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Modifications Incorporated in CpG Motifs of Oligodeoxynucleotides Lead to Antagonist Activity of Toll-like Receptors 7 and 9

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Oligodeoxynucleotides (ODNs) containing unmethylated CpG dinucleotides act as agonists of TLR9 and induce Th1-type immune responses. In the present study, we synthesized CpG containing ODNs in which C or G was substituted with 2′-*O*-methylribonucleotides, 5-methyl-dC, or 2′-*O*-methyl-5-methyl-C and studied their immune stimulatory activity alone and in combination with TLR agonists. In mouse and human primary cell-based assays, modified ODNs did not stimulate immune responses but inhibited TLR9 agonist-induced immune stimulatory activity. In mice, modified ODNs did not induce cytokines but inhibited immune responses induced by agonists of TLR7 and TLR9. Modified ODNs did not inhibit endosomal TLR3- or cell-surface TLR4-agonist-induced cytokines. This study demonstrates that ODNs incorporated with chemical modifications in CpG dinucleotides do not induce immune stimulatory activity but act as antagonists of TLR7 and TLR9 in vitro and in vivo. These types of modifications are commonly employed in antisense sequences and thereby may affect the intended mechanism of action.

Introduction

Toll-like receptors (TLRs[a]) are a family of transmembrane proteins that detect pathogen-associated molecular patterns and elicit pathogen-specific immune responses.[1] Of the 10 TLRs identified in humans, TLRs 3, 7, 8, and 9 are expressed in endosomal membranes and recognize pathogen-derived nucleic acid molecular patterns.[1] Synthetic, bacterial, and viral DNA containing unmethylated CpG dinucleotides act as ligands of TLR9 and are widely studied.[2−5]

We have extensively studied the structure-activity relationships of ODNs containing CpG motifs and identified certain nucleotide, backbone, and linker modifications that, upon site-specific incorporation in the flanking sequence 5′ or 3′ to the CpG dinucleotide, have significant influence on immune stimulatory activity.[6−13] Previously we reported that incorporation of 2′-*O*-methylribonucleotides in ODNs in the first or second nucleotide position adjacent to the CpG dinucleotide on the 5′-side abrogates immune stimulatory activity.[7,12,13] In recent studies, we observed that presence of 2′-*O*-methylribonuclotide substitutions in flanking sequences to CpG motif not only neutralize immune stimulatory activity but also act as antagonists of TLR7 and TLR9 in vitro and in vivo in mice.[14]

Recent studies have shown that TLR9 exists in dimer form and the binding of CpG ODN to the receptor causes conformational changes in the cytoplasmic signaling domain of the receptor, thus leading to the recruitment of adapter molecules and activation of immune signaling pathways.[15] ODNs lacking a CpG motif also bind to TLR9 but are not capable of bringing conformational changes in the signaling domain, thus failing to activate immune stimulation.[15] Our previous studies showed that substitution of either C or G of the CpG dinucleotide with 2′-*O*-methyl-C or -G, respectively, or C with 5-methyl-dC or a methylphosphonate linkage between C and G resulted in a significant decrease in immune stimulatory activity.[16] Such modifications are commonly employed in antisense ODNs containing CpG dinucleotides to minimize TLR9-mediated immune responses. On the basis of our most recent results,[14] we hypothesized that ODNs containing these substitutions in the CpG motif do not show TLR9-mediated immune responses because they do not bring conformational changes in the signaling domain of TLR9 but may act as antagonists of TLR9.

In the present study, we synthesized ODNs containing a CpG dinucleotide with 5-methyl-dC, 2′-*O*-methyl-C, or 2′-*O*-methyl-5-methyl-C substituted for C and/or 2′-*O*-methyl-G substituted for G and studied their immune stimulatory properties including antagonist activity. These studies were carried out in various cell-based assays including TLR9 transfected HEK293 cells, mouse spleen cell cultures, and in vivo in mice. We observed that these ODNs containing modified CpG dinucleotides are not immune stimulatory but act as antagonists of TLR7 and TLR9.

Materials and Methods

Synthesis and Purification of Oligodeoxynucleotides. All ODNs and their controls shown in Table 1 were synthesized, purified, and analyzed as previously reported.[14] All ODNs were characterized by capillary gel electrophoresis (CGE) or denaturing polyacrylamide gel electrophoresis (PAGE) and

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[a] Abbreviations: CGE, capillary gel electrophoresis; CpG, deoxycytidine−phosphate−deoxyguanosine; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; IFN, interferon; NF-κB, nuclear factor κB; ODN, oligodeoxynucleotide; PBMCs, peripheral blood mononuclear cells; PAGE, polyacrylamide gel electrophoresis; TLR, Toll-like receptor.

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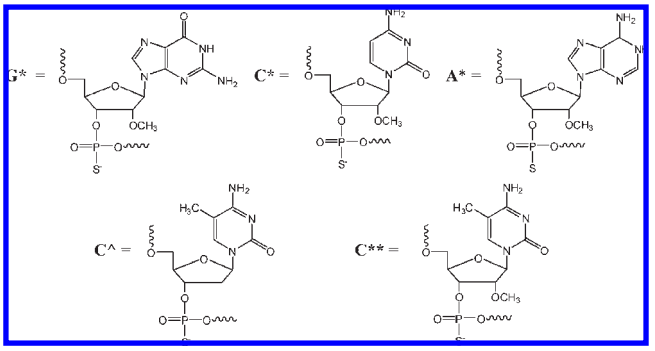
Table 1. Oligodeoxynucleotides Used in the Study and Their Characterization

		MW[b]		purity (%)[c]	
ODN	sequence and modification[a]	calculated	found	HPLC	CGE
1	5′-CTATCTGACGTTCTCTGT-3′ 5705	5706	98	97	
2	5′-CTATCTGAC**G***TTCTCTGT-3′	5735	5736	99	95
3	5′-CTATCTGA**C***GTTCTCTGT-3′	5735	5738	98	97
4	5′-CTATCTGA**C**∧GTTCTCTGT-3′	5719	5719	97	97
5	5′-CTATCTGA**C****GTTCTCTGT-3′	5749	5750	97	96
6	5′-CTATCTGA**C**∧**G***TTCTCTGT-3′	5749	5750	98	97
7	5′-CTATCTGA**C**∧CTTCTCTGT-3′	5679	5682	98	99
8	5′-CTATCT**G***A*CCTTCTCTGT-3′	5725	5724	97	95

[a] All are phosphorothioate oligodeoxynucleotides with chemical modifications shown below. [b] Molecular weight as calculated and determined (found) by MALDI-TOF mass spectrometry. [c] Purity of full-length



MALDI-TOF mass spectrometry (Waters MALDI microMX mass spectrometer) for purity and molecular mass, respectively (Table 1). The purity of full-length oligonucleotides ranged from 95% to 99% with the remainder found to lack one or two nucleotides by HPLC, CGE, and/or denaturing PAGE. All ODNs contained <0.075 EU/mg of endotoxin by the Limulus assay (Bio-Whittaker).

Mice. Five-to-eight-week-old C57BL/6 mice were obtained from Charles River Labs (Wilmington, MA) and maintained in the animal facility of Idera Pharmaceuticals. All of the experimental procedures were performed in accordance with the approved protocols and guidelines of the Institutional Animal Care and Use Committee of Idera Pharmaceuticals.

TLR Ligands. TLR ligands, polyI·polyC (TLR3) and lipopolysaccharide (LPS, TLR4), were purchased from InvivoGen (San Diego, CA) and Sigma (St. Louis, MO), respectively. An RNA-based agonist of TLR7, based on the structure reported earlier, was used.[17]

Cell Culture Assays of HEK293 Cells Expressing TLR9. HEK293 cells transfected with mouse TLR9 were obtained from InvivoGen. HEK293 cells expressing TLR9 were transiently transfected with the reporter gene Seap (Invivogen) for 6 h and stimulated with ODNs for 18 h. TLR-dependent reporter gene expression was determined according to the manufacturer's protocol and expressed as fold change in NF-κB activity.

J774 Cell Experiments. Murine J774 macrophage cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 IU/mL penicillin G and 100 μg/mL streptomycin). J774 cells were plated at a density of 5×10^6 cells/well in six-well plates, treated with ODNs alone or in combination for 1 h, and nuclear extracts were prepared and analyzed by native polyacrylamide gels as described earlier.[18] Gels were dried and exposed to HyBlot CL autoradiography films at −70 °C. Films were scanned, and the images were processed using Adobe imaging software.

Mouse Spleen Cell Cultures. Spleen cells from 5-to-8-week-old C57BL/6 mice were cultured in RPMI complete medium as described earlier.[14] Mouse spleen cells were plated in 96-well plates using 5×10^6 cells/mL. ODNs dissolved in PBS buffer were added alone or in combinations to the cell cultures. The cells were then incubated at 37 °C for 24 h, and the supernatants were collected for ELISA. The experiments were performed two or three times in duplicate for each concentration.

The secretion of IL-12 and IL-6 in cell culture supernatants was measured by sandwich ELISA as described previously.[18] The required reagents, including cytokine antibodies and standards for ELISA, were obtained from PharMingen.

Assessment of Mouse Serum Cytokine Levels. Female C57BL/6 mice, five to six weeks old ($n = 3$), were injected subcutaneously (sc) with modified ODNs. Blood was collected by retro-orbital bleeding 2 h after TLR agonist administration, and serum cytokines and chemokines were measured by Luminex multiplex assay or IL-12 levels by sandwich ELISA.

Multiplex Cytokine Assays. Serum samples from in vivo experiments were assayed using multiplex luminescent beads (Mouse Cytokine Twenty-Plex, Invitrogen, Camarillo, CA) according to the manufacturer's instructions and analyzed with a Luminex 100/200 instrument. Fluorescence intensity was transformed into cytokine concentration using StarStation software (Applied Cytometry Systems). Some serum samples were analyzed for IL-12 levels by ELISA as described above.

Results

Synthesis and Characterization of ODNs Containing Modifications in CpG Dinucleotide. ODN **1** containing a mouse-specific CpG motif was used as a parent compound (Table 1). ODNs **2** and **3** contained 2′-*O*-methyl-G and -C substitution, respectively, in the CpG dinucleotide. ODNs **4** and **5** contained a 5-methyl-dC or 2′-*O*-methyl-5-methyl-C substitution for C in the CpG dinucleotide. ODN **6** contained 5-methyl-dC and 2′-*O*-methyl-G for C and G, respectively, in the CpG dinucleotide. ODNs **7** and **8**, which contained chemical modifications but lacked a CpG dinucleotide,

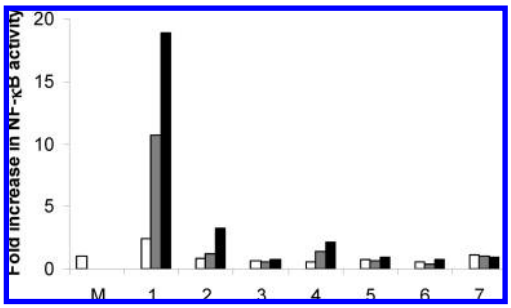


Figure 1. TLR9 activation by ODNs **1**−**7** at 0.1 (white), 0.3 (light shade), and 1 μg/mL (dark shade) in HEK293 cells expressing mouse TLR9. M stands for medium control. Data shown are representative of two independent experiments.

were used as controls. All ODNs were synthesized on an automated DNA/RNA synthesizer using β-cyanoethyl-phosphoramidite chemistry, purified, and characterized for purity by HPLC and CGE and sequence integrity by MALDI-TOF mass spectrometry (Table 1).

Activity of ODNs in HEK293 Cells Expressing TLR9. Activation of TLR9 by ODNs in cultures of HEK293 cells expressing mouse TLR9 was studied. ODN **1** activated TLR9, as measured by NF-κB levels induced in HEK293 cells (Figure 1). Control ODN **7** (without a CpG dinucleotide) had no activity in HEK293 cells. ODNs **2**−**6** (containing modifications) had no or little activity at the same concentrations, suggesting that the substitutions incorporated in CpG dinucleotide abrogate CpG-mediated immune stimulatory activity (Figure 1).

Activity of ODNs in J774 Cells. We also measured the ability of ODNs to activate NF-κB in J774 cells, a macrophage cell line. ODN **1** activated NF-κB as expected (Figure 2A). ODNs **2**−**6** showed no or little induction of NF-κB (Figure 2A). Control ODN **8** showed no activation of NF-κB (Figure 2A). When cells were co-incubated with ODN **1** and ODNs **3**−**6**, various degrees of inhibition of ODN **1**-activated NF-κB was observed, which was not observed with control ODN **8** (Figure 2B). ODN **2** showed the lowest inhibition of ODN **1**-activated NF-κB at the concentration studied.

Activity of ODNs in Mouse Spleen Cell Cultures. We then examined the ability of ODNs **1**−**6** to induce cytokines in C57BL/6 mouse spleen cell cultures. Consistent with HEK293 cell culture data, only ODN **1** induced dose-dependent IL-12 (Figure 3A) and IL-6 (Figure 3B) secretion and control ODN **7** had no activity. ODNs **2**−**6** induced significantly reduced cytokine levels compared with ODN **1**. ODNs **2** and **4**, which showed low levels of activity in HEK293 cells, induced levels of IL-12 and IL-6 that were slightly higher than background (Figure 3).

When spleen cells were co-incubated with ODN **1** and ODNs **2**−**7**, ODNs **2**−**6** inhibited ODN **1**-induced IL-12 (Figure 4A) and IL-6 secretion (Figure 4B). The inhibition was dependent on the concentration of ODNs, as shown for ODN **5** and ODN **6** (Figure 4C and Figure 4D). Control ODN **7** slightly inhibited ODN**1**-induced IL-12 and IL-6 production.

Activity of ODNs in Vivo in Mice. ODNs **1**−**7** were administered subcutaneously to mice at a dose of 2 mg/kg. Blood was collected 2 h after ODN administration, and serum cytokine and chemokine levels were determined by Luminex multiplex assay. ODN **1** induced MIP-1α,

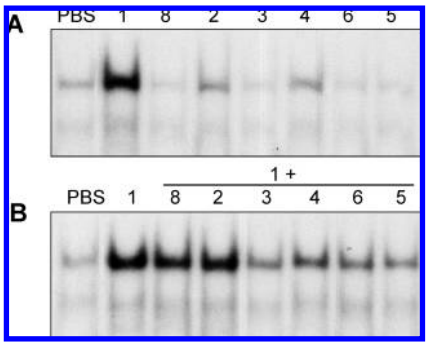


Figure 2. (A) Effect of ODNs **1**−**6** and **8** on NF-κB activation in J774 cells. The concentration of ODN **1** was 0.5 μg/mL, and ODNs **2**−**6** and **8** was 2.5 μg/mL. (B) Effect of ODNs containing chemical modifications on ODN **1**-induced NF-κB activation in J774 cells. ODN **1** (0.5 μg/mL) was co-incubated with (2.5 μg/mL) ODNs **2**−**6** or **8**. One hour later nuclear extracts were prepared and EMSA was carried out as described in the text.

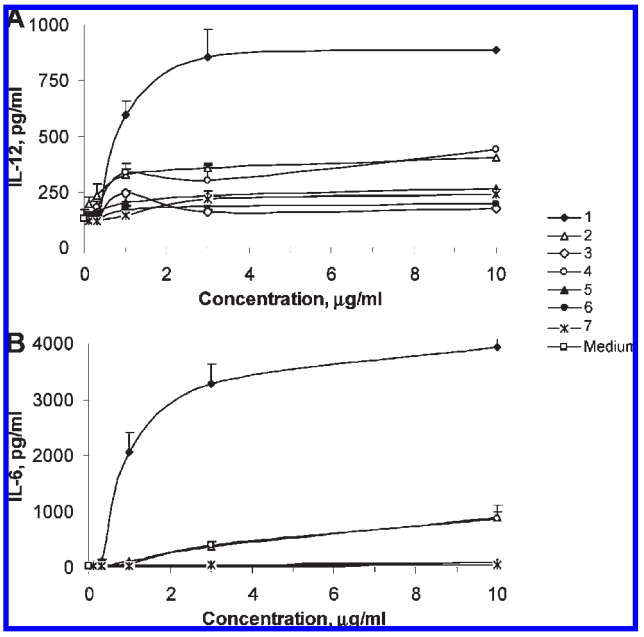


Figure 3. Immune stimulatory activity of ODNs in C57BL/6 mouse spleen cell cultures at 24 h; (A) IL-12 and (B) IL-6 induced by ODNs. Data shown are representative of three independent experiments.

TNF-α, IL-10, MCP-1, KC, and high levels of IL-12 (Figure 5A). At the same dose, ODNs **2**−**6** induced no or low levels of the same cytokines and chemokines (Figure 5A). Control ODN **7** induced background levels of cytokines (Figure 5A).

To study the in vivo inhibitory activity, ODNs **2**−**6** or **7** was administered at a dose of 2 mg/kg to mice subcutaneously in the left flank and 24 h later ODN **1** was administered at a dose of 0.5 mg/kg in the right flank. Blood was collected 2 h after ODN **1** administration, and serum cytokine and chemokine levels were measured by Luminex multiplex assay. ODNs **2**−**6** significantly inhibited ODN **1**-induced cytokine and chemokine production (Figure 5B). At a dose of 2 mg/kg ODNs, a 50−71% inhibition of IL-12 was observed. Control ODN **7** showed minimal inhibition of cytokines induced by ODN **1** (Figure 5B).

Dose-Dependent Effects of ODNs. Further studies were carried out with a representative ODN, ODN **6**, which had

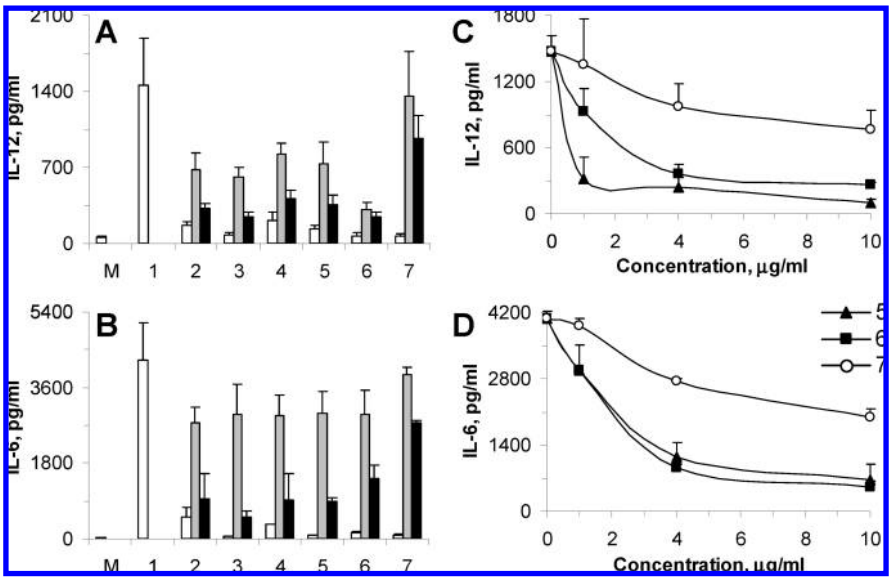


Figure 4. Effect of ODNs containing chemical modifications on ODN **1**-induced immune stimulation in mouse spleen cell cultures. ODNs **2**−**6** or control ODN **7** alone do not induce (A) IL-12 and (B) IL-6 but inhibit ODN **1**-induced IL-12 and IL-6 secretion in C57Bl/6 mouse splenocyte cultures. ODN **1** was used at 1 μg/mL in all experiments. ODNs **2**−**6** or control ODN **7** were at 4 μg/mL alone (white bars) or in combination with ODN **1** (1 μg/mL) at 1 μg/mL (light shaded bars) or 4 μg/mL (dark shaded bars) in both panels. M indicates medium control in both of the panels. Dose-dependent inhibition of ODN **1**-induced (C) IL-12 and (D) IL-6 are shown for ODNs **5** and **6** and control ODN **7**. Data point shown at 0 indicates IL-12 or IL-6 induction by ODN **1** alone at 1 μg/mL concentration. Data shown are representative of three independent experiments.

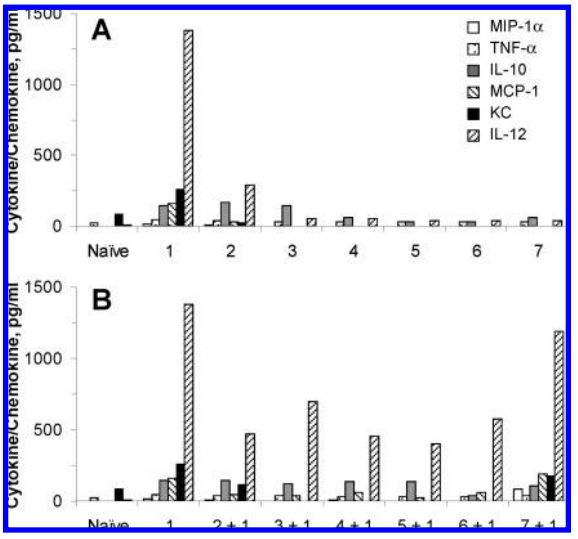


Figure 5. Serum cytokine and chemokine levels induced by (A) ODN **1** (0.5 mg/kg) and ODNs **2**−**6** and control ODN **7** (2 mg/kg) alone and (B) inhibition of ODN **1**-induced IL-12 by ODNs **2**−**6** and control ODN **7** in vivo in mice. For acute administration studies shown in panel A, C57BL/6 mice were injected with ODNs **1**−**7** at 2 mg/kg sc in the right flank. Blood was collected 2 h after ODN administration, and serum cytokine and chemokine levels were determined by Luminex multiplex assay. For inhibition experiments shown in panel B, 2 mg/kg ODNs **2**−**7** was administered sc to mice in the right flank and 24 h later 0.5 mg/kg ODN **1** was administered sc in the left flank. Blood was collected 2 h after ODN **1** administration, and the levels of serum cytokines and chemokines were determined by Luminex multiplex assay. Naive indicates serum from mice not injected with any agent. Data shown are representative of three independent experiments.

both C and G substituted with 5-methyl-C and 2′-*O*-methyl-G, respectively, in the CpG dinucleotide. To study the effect of ODN **6** dose on the inhibitory activity, ODN **6** was administered to mice at a dose of 2, 5, or 10 mg/kg subcutaneously in the right flank; 24 h later, 0.5 mg/kg ODN **1** was administered subcutaneously in the left flank. Two hours later, blood was collected and IL-12 levels in serum were determined by ELISA. ODN **6** inhibited ODN **1**-induced IL-12 in a dose-dependent manner (Figure 6A). The inhibition at 2, 5, and 10 mg/kg ODN **6** was 14%, 66%, and 93%, respectively.

We then studied the dose dependence of ODN **1** on the inhibitory activity of ODN **6** by administering 10 mg/kg ODN **6** subcutaneously in the right flank and 24 h later administering 0.25, 0.5, or 1.0 mg/kg ODN **1** sc in the left flank of mice. Serum levels of IL-12 were determined 2 h after ODN **1** administration. ODN **6** inhibited 85%, 66%, and 46% of ODN **1**-induced IL-12 at 0.25, 0.5, and 1.0 mg/kg doses, respectively. These results suggest that the inhibitory effect of ODN **6** was also dependent on the dose of ODN **1** administered (Figure 6B).

Duration of ODN Inhibitory Activity. To study the duration of the inhibitory activity of ODNs, a dose of 10 mg/kg ODN **6** was administered subcutaneously in the right flank of mice. ODN1 was administered subcutaneously in the left flank at a dose of 0.5 mg/kg 24, 48, or 72 h later. Two hours after ODN **1** administration, blood was collected and serum IL-12 levels were determined by ELISA. ODN **6** inhibited ODN **1**-induced IL-12 levels up to 72 h after ODN **6** administration (Figure 7). At 24, 48, and 72 h time points, 81%, 57%, and 35%, respectively, of IL-12 inhibition were observed.

Specificity of ODN Inhibitory Activity. We further studied the specificity of ODN **6** inhibitory activity for different TLR agonist-induced immune responses in vivo in C57BL/6 mice. ODN **6** was administered to mice at 10 mg/kg subcutaneously, and 24 h later an agonist of TLR 3, 4, 7, or 9 was administered in the opposite flank. Blood was collected 2 h after TLR agonist administration, and serum

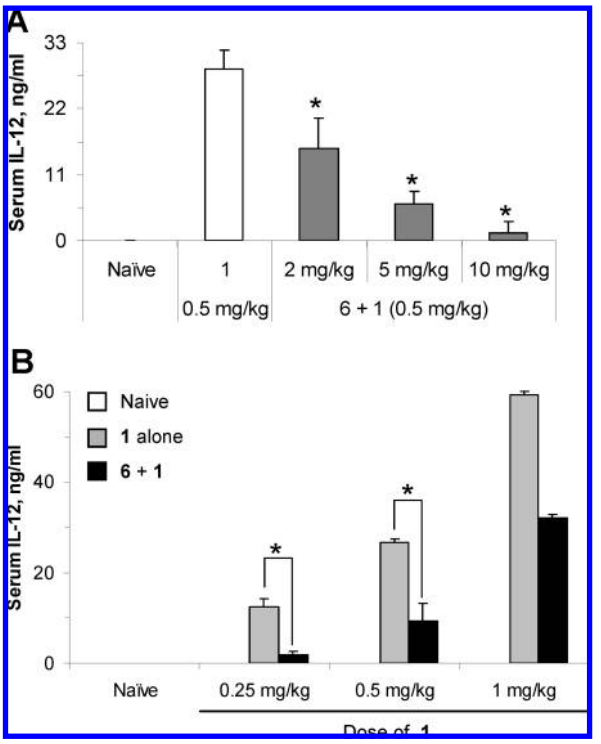


Figure 6. Inhibitory effect of ODN is dependent on the dose of (A) modified ODN **6** and (B) TLR9 agonist ODN **1**. In experiments shown in panel A, C57BL/6 mice were injected with 2, 5, or 10 mg/kg ODN **6** sc in the right flank and 24 h later 0.5 mg/kg ODN **1** in the left flank. Blood was collected 2 h after ODN **1** administration, and serum IL-12 levels were determined by ELISA. In experiments shown in panel B, C57BL/6 mice were injected with 10 mg/kg ODN **6** sc in the right flank and 24 h later 0.25, 0.5, or 1 mg/kg ODN **1** in the left flank. Blood was collected 2 h after ODN **1** administration, and serum IL-12 levels were determined by ELISA. Naive indicates serum from mice not injected with any agent. Data shown are representative of two independent experiments. The asterisk (∗) indicates $p < 0.05$.

cytokine levels were determined by Luminex multiplex assay. The agonists of TLRs 3, 4, 7, and 9 induced various levels of cytokines and chemokines. The levels and profiles of cytokines induced by each of the TLR agonists were distinct as expected (Figure 8). ODN **6** significantly inhibited (50−90%) production of cytokines and chemokines induced by TLR7 and TLR9, but not TLR3 or TLR4, agonists (Figure 8).

Discussion

Synthetic ODNs containing CpG motifs activate the immune system through TLR9 and induce Th1-type immune responses. Our previous studies showed that 2′-*O*-methylribonucleotides incorporated in the first two nucleotide positions adjacent to the CpG dinucleotide on the 5′-side neutralized immune stimulatory activity.[7,12,13] Recently, we showed that these compounds containing two 2′-*O*-methylribonucleotide substitutions adjacent to CpG in the 5′-flanking sequence did not stimulate immune responses but inhibited the activity of agonists of TLRs 7 and 9 in vitro and in vivo in mice, thereby acting as antagonists.[14]

In the mid-1990s, we had shown that substitution of the C or G of a CpG dinucleotide in ODNs with 2′-*O*-methylribonucleotide modifications or the C with 5′-methyl-dC abrogates immune stimulatory activity.[16] These types of

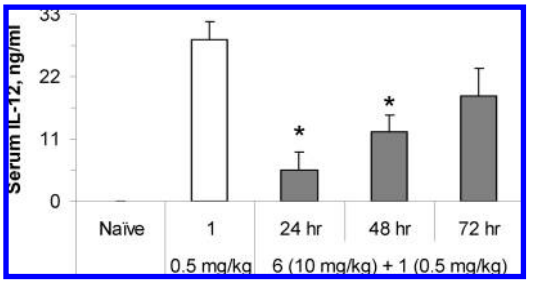


Figure 7. Duration of inhibitory effect of ODN **6** on ODN **1**-induced immune responses in mice. C57BL/6 mice were injected with 10 mg/kg ODN **6** sc in the right flank and then 24, 48, or 72 h later with 0.5 mg/kg ODN **1** sc in the left flank. Blood was collected 2 h after ODN **1** administration, and serum IL-12 levels were determined by ELISA. Naive indicates serum from mice not injected with any agent. Data shown are representative of three independent experiments. The asterisk (∗) indicates $p < 0.05$.

modifications are commonly being used in antisense ODN sequences containing CpG motif to minimize TLR9-mediated immune responses. However, it is not known if ODNs containing modified C and/or G may act as antagonists of TLR7 and TLR9 agonist-mediated immune responses. In the present study, we synthesized ODNs with 2′-*O*-methylribonucleotide, 5-methyl-dC, or 2′-*O*-methyl-5-methyl-C substitutions in place of C and/or G in the CpG dinucleotide of TLR9-stimulatory ODN **1**. We studied the immune stimulatory activity of these ODNs alone and their inhibitory activity in combination with TLR agonists in cell-based assays and in vivo in mice.

ODN **1** showed immune stimulatory activity through TLR9 as expected in TLR9-expressing HEK293 cells and mouse spleen cell cultures. Also as expected, ODNs **2**−**6** containing modifications in the CpG motif did not induce NF-κB activity in TLR9-transfected HEK293 cells compared with ODN **1**.

In J774-cell culture assays, ODNs **2**−**6** containing chemical modifications in the CpG dinucleotide did not activate NF-κB but inhibited NF-κB activation by ODN **1** when co-incubated. These results suggest that the interaction occurs upstream of NF-κB. More importantly, control ODN **7** lacking a CpG dinucleotide did not inhibit ODN **1**-activated NF-κB, suggesting that a CpG dinucleotide with chemical substitutions is required for antagonistic activity. Consistent with the results observed with ODNs **2**−**6**, an antisense sequence containing a 5-methyl-dCp2′-methoxyethyl-G dinucleotide modification[19] also did not activate NF-κB but showed strong inhibition of ODN **1**-activated NF-κB when co-incubated in J774 cells (data not shown).

In mouse spleen cell cultures, ODNs **2**−**6** containing 2′-*O*-methylribonucleotide, 5-methyl-dC, or 5-methyl-2′-*O*-methyl-C substitutions for C and/or G of CpG dinucleotide did not induce cytokine and chemokine secretion compared with ODN **1** as we have shown previously.[16] However, when co-incubated with ODN **1**, these ODNs inhibited ODN **1**-induced IL-12 and IL-6. The combination of ODN **1** with control ODN **7** lacking a CpG dinucleotide did not significantly inhibit ODN **1**-induced immune stimulatory activity in vitro. These results suggest that the presence of a CpG dinucleotide with specific chemical modifications in C and/or G is required for TLR9 antagonistic activity.

ODNs containing modifications in C and/or G (**2**−**6**) administered subcutaneously at doses up to 10 mg/kg showed

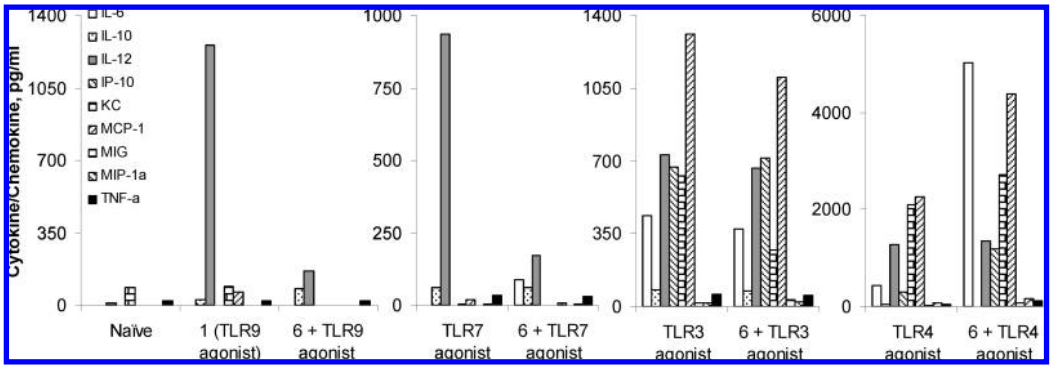


Figure 8. Specificity of inhibition of TLR agonist-induced immune responses by ODN **6** in C57BL/6 mice. C57BL/6 mice were injected sc with 10 mg/kg ODN **6** in the right flank, and 24 h later an agonist of TLR9 (ODN **1**, 0.5 mg/kg), TLR7 (RNA-based compound, 10 mg/kg), TLR3 (polyI·polyC, 25 mg/kg), or TLR4 (LPS, 0.25 mg/kg) was injected sc in the left flank. Blood was drawn 2 h after TLR agonist administration, and serum cytokine and chemokine levels were determined by luminex multiplex assay. Data shown are representative of two or more independent experiments.

no or minimal induction of cytokines and chemokines in mice, while TLR9 agonist ODN **1** at doses as low as 0.5 mg/kg induced higher levels of cytokine and chemokine production in mice. These results are consistent with our previous observations that chemical modifications introduced in the CpG dinucleotide lead to loss of immune stimulatory activity.[16] ODNs **2**−**6** inhibited the cytokine induction by TLR9 agonist ODN **1** when it was administered separately in the opposite flank. The inhibitory effect was dependent on the dose of the ODN containing modifications in the CpG dinucleotide as well as the TLR9 agonist. The inhibitory effect persisted for up to 72 h at a dose of 10 mg/kg. Importantly, control ODN had no inhibitory activity, suggesting that the inhibitory activity is dependent on the presence of a CpG dinucleotide.

TLRs 3, 7, 8, and 9 are expressed intracellularly in endosomal membranes, and the other TLRs are expressed on the plasma membrane. The compounds studied presently specifically inhibited cytokines and chemokines induced by agonists of TLR7 and TLR9 but not TLR3. The ODNs did not inhibit activity of the TLR4 agonist, LPS, suggesting that they do not act as inhibitors of surface TLRs.

Taken together, the present data show that ODNs containing 2′-*O*-methyl-C, 5-methyl-dC, or 5-methyl-2′-*O*-methyl-C substitutions for C and/or 2′-*O*-methyl-G or 2′-*O*-methoxyethyl-G for G of a CpG dinucleotide do not induce immune responses. Here, we report the novel observation that they instead inhibit TLR7- and TLR9-agonist-induced immune stimulatory activity in vitro and in vivo in mice. As these compounds act as antagonists of TLR7 and TLR9, they may have potential application in the treatment of certain autoimmune and inflammatory diseases in which immune complexes containing self-nucleic acids are shown to induce IFN-α and other cytokines through engagement of TLR7 and/or TLR9.[20] Further studies of these ODNs that have chemical modifications of the CpG dinucleotide are in progress in autoimmune disease models. Moreover, these studies shed light on the possible unintended mechanisms of action of antisense ODN sequences that contain chemically modified CpG dinucleotides.

References

(1) Akira, S.; Uematsu, S.; Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **2006**, *124*, 783–801.
(2) Yamamoto, S.; Kuramoto, E.; Shimada, S.; Tokunaga, T. In vitro augmentation of natural killer cell activity and production of interferon-alpha/beta and -gamma with deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. *Jpn. J. Cancer Res.* **1988**, *79*, 866–873.
(3) Hemmi, H.; Takeuchi, O.; Kawai, T.; Kaisho, T.; Sato, S.; Sanjo, H.; Matsumoto, M.; Hoshino, K.; Wagner, H.; Takeda, K.; Akira, S. A Toll-like receptor recognizes bacterial DNA. *Nature* **2000**, *408*, 740–745.
(4) Agrawal, S.; Kandimalla, E. R. Synthetic agonists of Toll-like receptors 7, 8, and 9. *Biochem. Soc. Trans.* **2007**, *35*, 1461–1467.
(5) Krieg, A. M. Therapeutic potential of Toll-like receptor 9 activation. *Nat. Rev. Drug Discovery* **2006**, *5*, 471–484.
(6) Yu, D.; Kandimalla, E. R.; Zhao, Q.; Cong, Y.; Agrawal, S. Immunostimulatory properties of phosphorothioate CpG DNA containing both 3′-5′- and 2′-5′-internucleotide linkages. *Nucleic Acids Res.* **2002**, *30*, 1613–1619.
(7) Agrawal, S.; Kandimalla, E. R. Antisense and/or immunostimulatory oligonucleotide therapeutics. *Curr. Cancer Drug Targets* **2001**, *1*, 197–209.
(8) Yu, D.; Kandimalla, E. R.; Zhao, Q.; Cong, Y.; Agrawal, S. Immunostimulatory activity of CpG oligonucleotides containing non-ionic methylphosphonate linkages. *Bioorg. Med. Chem.* **2001**, *9*, 2803–2808.
(9) Yu, D.; Kandimalla, E. R.; Zhao, Q.; Cong, Y.; Agrawal, S. Modulation of immunostimulatory activity of CpG oligonucleotides by site-specific deletion of nucleobases. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2263–2267.
(10) Yu, D.; Kandimalla, E. R.; Zhao, Q.; Bhagat, L.; Cong, Y.; Agrawal, S. Requirement of nucleobase proximal to CpG dinucleotide for immunostimulatory activity of synthetic CpG DNA. *Bioorg. Med. Chem.* **2003**, *11*, 459–464.
(11) Yu, D.; Kandimalla, E. R.; Cong, Y.; Tang, J.; Tang, J. Y.; Zhao, Q.; Agrawal, S. Design, synthesis, and immunostimulatory properties of CpG DNAs containing alkyl-linker substitutions: role of nucleosides in the flanking sequences. *J. Med. Chem.* **2002**, *45*, 4540–4548.
(12) Zhao, Q.; Yu, D.; Agrawal, S. Site of chemical modifications in CpG containing phosphorothioate oligodeoxynucleotide modulates its immunostimulatory activity. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3453–3458.
(13) Zhao, Q.; Yu, D.; Agrawal, S. Immunostimulatory activity of CpG containing phosphorothioate oligodeoxynucleotide is modulated by modification of a single deoxynucleoside. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1051–1054.
(14) Wang, D.; Bhagat, L.; Yu, D.; Zhu, F. G.; Tang, J. X.; Kandimalla, E. R.; Agrawal, S. Oligodeoxynucleotide-based antagonists for Toll-like receptors 7 and 9. *J. Med. Chem.* **2009**, *52*, 551–558.
(15) Latz, E.; Verma, A.; Visintin, A.; Gong, M.; Sirois, C. M.; Klein, D. C.; Monks, B. G.; McKnight, C. J.; Lamphier, M. S.; Duprex, W. P.; Espevik, T.; Golenbock, D. T. Ligand-induced conformational changes allosterically activate Toll-like receptor 9. *Nat. Immunol.* **2007**, *8*, 772–779.
(16) Zhao, Q.; Temsamani, J.; Iadarola, P. L.; Jiang, Z.; Agrawal, S. Effect of different chemically modified oligodeoxynucleotides on immune stimulation. *Biochem. Pharmacol.* **1996**, *51*, 173–182.

(17) Lan, T.; Kandimalla, E. R.; Yu, D.; Bhagat, L.; Li, Y.; Wang, D.; Zhu, F. G.; Tang, J. X.; Putta, M. R.; Trombino, A. F.; Sullivan, T.; Agrawal, S. Stabilized immune modulatory RNA compounds as agonists of Toll-like receptors 7 and 8. *Proc. Natl. Acad. Sci. U.S. A.* **2007**, *104*, 13750–13755.

(18) Kandimalla, E. R.; Bhagat, L.; Wang, D.; Yu, D.; Tang, J.; Wang, H.; Huang, P.; Zhang, R.; Agrawal, S. Divergent synthetic nucleotide motif recognition pattern: design and development of potent immunomodulatory oligodeoxyribonucleotide agents with distinct cytokine induction profiles. *Nucleic Acids Res.* **2003**, *31*, 2393–2400.

(19) Kastelein, J. J. P.; Wedel, M. K.; Baker, B. F.; Su, J.; Bradley, J. D.; Yu, R. Z.; Chuang, E.; Graham, M. J.; Crooke, R. M. Potent reduction of apolipoprotein B and low-density lipoprotein cholesterol by short-term administration of an antisense inhibitor of apolipoprotein B. *Circulation* **2006**, *114*, 1729–1735.

(20) Marshak-Rothstein, A. Toll-like receptors in systemic autoimmune disease. *Nat. Rev. Immunol.* **2006**, *6*, 823–835.