatory activity would be beneficial.

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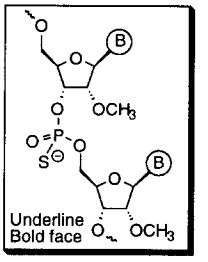
In the present study, we have made systematic modifications to PS-oligos containing CpG motif. We show that the site of incorporation of the modifications in the PS-oligo is a critical factor in modulating its immunostimulatory activity. Modifications described in this report involve substitution of a specific deoxynucleoside in the flanking region of the CpG motif of the PS-oligos with 2'-OMe. Some of these substitutions have no effect on the nuclease stability of the PS-oligo, while others provide a significant increase in nuclease stability.

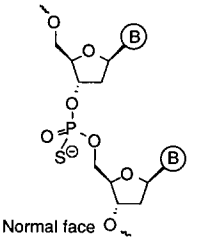
To study the impact of the site of chemical modification of PS-oligos containing CpG motif, we chose two oligonucleotides: Oligo **1**, which contains one CpG motif and Oligo **15**, which contains two CpG motifs. Both of these oligos have been studied earlier and are immunostimulatory.[14,16] To evaluate the immunostimulatory activity of oligonucleotides in the present study, we have used mouse spleen lymphocyte proliferation assay and splenomegaly in mice following single-dose administration.

All the oligonucleotides used in the present study were synthesized using an automated synthesizer and the phosphoramidite approach. At the desired site, incorporation of 2'-OMe was carried out using appropriate 2'-OMe phosphoramidite. The purity of each oligonucleotide was checked by capillary gel electrophoresis and the molecular weight was confirmed by MALDI-TOF Mass spectra analysis.[21]

Table 1. Oligodeoxynucleotide Phosphorothioates and Site of Modifications

Oligo No.	*Sequence & Modification (5'-3')*
1	TCCATGACGTTCCTGATGC
2	TCCAT**GA**CGTTCCTGATGC
3	TCCA**UG**ACGTTCCTGATGC
4	TCC**AU**GACGTTCCTGATGC
5	TC**CA**TGACGTTCCTGATGC
6	TCCATGACG**UU**CCTGATGC
7	TCCATGACGT**UC**CTGATGC
8	TCCATGACGTT**CC**TGATGC
9	TCCATGACGTTC**CU**GATGC
10	TCCATGACGTTCC**UG**ATGC
11	TCCATGACGTTCCT**GA**TGC
12	TC**CA**TGACGTTC**CU**GATGC
13	TCCATGACGTTCCTG**AUGC**
14	TC**CA**TGACGTTCCTG**AUGC**
15	TCCATGACGTTCCTGACGTT
16	TCC**AU**GACGTTCCTGACGTT
17	TCCATGACGTTC**CU**GACGTT
18	TCC**AU**GACGTTC**CU**GACGTT





Mouse (CD1) spleen lymphocytes were cultured with oligonucleotides at concentrations of 0.1, 1, and 10 μg/mL for 48 h and cell proliferation was determined by ^{3}H-uridine incorporation as previously described.[10] Oligo **1** induced a dose-dependent effect on cell proliferation. At 0.1 μg/mL, the proliferation index was 2.87 ± 0.28 (Fig. 1). We selected the 0.1 μg/mL concentration for a comparative study with the oligonucleotides listed in Table 1. Substitution of the 5'-flanking GA deoxynucleosides of the CpG motif of Oligo **1** with 2'-OMe (Oligo **2**), resulted in complete suppression of cell proliferation at all concentrations

used (Fig 1). At 0.1 μg/mL, the cell proliferation index was similar to that observed with medium alone. Substitution of the 3'- flanking TT deoxynucleosides of the CpG motif of Oligo **1** with 2'-OMe (Oligo **6**) did not affect cell proliferation: the proliferation index with Oligo **6** was 2.07 ± 0.55 (Fig. 1).

To further understand if substitution of two deoxynucleosides in the 5'-flanking region with 2'-OMe away from the CpG motif would have any effect on cell proliferation, we synthesized Oligos **3**, **4** and **5** (Table 1). Two deoxynucleosides were substituted with 2'-OMe leaving one, two and three deoxynucleosides respectively, between the site of substitution and the CpG motif. The proliferation indices of Oligo **3**, **4**, and **5** were 3.42 ± 0.41, 8.44 ± 2.08, and 10.38 ± 1.15, respectively. These values represent increases of 29%, 297% ($P < 0.05$) and 400% ($P < 0.01$), respectively, compared with Oligo **1** (Fig. 1). Substitution of the remaining deoxynucleosides closer to the 5'-end than in oligo **5** produced no further increase in the proliferation index (data not shown).

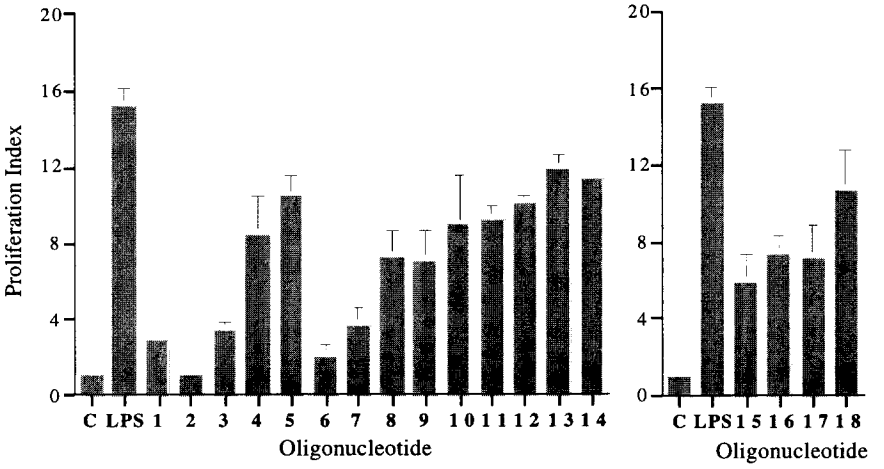


Figure 1. Proliferation indices of mouse spleen lymphocytes cultured in the presence of medium (C), LPS (lipopolysacharide) at 10 μg/mL and various oligonucleotides (Oligos **1-18**) at 0.1 μg/mL concentration. The cell proliferation assay was performed under the same experimental conditions as reported earlier.[10] Data are presented as proliferation index compared to cells cultured with medium alone. The assays were performed in triplicate for at least three times.

Similarly, substitutions were made in oligo **1** in the 3'- flanking region to the CpG motif. Oligo **7**, **8**, **9**, **10**, and **11** were synthesized, in which two deoxynucleosides were substituted with 2'-OMe, leaving one, two, three, four, and five deoxynucleosides respectively, between the CpG motif and the 2'-OMe substitution. The proliferation indices of Oligo **7**, **8**, **9**, **10** and **11** were 3.63 ± 0.89, 7.22 ± 1.37, 7.01 ± 1.63, 8.85 ± 2.63, and 9.24 ± 0.65, respectively. Compared to Oligo **1**, the increases in proliferation index for Oligo **7**, **8**, **9**, **10**, and **11** were 39%, 231% ($P < 0.05$), 221% ($P < 0.05$), 317%, and 338% ($P < 0.01$), respectively, compared with Oligo **1**.

From these results, it is evident that substitution of two deoxynucleotides in the flanking sequence at either the 5'-end or the 3'-end away from the CpG motif (e.g. Oligo **5** or **9**) increases the immunostimulatory

activity. To test whether the substitutions made in Oligo **5** and Oligo **9** have additive effects to further increase the immunostimulatory activity, we synthesized Oligo **12**, which had two deoxynucleosides that were substituted with 2'-OMe at both the 3'- and 5'-end. Oligo **12** did not show a further increase in the proliferation index when compared with Oligo **5**, but it had a higher proliferation index than did Oligo **9**.

To explore if the above observations made with the use of Oligos **3** to **5** and Oligos **7** to **11** are sequence specific or general, we made the same modifications to Oligo **15**, which contains two CpG motifs. Oligo **15** had a proliferation index of 5.83 ± 1.46 at a concentration of 0.1 μg/mL. Substitution of two deoxynucleosides at 5'- ends of individual CpG motifs leaving two deoxynucleosides between the CpG motif and substitution with 2'-OMe produced Oligos **16** and **17**, which had proliferation indices of 7.34 ± 0.99 and 7.13 ± 1.67, respectively, an increase of 20% compared with Oligo **15**. An oligonucleotide with two deoxynucleosides substituted in the 5'- flanking region of both CpG motifs (Oligo **18**) had a higher proliferation index 10.62 ± 2.12, which is an increase of about 50% compared with Oligo **15**.

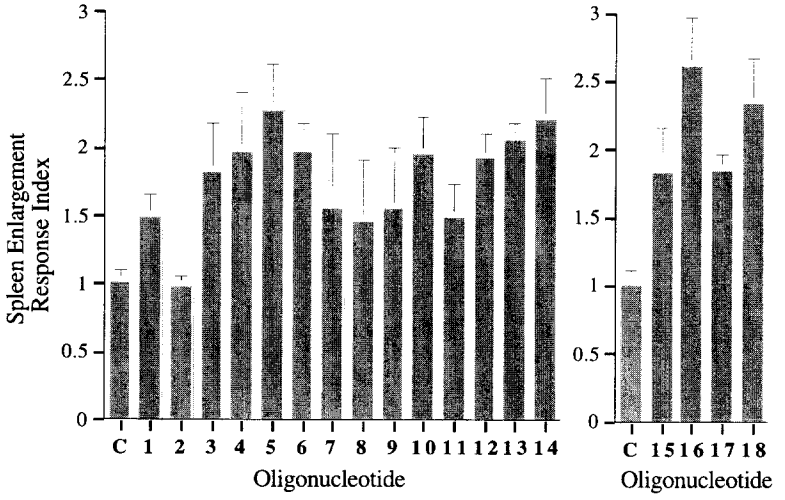


Figure 2. Spleen enlargement of mice following administration of various oligonucleotides (Oligos **1-18**). C-represents mice received vehicle alone. Female CD1 mice (4-5 weeks, 20-22 g) were injected intraperitoneally with a dose of 10 mg/kg of Oligos **1-14** and 5 mg/kg of Oligos **15-18** in 0.25 mL of sterile PBS. At least three animals were used for each oligonucleotide. Mice were sacrificed 72 h later, spleens were removed, blotted to dryness and weighed. Spleen enlargement response index is calculated based on spleen weight of mice received oligonucleotides compared to mice received vehicle alone.

In addition to investigating the effects of site-specific substitution, we tested whether the increased metabolic stability of PS-oligos containing CpG motifs would result in increased cell proliferation and whether that could be combined with 5'-substitutions to further increase the cell proliferation activity. Oligo **13** was synthesized, in which four continuous deoxynucleosides at the 3'- end of Oligo **1** were substituted with 2'-OMe, which results in a significant increase in stability towards nucleases.[22] Oligo **13** had a proliferation index of 11.92 ± 0.65 ($P < 0.01$), which is an increase of about 480% compared with Oligo **1**

(Fig. 1). Further modification of Oligo **13** by substitution of two deoxynucleosides at the 5'-end (Oligo **14**) did not result in a further increase in the proliferation index.

After observing that the above substitutions in PS-oligos modulate the immunostimulatory activity as observed in the cell culture assay, we administered the oligonucleotides listed in Table 1 intraperitoneally to mice and measured the spleen weights to confirm that if substitutions have same effect *in vivo*. Administration of Oligo **1** caused about 50% increase in spleen weight (Fig. 2). Oligo **2**, which had shown no immunostimulatory activity in cell culture assay, produced no significant increase in spleen weights (Fig. 2). Substitution of two deoxynucleosides away from the CpG motif towards the 5'-end, Oligo **3**, **4**, and **5** produced progressive increases in spleen weights, which were 67%, 95% ($P < 0.05$) and 157% ($P < 0.05$) greater, respectively, than those of mice treated with Oligo **1** (Fig. 2). This results supports previous observations that these oligonucleotides have greater immunostimulatory activity than does Oligo **1**. Substitution of two deoxynucleosides with 2'-OMe toward the 3'-end of the CpG motif produced in general a less significant increase in spleen weight and correlation with cell proliferation data. Only oligo **6** and **11** caused an increase in spleen weight of 97% and 95% compared to Oligo **1**. Oligo **12**, which had substitutions made at both the 3'- and 5'-end, showed 95% increase in spleen weight compared with Oligo **1** (Fig. 2).

Oligos **15**, **16**, **17**, and **18**, all of which contain two CpG motifs, produced increases in spleen weights following administration of a dose of 5 mg/kg. Oligo **15** caused a 175% increase in spleen weight compared with untreated mice. Oligo **16**, in which two of the 5'-end CpG motifs were substituted with 2'-OMe, caused a 93% increase in spleen weight compared with Oligo **15** (Fig. 2). Oligo **17**, which had a substitutions at the 5'-end of one CpG (and at the 3'-end of the other CpG motif) produced no further increase in spleen weight. An oligonucleotide with substitutions at the 5'-ends of both CpG motifs (Oligo **18**) did not produce further increases in spleen weight compared with oligo **16** and produced only about a 60% increase over that seen with Oligo **15** (Fig. 2).

Oligo **13**, which is more metabolically stable than Oligo **1**, produced 114% ($P < 0.05$) increase in spleen weight compared with Oligo **1**. This observation supports the cell proliferation data. Similar results were observed with Oligo **14**, which has greater metabolic stability and is substituted at the 5'-end of the CpG motif (Fig. 2).

The above results suggest that the immunostimulatory activity of PS-oligos containing CpG motifs can be modulated by introducing modifications in their flanking sequences at the 3'-end or 5'-end. The site of modification determines the effect of the modification on increasing or suppressing immunostimulatory activity. Substitution of the deoxynucleoside immediately next to the CpG motif at the 5'-end significantly suppressed the immunostimulatory activity, while similar substitutions at the 3'-end had no suppressive effect. Substitutions made in the 5'- or 3'-flanking region produced significant increases in the immunostimulatory activity of the PS-oligo. Similarly, modifications that increase the nuclease stability also increased the immunostimulatory activity.

The results described here are important because the increased potency of CpG-containing PS-oligos may be beneficial as these oligonucleotides are used as immune stimulators. At the same time, PS-oligos that are being used as antisense agents may be burdened with nonsequence-specific effects as a result of the immunostimulatory activity of CpG motifs and modifications. Based on the results described here, while

making modification of PS-oligos containing CpG motif, to improve antisense, special consideration should be given. Further studies are ongoing to understand the impact of substitution of other 2'-*O*-alkylribonucleosides on immunostimulatory activity of PS-oligos.

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21. The oligonucleotides were synthesized in 1 μmol scale on an automated DNA synthesizer (Expedite 8909 PerSeptive Biosystems). All nucleoside phosphoramidites were obtained from PerSeptive Biosystems. Mass spectra were recorded on a Bruker Proflex III MALDI-TOF mass spectrometer with 337 nm N_2 laser. Molecular weight observed and calculated (in brackets) for each oligonucelotide were as follows: Oligo **1**, 6041, (6043); Oligo **2**, 6103, (6103); Oligo **3**, 6091, (6089); Oligo **4**, 6091, (6089); Oligo **5**, 6105,(6103); Oligo **6**, 6075, (6075); Oligo **7**, 6087, (6089); Oligo **8**, 6102, (6103); Oligo **9**, 6089, (6089); Oligo **10**, 6090, (6089); Oligo **11**, 6102, (6103); Oligo **12**, 6151, (6149); Oligo **13**, 6149, (6149); Oligo **14**, 6181, (6209); Oligo **15**, 6364, (6363); Oligo **16**, 6410, (6409); Oligo **17**, 6407, (6409); Oligo **18**, 6458, (6455).
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