Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 2585–2588

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Accessible 5′-End of CpG-Containing Phosphorothioate Oligodeoxynucleotides is Essential for Immunostimulatory Activity

Dong Yu, Qiuyan Zhao, Ekambar R. Kandimalla and Sudhir Agrawal*

Hybridon, Inc., 345 Vassar Street, Cambridge, MA 02139, USA

Received 13 July 2000; accepted 1 September 2000

Abstract—In our ongoing efforts to decipher the sequence and structural requirements in the flanking region of the CpG motif in phosphorothioate oligodeoxynucleotides (PS-oligos), we have examined the requirement of free 5′- and 3′-ends of PS-oligos on immune stimulation. Our model studies using 3′–3′-linked (containing two free 5′-ends) and 5′–5′-linked (containing two free 3′-ends) CpG-containing PS-oligos demonstrate that immunostimulatory activity is significantly reduced when the 5′-end of the PS-oligo is not accessible, rather than the 3′-end, suggesting that the 5′-end plays a critical role in immunostimulatory activity.

Bacterial DNA and synthetic single-stranded oligodeoxynucleotides containing unmethylated CpG motifs activate cells of the innate immune system. Tokunaga et al. originally reported that mycobacterial DNA and oligonucleotides containing palindromic sequences stimulate murine MK cells, induce interferons, and have antitumor activity.[1–3] In subsequent studies, it was reported that bacterial DNA induces proliferation of murine B cells[4] similar to that observed with synthetic PS-oligos containing unmethylated CpG dinucleotides (CpG-PS-oligos) with appropriate flanking sequences.[5] Activation of immune cells (B-cells, macrophages, and dendritic cells) appears to occur upon CpG-oligo uptake, resulting in the activation of the stress kinase pathways and NF-κB[6,7] and induction of various cytokines including IL-6, IL-12, γ-IFN, and TNF-α.[8–11] In a number of recent studies the use of CpG-oligos as antiviral and anticancer agents, and as vaccine adjuvants, has been demonstrated.[12–15]

Our laboratory has been studying the impact of chemical modifications on the immunostimulatory activity of CpG-PS-oligos.[16–19] Modification of the phosphorothioate internucleotide linkages between the CpG motif of CpG-PS-oligo or substitution of the CpG motif with 2′-*O*-methylribonucleoside resulted in a significant loss of immunostimulatory activity.[16] Our recent studies indicated that substitution of the first, second, or both deoxynucleosides in the 5′-flanking region immediately next to the CpG motif in CpG-PS-oligos with 2′-*O*-methylribonucleosides also caused a decrease in immunostimulatory activity, while the same substitutions in the 3′-flanking deoxynucleoside had no significant effect.[18] Substitution of the deoxynucleoside immediately next to the CpG motif in CpG-PS-oligo in both the 5′- and 3′-ends with 3′-*O*-methylribonucleoside resulted in the loss of immunostimulatory activity. Substitution of one or two deoxynucleosides away from the CpG motif in CpG-PS-oligo in the 5′-flanking region, either by 2′-substituted ribonucleosides (2′-*O*-methylribonucleoside, 2′-*O*-methyoxyethoxyribonucleoside) or 3′-*O*-methylribonucleoside, resulted in increased immunostimulatory activity. Similar substitution in the 3′-flanking region had less of an impact on the immunostimulatory activity than did 5′-substitutions. These observations suggest that the 5′-flanking region of CpG-PS-oligos plays an important role in immunostimulatory activity.

To confirm this observation, we carried out the study described herewith in which the 5′- or 3′-end of CpG-PS-oligo was blocked and studied its impact on immunostimulatory activity. We chose two oligonucleotide sequences, oligos **1** and **4** (Table 1), each containing two CpG motifs. Both oligos **1** and **4** have shown immunostimulatory activity in earlier studies. We have synthesized oligos **2**–**4** and **6**–**8** in which two units of oligo **1** or **4**, respectively, are attached by 3′–5′- or 5′–5′- or

*Corresponding author. Tel.: +1-617-679-5501; fax: +1-617-679-5582; e-mail: sagrawal@hybridon.com

0960-894X/00/$ - see front matter
PII: S0960-894X(00)00537-0

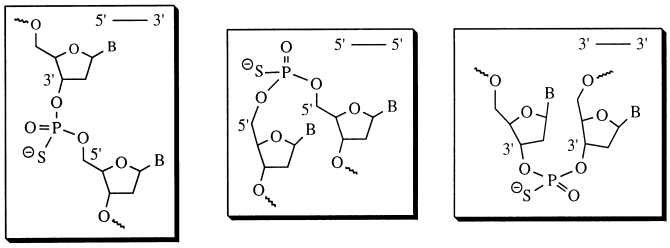
3′–3′-linkage. We have evaluated these oligos for immunostimulatory activity using mouse spleen lymphocyte proliferation assay and splenomegaly in mice following single-dose administration.[16]

Synthesis of CpG-PS-oligos

CpG-PS-oligos used in the present study (Table 1) were synthesized using an automated synthesizer and phosphoramidite approach as outlined in Figure 1. Oligo **1** (16-mer) was synthesized using nucleoside-5′-β-cyanoethylphosphoramidites. Oligo **2**, a 32-mer, was synthesized using nucleoside-3′-β-cyanoethylphosphoramidites and controlled pore glass support (CPG-solid support) with a 3′-linked nucleoside in which the 16-mer sequence of oligo **1** was repeated twice; therefore, oligo **2** had two 16-mers (oligo **1**) linked by a normal 3′–5′-linkage (Fig. 1). Oligo **3**, a 32-mer, was synthesized with two 16-mers (oligo **1**) linked by a 5′–5′-linkage, so oligo **3** had two 3′-ends and no 5′-end. Synthesis of oligo **3** was carried out in two steps: the first 16-mer was synthesized

Table 1. Oligodeoxynucleotide phosphorothioates and modification of linkages

Oligo no.	Sequence and modification	Molecular weight	
		Calculated	Found
1	5′-G A G A A C G C T C G A C C T T-3′	5106	5108
2	5′-G A G A A C G C T C G A C C T T-3′ —— 5′-G A G A A C G C T C G A C C T T-3′	10,290	10,288
3	3′-T T C C A G C T C G C A A G A G-5′ —— 5′-G A G A A C G C T C G A C C T T-3′	10,290	10,288
4	5′-G A G A A C G C T C G A C C T T-3′ —— 3′-T T C C A G C T C G C A A G A G-5′	10,290	10,290
5	5′-T C T C C C A C G T G C G C C A T-3′	5683	5685
6	5′-T C C C A G C G T G C G C C A T-3′ —— 5′-T C C C A G C G T G C G C C A T-3′	10,194	10,191
7	3′-T A C C G C G T G C G A C C C T-5′ —— 5′-T C C C A G C G T G C G C C A T-3′	10,194	10,192
8	5′-T C C C A G C G T G C G C C A T-3′ —— 3′-T A C C G C G T G C G A C C C T-5′	10,194	10,196



⟶ 5′–3′-direction of CpG motif.

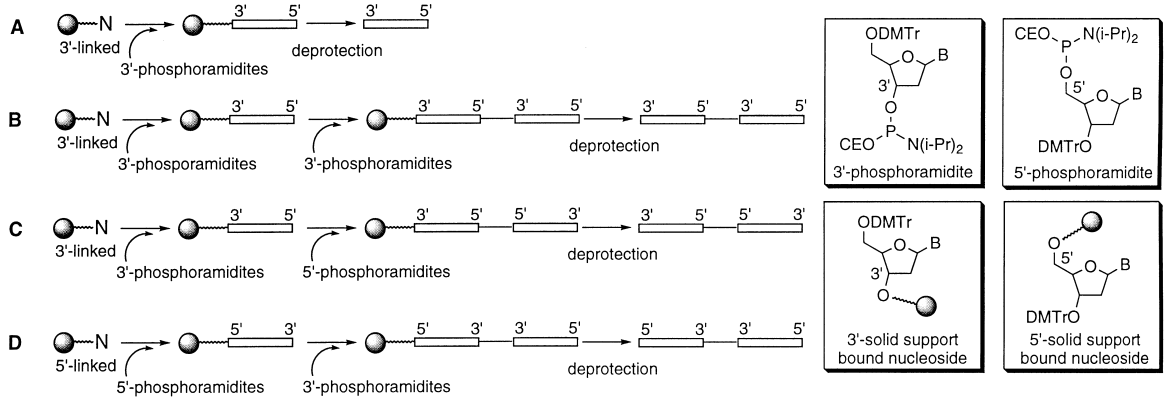


Figure 1. Schematic representation of synthetic cycles followed for the synthesis of oligos **1** and **5** (A), oligos **2** and **6** (B), oligos **3** and **7** (C), and oligos **4** and **8** (D). Structures of the two different phosphoramidites and nucleoside-linked CPG solid supports used for the synthesis of oligos **1**–**8** are shown in boxes.

using nucleoside-3′-β-cyanoethylphosphoramidites and solid support with a 3′-linked nucleoside, and then synthesis of the second 16-mer segment was continued using nucleoside-5′-β-cyanoethylphosphoramidites (Fig. 1). Oligo **4**, a 32-mer, comprised two 16-mers (oligo **1**) linked by a 3′–3′-linkage, so oligo **4** had two 5′-ends and no 3′-end. Synthesis of oligo **4** was carried out in two steps: the first 16-mer was synthesized using nucleoside-5′-β-cyanoethylphsphoramidites and solid support with a 5′-linked nucleoside, and the synthesis of the second 16-mer segment was continued using nucleoside-3′-β-cyanoethylphosphoramidites. Synthesis of oligos **5**–**8** was carried out by using the same nucleoside-β-cyanoethylphosphoramidites as for oligos **1**–**4**, respectively. At the end of the synthesis, oligos **1**–**8** were deprotected with concentrated ammonia solution, purified by reversed phase HPLC, detritylated, desalted and dialyzed. The purity of each PS-oligo was checked by CGE and the molecular weight was confirmed by MALDI-TOF mass spectral analysis (Table 1). The sequence integrity and directionality of 5′-CpG motif in oligos **1**–**8** were confirmed by recording melting temperatures (T_ms) of the duplexes with their respective DNA complementary strands (5′-AAGGTCGAGCGTTCTC-3′ for oligos **1**–**4**, and 5′-ATGGCGCACGCTGGGAGA-3′ for oligos **5**–**8**) as described earlier. The T_ms of these duplexes were 53.9±0.9 °C (oligos **1**–**4**), 61.8 °C (oligo **5**), and 58.8±0.6 °C (oligos **6**–**8**) (note that oligo **5** was a 18-mer and oligos **6**–**8** were 32-mers but not 36-mers).

Mouse Spleen Lymphocyte Proliferation Assay

Immunostimulatory activity of CpG-PS-oligos was studied initially in a lymphocyte proliferation assay. Typically, mouse (Balb-C) spleen lymphocytes were cultured with CpG-PS-oligos at concentrations of 0.1, 1.0, and 10.0 μg/mL for 48 h and cell proliferation was determined by ^{3}H-uridine incorporation, as described previously.[16]

Oligo **1** induced a dose-dependent effect on cell proliferation; at a concentration of 10 μg/mL (~2.0 μM), the proliferation index was 5.0±0.32 (Fig. 2). Oligo **2**, which consisted of two units of oligo **1** linked by a 3′–5′-linkage, had a proliferation index of 5.8±0.28 at the same dose (~1.0 μM). Oligo **3**, which consisted of two units of oligo **1** linked by a 5′–5′-linkage, had a proliferation index of 2.0±0.26, reflecting a significantly lower immunostimulatory activity than observed with oligos **1** and **2**. Oligo **4**, which consisted of two units of oligo **1** linked by a 3′–3′-linkage, had a proliferation index of 7.2±0.5, reflecting a greater immunostimulatory activity than observed with oligos **1** and **2**.

Similar results were obtained with oligos **5**–**8** (Fig. 2). Oligo **5** had a proliferation index of 3.9±0.12. Oligos **6**–**8**, in which two units of oligo **5** are linked by a 3′–5′-linkage (oligo **6**), 5′–5′-linkage (oligo **7**), and 3′–3′-linkage (oligo **8**) had proliferation indices of 4.9±0.2, 1.74±0.21, and 7.7±0.82, respectively. Comparison of the results obtained with oligos **6**–**8** show that oligos **6** and **8**, in which two oligo **5** sequences were linked by a 3′–5′-linkage or a 3′–3′-linkage had greater immunostimulatory activity, while oligo **7**, in which two units of oligo **5** were linked by a 5′–5′-linkage had significantly less immunostimulatory activity, than did oligo **5**.

Based on lymphocyte proliferation results of oligos **1**–**8**, it is clear that when oligos are linked through their 5′-ends, there is a significant loss of immunostimulatory activity, while if they are linked through their 3′-ends, there is an increase in immunostimulatory activity. It is important to note that 3′–3′-linked oligos have shown substantially greater stability towards degradation by exonucleases than the oligos that contained a free 3′-end,[20] which could also result in increased immunostimulatory activity. The lower immunostimulatory activity of oligos **3** and **7**, in which the 5′-end of oligos is blocked, suggests that accessibility to the 5′-end of oligo is essential for immunostimulatory activity of CpG-PS-oligos.

Splenomegaly in Mice

To confirm the immunostimulatory activity of oligos **1**–**8** in vivo, a dose of 5 mg/kg of oligonucleotides was injected intraperitoneally to Balb-C mice. The mice were sacrificed 72 h post-administration, spleens were removed, blotted to dryness, and weighed. Change in spleen weight in treated and untreated mice was used as a parameter for immunostimulatory activity.

Administration of a 5 mg/kg dose of oligo **1** caused about 40% increase in spleen weight compared with the control

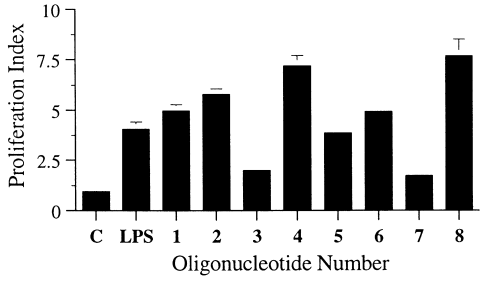


Figure 2. Proliferation indices of oligos **1**–**8** in mouse spleen lymphocyte cultures at 10 μg/mL dose. C and LPS represent proliferation in the presence of medium (control) and lypopolysaccharide, respectively. The assays were performed in triplicate at least three times.

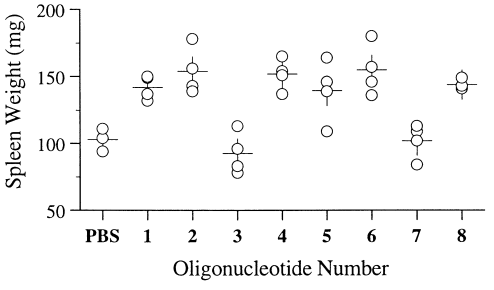


Figure 3. Spleen enlargement in mice following administration of oligos **1**–**8** at a dose 5 mg/kg intraperitoneally. Control mice received vehicle (PBS). Four animals were used for each oligo treatment. Average values are indicated by a large + sign.

mice that received PBS (Fig. 3). Administration of oligos **2** and **4** also caused about 50% increase in spleen weight. Administration of oligo **3** caused no difference in spleen weight compared with control mice. These results further support the observation that oligo **3**, in which the 5′-end was blocked, had significantly less immunostimulatory activity compared to oligos that had an accessible 5′-end. These results were also confirmed with the administration of oligos **5**–**8**. Administration of oligos **5**, **6**, and **8** caused about 40–50% increase in spleen weight, whereas no change in spleen weight was observed following the administration of oligo **7**.

The above results suggest that the immunostimulatory activity of PS-oligos containing a CpG motif is significantly minimized if the 5′-end of the oligo is not accessible. This loss in immunostimulatory activity of oligos **3** and **7** cannot be explained based on nuclease stability, as both oligos have two 3′-ends and are not more susceptible to 3′-exonuclease degradation than are oligos **1**, **2**, **5**, and **6**, which have one 3′-end. PS-oligos **4** and **8**, which have their 3′-ends blocked and are very stable to degradation by exonucleases, showed similar immunostimulatory activity. Oligos **4** and **8** may show sustained immunostimulatory activity due to their increased in vivo stability, which is not evident in the present study as mice were sacrificed at only 72 h after administration. Studies are in progress in which mice will be sacrificed at times later than 72 h after administration.

The results described here are intriguing and suggest that the 5′-end of CpG-PS-oligos is critical for immunostimulatory activity. In our previous studies, we have shown that substitution of deoxynucleosides in 5′-flanking regions by modified 2′- or 3′-substituted ribonucleosides resulted in increased immunostimulatory activity. In addition, substitution of deoxynucleosides immediately upstream (5′-end) to the CpG motif caused a significant suppression, and substitution of deoxynucleosides immediately downstream (3′-end) to the CpG motif had no effect on immunostimulatory activity. Taken together, these results suggest that the enzyme/receptor responsible for the immunostimulation recognizes the CpG motif in oligos from the 5′-end and requires accessibility to the 5′-end. Detailed studies are under way to examine this hypothesis. Conjugation of antigens or allergens to immunostimulatory CpG-oligonucleotides has been shown to enhance immunotherapeutic potential of antigens several fold compared to administration of unconjugated antigen and CpG oligonucleotide mixture.[21,22] Our present studies suggest that it would be more appropriate to conjugate antigens or allergens to the 3′-end of the immunostimulatory CpG-oligonucleotides rather than 5′-end for optimal immunostimulatory activity.

References

1. Tokunaga, T.; Yamamoto, H.; Shimada, S.; Abe, H.; Fukuda, T.; Fijisawa, Y.; Furutani, Y.; Yano, O.; Kataoka, T.; Sudo, T.; Makiguchi, N.; Suganuma, T. *J. Natl. Cancer Inst*. **1984**, *72*, 955.
2. Tokunaga, T.; Yano, O.; Kuramoto, E.; Kimura, Y.; Kataokam, T.; Yamamoto, S. *Microbiol. Immunol*. **1992**, *36*, 55.
3. Tokunaga, T.; Yamamoto, T.; Yamamoto, S. *Jpn. J. Infect. Dis*. **1999**, *52*, 1.
4. Messina, J. P.; Gilkeson, G. S.; Pisetsky, D. S. *J. Immunol*. **1991**, *147*, 1759.
5. Krieg, A. M.; Yi, A. K.; Matson, S.; Waldschmidt, T. J.; Bishop, G. A.; Teasdale, R.; Koretzky, G. A.; Kliman, D. M. *Nature* **1995**, *374*, 546.
6. Hacker, H.; Mischak, H.; Miethke, T.; Liptay, S.; Schimd, R.; Sparwasser, T.; Heeg, K.; Lipford, G. B.; Wagner, H. *EMBO J*. **1998**, *17*, 6230.
7. Yi, A. K.; Krieg, A. M. *J. Immunol*. **1998**, *161*, 4493.
8. Sparwasser, T.; Miethke, T.; Lipford, G. B.; Erdmann, A.; Haecker, H.; Heeg, K.; Wagner, H. *Eur. J. Immunol*. **1997**, *27*, 1671.
9. Sparwasser, T.; Koch, E. S.; Vabulas, R. M.; Lipford, G. B.; Heeg, K.; Ellart, J. W.; Wagner, H. *Eur. J. Immunol*. **1998**, *28*, 2045.
10. Klinman, D. M.; Yi, A. K.; Beaucage, S. L.; Conover, J.; Krieg, A. M. *Proc. Natl. Acad. Sci. U.S.A*. **1996**, *93*, 2879.
11. Zhao, Q.; Temsamani, J.; Zhou, R. Z.; Agrawal, S. *Antisense Nucleic Acid Drug Dev*. **1997**, *7*, 495.
12. Dunford, P. J.; Mulqueen, M. J.; Agrawal, S. *Antisense 97: Targeting the Molecular Basis of Disease* (Nature Biotechnology) Conference Abstract, 1997; p 40.
13. Agrawal, S.; Kandimalla, E. R. *Mol. Med. Today* **2000**, *6*, 72.
14. Chu, R. S.; Targoni, O. S.; Krieg, A. M.; Lehmann, P. V.; Harding, C. V. *J. Exp. Med*. **1997**, *186*, 1623.
15. Zimmermann, S.; Egeter, O.; Hausmann, S.; Lipford, G. B.; Rocken, M.; Wagner, H.; Heeg, K. *J. Immunol*. **1998**, *160*, 3627.
16. Zhao, Q.; Temsamani, J.; Idarola, P.; Jiang, Z.; Agrawal, S. *Biochem. Pharmacol*. **1996**, *51*, 173.
17. Agrawal, S.; Zhao, Q. *Antisense Nucleic Acid Drug Dev*. **1998**, *8*, 135.
18. Zhao, Q.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett*. **1999**, *9*, 3453.
19. Zhao, Q.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett*. **2000**, *10*, 1051.
20. Chaix, C.; Iyer, R. P.; Agrawal, S. *Bioorg. Med. Chem. Lett*. **1996**, *6*, 827.
21. Shirota, H.; Sano, K.; Kikuchi, T.; Tamura, G.; Shirato, K. *J. Immunol*. **2000**, *164*, 5575.
22. Togje, H.; Takabayashi, K.; Schwartz, D.; Van Nest, G.; Tuck, S.; Eiden, J. J.; Kagey-Sobotka, A.; Creticos, P. S.; Lichtenstein, L. M.; Spiegelberg, H. L.; Raz, E. *J. Allergy Clin. Immunol*. **2000**, *106*, 124.