# Immune-Stimulatory Dinucleotide at the 5′-End of Oligodeoxynucleotides Is Critical for TLR9-Mediated Immune Responses

Mallikarjuna R. Putta, Lakshmi Bhagat, Daqing Wang, Fu-Gang Zhu, Ekambar R. Kandimalla, and Sudhir Agrawal*

Idera Pharmaceuticals, Inc., 167 Sidney Street, Cambridge, Massachusetts 02139, United States

**ABSTRACT:** Oligodeoxynucleotides (ODNs) containing a CpG or certain synthetic dinucleotides, referred to as immune-stimulatory dinucleotides, induce Toll-like receptor 9 (TLR9)-mediated immune responses. Chemical modifications such as 2′-O-methylribonucleotides incorporated adjacent to the immune-stimulatory dinucleotide on the 5′-side abrogate TLR9-mediated immune responses. In this study, we evaluated the effect of the location of immune-stimulatory dinucleotides in ODNs on TLR9-mediated immune responses. We designed and synthesized ODNs with two immune-stimulatory dinucleotides, one placed toward the 5′-end region and the other toward the 3′-end region, incorporated 2′-O-methylribonucleotides selectively preceding the 5′- or 3′-immune-stimulatory dinucleotide or both, and studied TLR9-mediated immune responses of these compounds in cell-based assays and in vivo in mice. These studies showed that an immune-stimulatory dinucleotide located closer to the 5′-end is critical for and dictates TLR9-mediated immune responses. These studies provide insights for the use of ODNs when employed as TLR9 agonists and antagonists or antisense agents.

**KEYWORDS:** *immune stimulation, immune-stimulatory dinucleotide, NF-κB, oligodeoxynucleotides, Toll-like receptor 9*
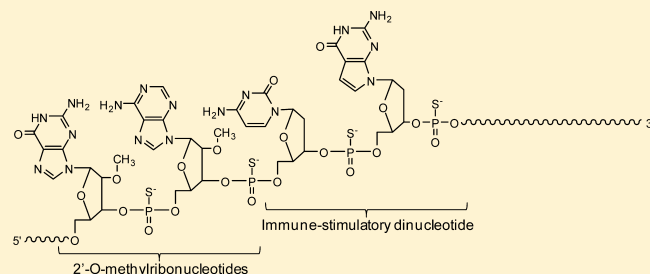


Synthetic phosphorothioate (PS) oligodeoxynucleotides (ODNs) are widely used as antisense agents.[1,2] The use of PS-ODNs for antisense applications has been limited, however, by the presence of unmethylated cytosine-phosphate-guanine (CpG) dinucleotides, referred to as immune-stimulatory dinucleotides, which induce Toll-like receptor 9 (TLR9)-mediated immune responses, including Th1-type cytokine secretion, B-cell proliferation, and up-regulation of costimulatory molecules.[3] TLR9 belongs to a family of pattern recognition receptors (PRRs) of the innate immune system and is involved in the recognition of pathogen-associated molecular patterns. TLR9 is expressed in the endolysosomal compartments of human B cells and plasmacytoid dendritic cells.[3,4] ODNs containing CpG dinucleotides and other ODNs, which contain synthetic immune-stimulatory dinucleotides in which C is replaced with 5-OH-dC, furano-dT, pyrrolo-dC, 4-thio-dU, N³-methyl-dC, N⁴-ethyl-dC, or arabinoC or G is replaced with 7-deaza-dG, N¹-methyl-dG, dI, 8-O-methyl-dG, or arabinoG, induce TLR9-mediated immune responses.[5−11]

In the course of our efforts to develop immune-stimulatory ODNs, we have shown that 5′-end accessibility is important and that ODNs lacking an accessible 5′-end do not induce TLR9-mediated immune responses.[5,12−15] In fact, our studies have shown that immune-stimulatory ODNs containing two 5′-ends induce greater TLR9-mediated immune responses than do ODNs containing a single 5′-end.[5,8,9,12,14] ODNs attached through their 5′-ends, which do not have an accessible 5′-end, do not induce TLR9-mediated immune responses.[12,14]

Our systematic studies have shown that chemical modifications, such as methylphosphonate linkages, 2′-O-alkyl-ribonucleotides, 3′-deoxy/O-alkyl-ribonucleotides, non-nucleotide linkers, or abasic nucleotides incorporated site specifically in the flanking sequence 5′ or 3′ to the immune-stimulatory dinucleotide, have a significant impact on immune-stimulatory activity.[5,16−23] 2′-O-Alkyl-ribonucleotide modifications introduced at the fourth to sixth nucleotide positions 5′ to the immune-stimulatory dinucleotide significantly enhance the immune-stimulatory activity of the ODN.[5,16,17,23] By contrast, ODNs with 2′-O-alkyl-ribonucleotide modifications incorporated adjacent to the immune-stimulatory dinucleotide on the 5′-side do not produce immune responses.[5,16,17,23] ODNs with 2′-O-alkyl-ribonucleotide modifications incorporated in the 3′-flanking sequence distal to the immune-stimulatory dinucleotide have immune-stimulatory activity equal to or greater than that of the unmodified parent ODN.[5,16,17,23]

Many CpG ODNs reported in the literature contain multiple immune-stimulatory dinucleotides.[24] It is not clear, however, how the position of each immune-stimulatory dinucleotide influences activity and which immune-stimulatory dinucleotide in the sequence is critical for TLR9-mediated immune responses. The present study was undertaken to elucidate the role of immune-stimulatory dinucleotide located closer to the 5′-end or the 3′-end of the ODN on the induction of TLR9-mediated immune responses in vitro and in vivo in mice.
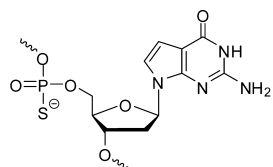
**Table 1. ODNs Used in the Study and Their Analytical Characterization**

G* = 2′-deoxy-7-deazaguanosine          G/A = 2′-O-methyl-ribonucleotide

| ODN no. | sequence[a] | mol. wt.[b] | | purity (% FLP)[c] | |
|---|---|---|---|---|---|
| | | calcd | obsd | HPLC | CGE |
| 1 | 5′-TCTGACG\*TTTTTTGACG\*TTCT-3′ | 6705 | 6707 | 97 | 96 |
| 2 | 5′-TCT<u>GA</u>CG\*TTTTTTGACG\*TTCT-3′ | 6766 | 6765 | 96 | 95 |
| 3 | 5′-TCTGACG\*TTTTTT<u>GA</u>CG\*TTCT-3′ | 6766 | 6768 | 97 | 95 |
| 4 | 5′-TCT<u>GA</u>CG\*TTTTTT<u>GA</u>CG\*TTCT-3′ | 6826 | 6826 | 96 | 95 |
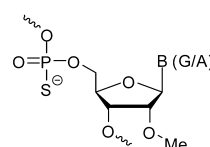
[a]All ODNs contain a PS backbone. G* = 7-deaza-dG; <u>G</u>/<u>A</u> = 2′-O-Me-G/A (structures are shown above). [b]Molecular weights of compounds as calculated (calcd) and determined (obsd) by MALDI-ToF mass spectral analysis. [c]Purity of ODNs as determined by anion-exchange HPLC and capillary gel electrophoresis (CGE); FLP is full-length product.

Synthetic ODNs containing unmethylated CpG* (G* = 7-deaza-dG) dinucleotides induce TLR9-mediated immune responses, including Th1-type cytokine secretion in vitro and in vivo.[5,6,8,9,11] In the present study, we designed 21-mer ODNs containing CpG* (ODNs 1−4) immune-stimulatory dinucleotides (Table 1). Each ODN contains two immune-stimulatory dinucleotides, one at the 5′-end and the second near the 3′-end, referred to as 5′- and 3′-immune-stimulatory dinucleotides, respectively (Table 1). 2′-O-Methylribonucleotide modifications incorporated preceding immune-stimulatory dinucleotides abrogate the immune-stimulatory activity of immune-stimulatory dinucleotide-containing ODNs.[5,16,17,23] Therefore, 2′-O-methyl-G and -A modifications were introduced in place of dG and dA nucleotides, respectively, preceding the immune-stimulatory dinucleotides to study the role of each immune-stimulatory dinucleotide in TLR9-mediated immune responses. ODN 1 contained no 2′-O-methylribonucleotide substitutions and served as a TLR9 agonist. ODN 2 had 2′-O-methyl-G and -A substitutions preceding the 5′-immune-stimulatory dinucleotide. ODN 3 contained 2′-O-methyl-G and -A substitutions preceding the 3′-immune-stimulatory dinucleotide. ODN 4 contained 2′-O-methylribonucleotide substitutions preceding both of the immune-stimulatory dinucleotides (Table 1). All four ODNs were synthesized on solid support using β-cyanoethylphosphoramidite chemistry on automated DNA synthesizers with a PS backbone. All ODNs contained ≥95% full-length product as characterized by anion-exchange HPLC and CGE (Table 1). The sequence integrity was determined by MALDI-ToF mass spectral analysis (Table 1).

We evaluated dose-dependent TLR9 stimulation by ODNs 1−4 as a function of NF-κB activation in HEK293 cells expressing mouse TLR9 and in control (LacZ) cells. The results obtained in HEK293 cells expressing TLR9 are presented as a fold increase in NF-κB activation over cells incubated with media alone (Figure 1). ODN 1 showed a dose-dependent TLR9-mediated NF-κB activation (Figure 1). ODN 2, in which the 5′-immune-stimulatory dinucleotide is preceded with 2′-O-methylribonucleotide substitutions, did not activate NF-κB, suggesting that the immune-stimulatory dinucleotide present toward the 5′-end region of the ODN dictates TLR9-mediated immune responses. ODN 3, which contained 2′-O-methylribonucleotide substitutions preceding the 3′-immune-stimulatory dinucleotide, activated NF-κB similar to ODN 1. ODN 4 did not cause TLR9-mediated NF-κB activation. None of the ODNs activated NF-κB in LacZ control cells at any concentration studied (data not shown).



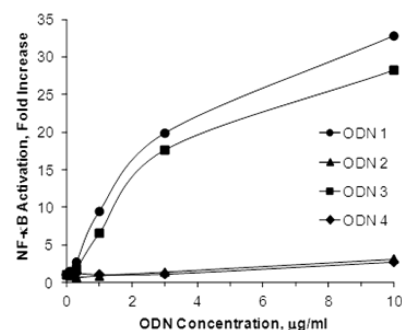**Figure 1.** Dose-dependent NF-κB activation by ODNs in HEK293 cells expressing mouse TLR9. Data shown are representative of three independent experiments.

We assessed NF-κB activation in murine macrophage J774 cells by electrophoretic mobility-shift assay. ODN 1 induced NF-κB activation as expected (Figure 2). ODN 2 failed to
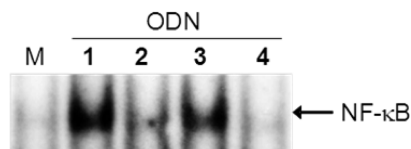


**Figure 2.** Activation of the transcription factor NF-κB in murine macrophage J774 cells. J774 cells were treated with PBS (M; medium) or 10 μg/mL ODN 1−4 (lanes as labeled with ODN numbers). Data shown are representative of two independent experiments.

induce NF-κB activation, suggesting that the presence of 2′-O-methylribonucleotide substitutions preceding an immune-stimulatory dinucleotide abrogates immune-stimulatory activity and that the 5′-immune-stimulatory dinucleotide is critical for TLR9-mediated immune stimulation (Figure 2). ODN 3 induced NF-κB activation at a level similar to that produced by ODN 1 (Figure 2). ODN 4 did not induce NF-κB activation (Figure 2). These results are consistent with the NF-κB activation results observed in HEK293 cell assays.

TLR9 agonists induce cytokine secretion in mouse spleen cell cultures. Cytokine induction by ODNs in mouse spleen cells was evaluated following incubation of spleen cells with 0−10 μg/mL ODN for 24 h and determining IL-12 and IL-6 levels by ELISA. ODN 1 strongly induced IL-6 and IL-12

production in mouse spleen cells (Figure 3). Comparison of the cytokine levels induced by ODNs **2** and **3** showed that ODN **2** induced significantly lower levels of both IL-6 and IL-12 as compared with ODN **3** (Figure 3). ODN **3** induced production
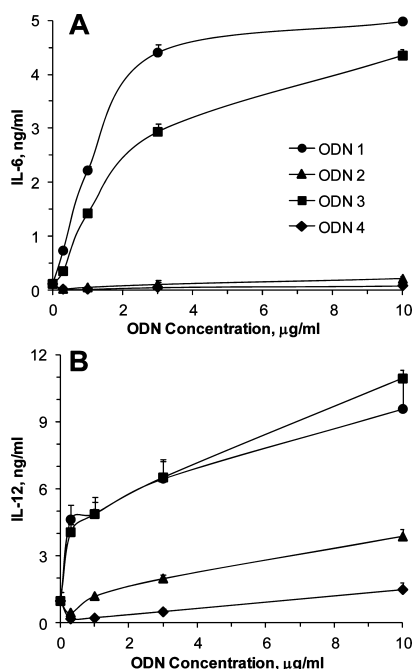


**Figure 3.** Dose-dependent (A) IL-6 and (B) IL-12 induction by ODNs in mouse spleen cell cultures. Spleen cells were cultured in the absence or presence of ODNs at various concentrations for 24 h, and supernatants were analyzed by ELISA for IL-6 and IL-12 levels. Each value is an average of three replicate wells ± SDs, and the data shown are representative of three independent experiments.

of IL-6 and IL-12 at levels comparable to ODN **1** (Figure 3). As expected, ODN **4** did not induce production of IL-6 and IL-12 at any concentration studied (Figure 3). These results are consistent with the data observed in HEK293 and J774 cell studies.

We studied the in vivo activity of ODNs in C57BL/6 mice. Mice were injected subcutaneously (sc) with ODNs **1**−**4** (1 or 20 mg/kg), and serum IL-12 levels were measured 2 h after the ODN administration. ODN **1** administration to mice led to elevated levels of IL-12 in serum (Figure 4). As observed in HEK293 and spleen cell cultures, ODN **2** did not induce TLR9-mediated IL-12 production in mice at doses of up to 20 mg/kg. ODN **3** induced TLR9-mediated IL-12 production at doses similar to ODN **1** (Figure 4). ODN **4** did not induce IL-12 production at any dosage studied (Figure 4).

Together, these results suggest that the immune-stimulatory dinucleotide present towards the 5′-end, but not the 3′-end, of the ODN is critical for and dictates TLR9-mediated immune responses. Incorporation of 2′-*O*-methylribonucleotide substitutions preceding the immune-stimulatory dinucleotides leads to loss of TLR9-mediated immune-stimulatory activity. These results further emphasize that the 5′-ends of ODNs play a key role in TLR9-mediated immune responses as we have demonstrated previously.[12−15]

## EXPERIMENTAL PROCEDURES

All ODNs were synthesized on a MerMade 6 DNA/RNA synthesizer (Bioautomation, Inc., Plano, TX) with a PS backbone using β-cyanoethylphosphoramidite chemistry on a 10 μmol scale. After the synthesis, ODNs were cleaved from the solid support, deprotected,
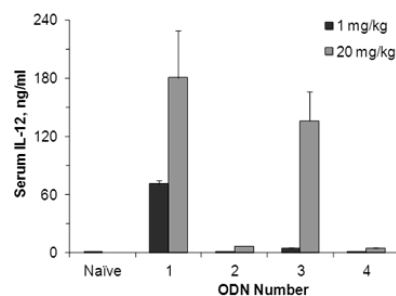


**Figure 4.** IL-12 induction by ODNs in vivo in C57BL/6 mice following sc administration at a dose of 1 and 20 mg/kg. Blood was collected 2 h after ODN administration, and serum IL-12 levels were determined by ELISA. Each bar represents the mean of three mice ± SD. Data shown are representative of two independent experiments.

and purified on an anion-exchange HPLC system using standard protocols.[25] ODNs were desalted on a C18 reverse-phase HPLC, dialyzed, and lyophilized. The purity of lyophilized ODNs was found to be ≥95% with the remainder being shorter by one or two nucleotides ($n − 1$ and $n − 2$), as determined by analytical anion-exchange HPLC and capillary gel electrophoresis (Table 1). The sequence integrity of ODNs was determined by MALDI-ToF mass spectrometry (Micro MX, Waters Co., MA), and the data are shown in Table 1.

HEK293 cells stably expressing mouse TLR9 or LacZ (Invivogen, San Diego, CA) were cultured in 96-well plates and transiently transfected with a SEAP reporter gene.[10] Following transfection, ODNs were added to the cells, and the cultures were continued for 18 h. Aliquots of culture supernatant from each well were incubated with QuantiBlue substrate (Invivogen), and the blue color generated was measured using a plate reader at 620−645 nm. The data are shown as fold increase in NF-κB activity over PBS control.

J774 cells were plated at a density of $5 × 10^6$ cells per well in six-well plates and treated with ODNs at 10 μg/mL for 1 h, at which time nuclear extracts were prepared and analyzed on a 6% native polyacrylamide gel in TBE buffer at 140 V for 2−3 h.[8] Gels were dried and exposed to Hyblot autoradiography film at −70 °C. Films were scanned, and the images were processed with Adobe Photoshop imaging software.

Spleen cells obtained from 6- to 8-week-old female C57BL/6 mice were prepared and cultured in RPMI complete medium consisting of RPMI 1640 with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Mouse spleen cells were plated in 96-well plates at $1 × 10^6$ cells/mL and incubated with 0−10 μg/mL ODNs for 24 h. Supernatants were collected and assayed for IL-6 and IL-12 levels by ELISA.[14]

Six- to eight-week-old female C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). All of the animal studies were carried out in accordance with the Idera's IACUC-approved animal protocols and guidelines. Mice ($n = 3$) were injected sc with ODNs at 1 or 20 mg/kg. Blood was collected by retro-orbital bleeding 2 h after ODN administration, and serum IL-12 levels were determined by ELISA as described above.

## AUTHOR INFORMATION

**Corresponding Author**
*Tel: 1-617-679-5500. Fax: 1-617-679-5572. E-mail: sagrawal@iderapharma.com.

**Notes**
The authors declare the following competing financial interest(s): All authors are/were employees of Idera Pharmaceuticals and hold stock options.

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