Oligodeoxyribonucleotide-Based Antagonists for Toll-Like Receptors 7 and 9

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Oligodeoxyribonucleotides containing unmethylated CpG motifs act as TLR9 agonists. In this study, we evaluated oligonucleotides containing an unmethylated CpG motif in which two nucleotides adjacent to the CpG dinucleotide were substituted with 2′-*O*-methylribonucleotides, resulting in TLR7 and TLR9 antagonists. In mouse and human cell cultures, antagonists did not stimulate immune activation but inhibited TLR7 and TLR9 agonist-induced activity. In mice, antagonists inhibited immune responses induced by TLR9 agonists for up to several days, and the inhibition was dose-dependent. Antagonists also inhibited immune responses induced by an RNA-based TLR7/8 agonist but not TLRs 2, 3, 4, or 5 agonists in mice. Additionally, antagonist inhibited TLR9 agonist-induced IL-6 in lupus-prone MRL/lpr mouse spleen cell cultures. These results indicate that antagonists described herein can suppress immune responses induced by TLR7 and TLR9 agonists. Antagonists may be suitable candidates for treating inflammatory and autoimmune diseases where inappropriate or uncontrolled TLR activation has been implicated.

Introduction

Toll-like receptors (TLRs)[a] are highly conserved transmembrane proteins that detect pathogen-associated molecular patterns and elicit pathogen-specific immune responses.[1] Of the 10 TLRs identified to date, TLRs 3, 7, 8, and 9 are expressed in endosomal membranes within the cell and recognize pathogen-derived nucleic acid molecular patterns.[1] Synthetic and bacterial DNA containing unmethylated CpG motifs are shown to be ligands for TLR9 and are widely studied.[2,3] Several TLR9 agonists containing CpG or synthetic immunostimulatory motifs are being tested in clinical trials as potential treatment for various diseases, including cancers, asthma, allergies, and infectious diseases, and as adjuvants with vaccines.[4,5]

Recent studies have shown that TLRs play a role in the development of autoimmune diseases.[6] Especially, nucleic acid recognizing TLR7 and TLR9, have been shown to recognize circulating immune complexes containing self-RNA or -DNA, respectively, and induce IFN-α and other inflammatory cytokines leading to the progression of autoimmune diseases, including lupus, rheumatoid arthritis, multiple sclerosis, and psoriasis.[6,7] Therefore, compounds that act as antagonists of TLR7 and TLR9 would have wide application in the treatment of autoimmune and inflammatory diseases.

Certain synthetic oligodeoxyribonucleotides containing dG-rich sequences along with sequence motifs other than CpG have been shown to act as antagonists and inhibit TLR9 activation by CpG oligos.[8−13] The mechanism of action of poly dG-based antagonists of TLR9 is not well understood, but the evidence suggests that they may interfere with STAT1, -3, and -4 and/or other downstream factors proximal to NF-κB activation involved in the signaling pathways of TLR9.[13,14] These types of oligonucleotides, depending on the nucleotide sequence, are also known to act as agonists of TLR9[15,16] to have antitumor and anti-infective activities.[17,18] Poly dG-based oligonucleotides lack pharmaceutical attributes due to unpredictable secondary structure formation depending on the experimental conditions, non-sequence-specific protein binding, including immune stimulation.[19,20]

We have extensively studied the structure−activity relationships of immune stimulatory oligodeoxynucleotides and shown that certain nucleotide and backbone modifications incorporated site-specifically in the flanking sequence 5′- or 3′- to the CpG dinucleotide have a significant influence on immunostimulatory activity.[21−28] A 2′-*O*-methyl ribonucleotide incorporated in the first or second nucleotide position adjacent to the CpG dinucleotide in the 5′-flanking region abrogates immune stimulatory activity and substitutions three or more nucleotides away from CpG in the 5′-flanking sequence enhance activity.[22,27,28] On the contrary, a 2′-*O*-methyl-ribonucleotide incorporated at the first or second position adjacent to the CpG in the 3′-flanking sequence retains or enhances activity compared with the unmodified parent compound.[22]

In the present study, we examined the ability of oligodeoxynucleotides containing an unmethylated CpG or a synthetic immunostimulatory motif, CpR (R = 7-deaza-dG), and two 2′-*O*-methyl-ribonucleotide substitutions in the first and second positions adjacent to the immunostimulatory dinucleotide in the 5′-flanking region to inhibit immune activation mediated through TLRs.

Materials and Methods

Synthesis and Purification of Oligonucleotides. All oligonucleotides and their controls shown in Table 1 were synthesized, purified, and analyzed as previously described.[29] All oligonucleotides were characterized by capillary gel electrophoresis (CGE) or denaturing polyacrylamide gel electrophoresis (PAGE) and MALDI-ToF mass spectrometry (PerSeptive Biosystems Voyager DEStar MALDI-ToF mass spectrometer with 337 nm N_2 laser) for purity and molecular mass, respectively (Table 1). The purity of full-length oligonucleotides ranged from 93−98%, with the remainder found to lack one or two nucleotides by HPLC, CGE, and/or denaturing PAGE (Supporting Information Table 1). All oligonucleotides contained <0.075 EU/mg of endotoxin by the Limulus assay (Bio-Whittaker).

Mice. Female C57BL/6 mice, 6−8 weeks old, and MRL/lpr mice were obtained from Charles River Laboratories, (Wilmington, MA)

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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; PBMCs, peripheral blood mononuclear cells; PAGE, polyacrylamide gel electrophoresis; TLR, Toll-like receptor.

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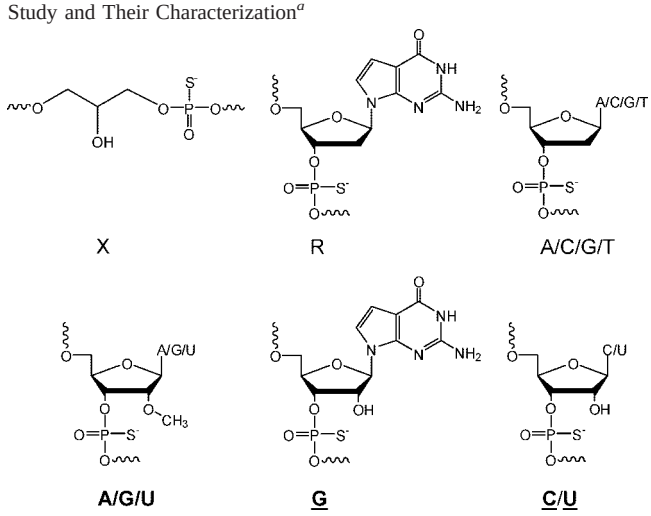
Table 1. Antagonists and Agonists of TLR7 and TLR9 Used in the Study and Their Characterization[a]



no.	sequence and modification[a]	mol. wt.[b] calculated	found
	TLR9 Agonists		
1	5'-CTATCTGACGTTCTCTGT-3'	5705	5695
2	5'-CTATCTGACG**U**UCTCTGT-3'	5736	5730
3	5'-TCTGACRTTCT-X-TCTTRCAGTCT-5'	7164	7164
4	5'-CTATCTGTCGTTCTCTGT-3'	5695	5687
5	5'-TCTGTCRTTCT-X-TCTTRCTGTCT-5'	7146	7148
	Antagonists		
6	5'-CTATCT**GA**CGTTCTCTGT-3'	5765	5767
7	5'-CTATCT**GU**CGTTCTCTGT-3'	5741	5732
8	5'-CTATCT**GA**CRTTCTCTGT-3'	5764	5763
9	5'-CTATCT**GU**CRTTCTCTGT-3'	5741	5733
	Controls		
10	5'-CTATCTCACCTTCTCTGT-3'	5624	5621
11	5'-CTATCT**GA**CCTTCTCTGT-3'	5725	5722
	Poly-dG-based antagonists		
12	5'-TCCTGGAGGGGAAGT-5'	--	ND
13	5'-TCCTGGCGGGGAAGT-5'	--	ND
	TLR7/8 agonist		
14	5'-**UGCUGUUCUG**-X-**GUCUUGUCGU**-5'	7404	7402

[a] Sequences **1**−**13** are oligodeoxyribonucleotides and sequence **14** is an oligoribonucleotide. All oligonucleotides contain phosphorothioate backbone. R and X represent 2′-deoxy-7-deazaguanosine and glycerol linker, respectively; **G**/**A**/**U** indicate 2′-O-methyl-ribonucleotide modifications; **G** represents 7-deazaguanosine (structures shown above). [b] Molecular weight as calculated and determined (found) by MALDI-ToF mass spectrometer. ND: Not determined.

and The Jackson Laboratory (Bar Harbor, ME), respectively. All the experimental procedures were performed according to the approved protocols and guidelines of the Institutional Animal Care and Use Committee of Idera Pharmaceuticals.

TLR Ligands. TLR ligands were purchased from the following sources: macrophage-activating lipopeptide-2 (MALP-2, TLR2), Axxora (San Diego, CA), PolyI.PolyC (TLR3), InvivoGen (San Diego, CA), and lipopolysaccharide (LPS, TLR4), Sigma (St. Louis, MO). Flagellin from *Salmonella muenchen* (TLR5) was a gift from Inotek Pharmaceuticals (Beverly, MA).

NF-κB Activation Assays in HEK293 Cells Expressing TLR9. HEK293 cells stably transfected with mouse TLR9 were obtained from InvivoGen. HEK293 cells expressing TLR9 were transiently transfected with reporter gene SEAP (Invivogen) for 6 h and stimulated with agonists in the presence or absence of antagonist for 18 h. TLR-dependent reporter gene expression was determined using the Quanti-Blue substrate, according to the manufacturer's protocol and expressed as fold change in NF-κB activity or % NF-κB activity.

MRL/lpr Mouse Spleen Cell Cultures. MRL/lpr mouse splenic B lymphocytes were purified using a B-cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of B cells was assessed by staining them with FITC-labeled antimouse B220 (BD Biosciences, San Diego, CA) and analyzing with flow cytometry (>95%). Spleen B cells in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin were cultured at 5×10^5 cells/200 μL in 96-well culture plates with 1 μg/mL agonist **3** in the presence or absence of 0.3, 1, or 3 μg/mL antagonist **8**, or 3 μg/mL antagonist **8** alone. Cell culture supernatants were collected after 24 h for cytokine measurements by ELISA.

The secretion of IL-12 and IL-6 in cell culture supernatants was measured by sandwich ELISA as described previously.[29] The required reagents, including cytokine antibodies and standards for ELISA, were obtained from BD Biosciences.

Assessment of Mouse Serum Cytokine Levels. Female C57BL/6 mice, 6−8 weeks old, ($n = 3$) were injected subcutaneously (sc) with agonists in the absence or presence of antagonist. Blood was collected by retro-orbital bleeding and IL-12 levels in the serum were determined by sandwich ELISA or mouse multiplex Luminex assay as described below.

Preparation of J774 Cell Extracts for NF-κB Activation. Murine J774 macrophage cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS and antibiotics (100 IU/mL of penicillin/streptomycin). For NF-κB activation, J774 cells were plated at a density of 5×10^6 cells/well in six-well plates, treated with agonist, antagonist, or combinations for 1 h after adding the agonist, and nuclear extracts were prepared and analyzed by electrophoretic mobility shift assay (EMSA) on native polyacrylamide gels as described earlier.[29] 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.

Isolation of Human PBMCs and B Cells. Peripheral blood mononuclear cells (PBMCs) from freshly drawn healthy volunteer blood (Research Blood Components, Brighton, MA) were isolated by Ficoll density gradient centrifugation method (Ficoll-Paque PLUS, GE Health Care). The culture medium used for the assay consisted of RPMI 1640 medium supplemented with 1.5 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μM 2-mercaptoethanol, 100 IU/mL penicillin−streptomycin mix, and 10% heat-inactivated FBS.

B cells were isolated from PBMCs by positive selection using the CD19 cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions.

Human B-Cell Proliferation Assay. About 1×10^5 B cells were stimulated with different concentrations of agonist and antagonist for 64 h, then pulsed with 0.75 μCi of [^{3}H]-thymidine and harvested 8 h later. The incorporation of [^{3}H]-thymidine was measured using a scintillation counter, and the data are shown as counts per minute (cpm).

Human PBMC Cultures for NF-κB Activation and Cytokine Measurements. A total of 5×10^6 PBMCs per mL (i.e., 1×10^6/200 μL/well) were stimulated in 96-well flat bottom plates with agonist and/or antagonist, dissolved in PBS, for a period of 24 h. Unstimulated cells served as controls. At the end of the

incubation period, supernatants were harvested and stored frozen until the time of assay by Luminex multiplex technology.

For NF-κB activation, human PBMCs were plated at a density of 1×10^7 cells/well in six-well plates, treated with agonist, antagonist, or combinations for 2 h after the addition of the agonist, and nuclear extracts were prepared and run in gel shift assays as described above for J774 cells.

Multiplex Cytokine Assays. Selected serum samples from in vivo experiments and human cell culture supernatants were assayed using multiplex antibody bead assay kits (mouse cytokine 20-plex and human cytokine 25-plex bead kits, Invitrogen, Camarillo, CA) according to the manufacturer's instructions and analyzed with a Luminex 100 or 200 instrument (Applied Cytometry Systems). Fluorescence intensity was transformed into cytokine concentration using StarStation software (Applied Cytometry Systems).

Statistical Analysis. All results are expressed as mean ± SEM. Student's *t* test was used to compare differences between the antagonists and vehicle (PBS) treated mice. Values of $p < 0.05$ were considered significant and indicated with * in figures.

Results

Design of Antagonists for TLR9. Oligonucleotides **1**−**9** contained a CpG or CpR (R = 7-deaza-dG) immune stimulatory dinucleotide (Table 1). Oligodeoxynucleotides **1**−**5** were TLR9 agonists, and oligoribonucleotide **14** was a TLR7/8 agonist.[4] Oligonucleotides **6**−**9** contained the same nucleotide sequences as **1** or **4** except that the first two nucleotides adjacent to CpG or CpR were substituted with 2′-*O*-methylribonucleotides and were examined for their ability to act as TLR9 antagonists. Oligonucleotide **2** had two 2′-*O*-methylribonucleotide substitutions adjacent to the CpG in the 3′-flanking sequence to study the effect of site-specific chemical modifications. Oligonucleotides **10** and **11** were controls without CpG or CpR motif. All of the compounds contained 93−98% full-length product as characterized by capillary gel electrophoresis and anion-exchange HPLC (Supporting Information Table 1 and HPLC profiles). Sequence integrity was determined by MALDI-ToF spectral analysis (Table 1).

TLR9 Activation by Oligonucleotides in HEK293 Cell Cultures. Activation of TLR9 by oligonucleotides in cultures of HEK293 cells expressing mouse TLR9 was examined. Agonists **1**−**4**, but not antagonists **6**−**9** or controls **10** and **11**, showed TLR9-mediated NF-κB activation in HEK293 cells (Figure 1A). Treatment of HEK293 cells with combinations of agonist and antagonist showed that **6**−**9** inhibited TLR9 activation by agonists (Figure 1B).

Site-Specific Chemical Modifications are Required for Antagonist Activity. We studied the site of chemical modifications (5′- vs 3′-) necessary for antagonist activity of **6**. HEK293 cells expressing TLR9 were incubated with 0.25 μg/mL of agonist **1** and increasing concentrations of antagonist **6** or compound **2**, which has the same number of 2′-*O*-methylribonucleotide modifications in the 3′-flanking region of the CpG dinucleotide as antagonist **6** has in the 5′-flanking region. Compound **2** did not inhibit the activity of agonist **1** but showed additive activity (Figure 1C). On the contrary, antagonist **6** inhibited the activity of agonist **1** in a dose-dependent manner and almost complete inhibition was observed at the highest dose (Figure 1C).

Antagonists Inhibit NF-κB Activation Induced by Agonist in J774 Cells. We further studied the inhibitory activity of antagonists in mouse macrophage cell line J774. When J774 cells were incubated with agonist **3**, antagonist **8**, or control oligonucleotide **11** alone, as expected, agonist **3**, but not antagonist **8** or control oligonucleotide **11**, showed NF-κB activation (Figure 2A). Further, antagonist **8** inhibited NF-κB activation by agonist **3** in a dose-dependent fashion when cells were preincubated with the antagonist prior to the addition of the agonist (Figure 2A). On the contrary, control oligonucleotide **11** did not show inhibition of agonist **3** induced NF-κB activation under the same experimental conditions (Figure 2A). Additionally, we studied the ability of antagonist **8** to inhibit TLR7/8 agonist (**14**)- and TLR4 agonist (LPS)-induced NF-κB activation in J774 cells. Antagonist **8** showed inhibition of NF-κB activity induced by TLR7/8 agonist but not TLR4 agonist (Figure 2B,C). No significant inhibition of NF-κB activation induced by agonists of TLR2 and 5 was observed (data not shown).

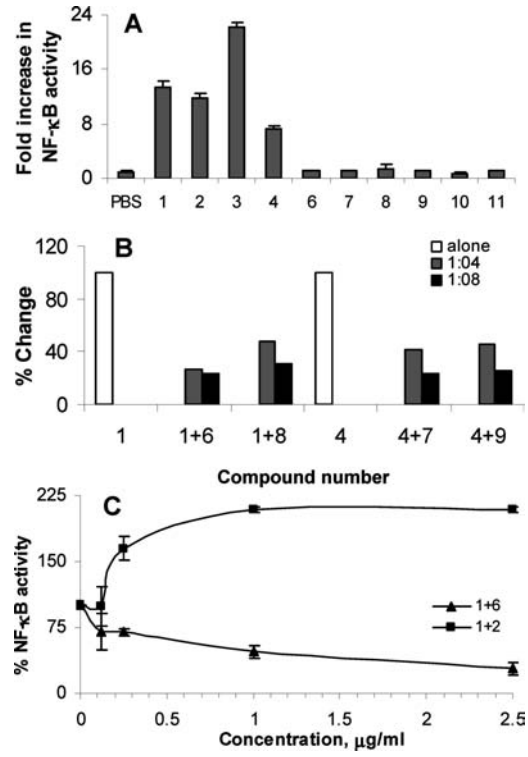


Figure 1. (A) NF-κB activation by agonists and antagonists at 10 μg/mL concentration in HEK293 cells expressing mouse TLR9. (B) Coaddition of antagonist with agonist in HEK293 cells at the indicated ratios inhibits agonist-induced NF-κB activation. The concentration of agonist **1** was 0.5 μg/mL. The ratios of agonist to antagonist used are shown. (C) Effect of compound **2** and antagonist **6** on agonist **1**-induced NF-κB activation in HEK293 cells expressing mouse TLR9. HEK293 cells were stimulated with agonist **1** (0.25 μg/mL) in the absence or presence of compound **2** or antagonist **6** at increasing concentrations. Agonist **1** alone-induced activity in the absence of **2** or **6** is taken as 100%.

To understand the effect of sequential addition of antagonist on agonist-induced NF-κB activity, antagonist was added at the same time as or before or after addition of agonist to J774 cells. Antagonist **8** inhibited NF-κB activation by **3** when the antagonist was added to cells at the same time as or 30 min before agonist addition (Figure 2D). Activation of NF-κB by **3** was also inhibited to some extent when **8** was added 30 min after agonist addition (Figure 2D).

Inhibitory Activity of Antagonists is Reversible. To study the reversible nature of inhibitory effects of antagonists, a fixed concentration of **8** was titrated with increasing concentrations of agonist **3** in HEK293 and J774 cell cultures. As the concentration of **3** was increased, agonist overcame antagonist inhibition, resulting in an increase in NF-κB activity both in HEK293 (Figure 3A) and J774 cells (Figure 3B).

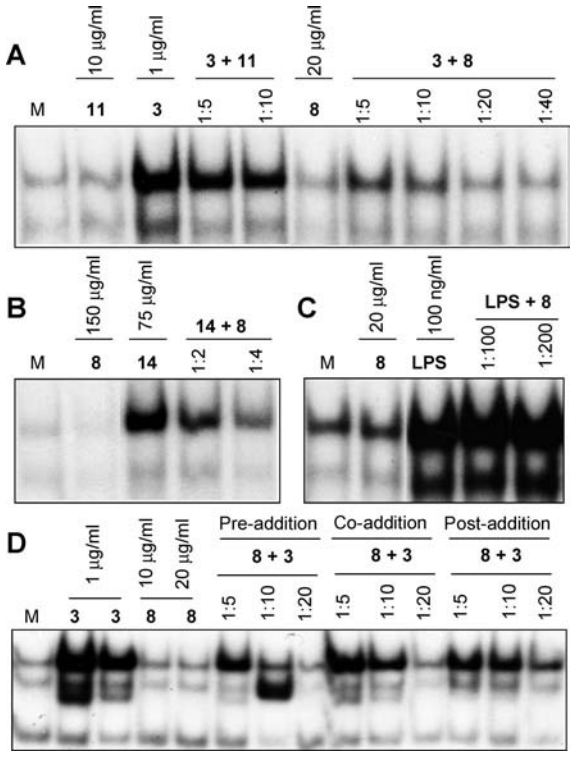


Figure 2. Effect of antagonist on agonist-induced NF-κB activation in J774 cells. (A) Antagonist **8**, but not the control oligonucleotide **11**, inhibits TLR9 agonist-induced NF-κB activation in a dose-dependent manner. Antagonist **8** (5, 10, 20, 40 μg/mL) or control oligonucleotide **11** (5, 10 μg/mL) was added 1 h prior to the addition of agonist **3** (1 μg/mL). (B) Antagonist inhibits RNA-based TLR7/8 agonist **14**-induced NF-κB activation in a dose-dependent manner. (C) Antagonist does not inhibit TLR4 agonist (LPS, 100 ng/mL)-induced NF-κB activation. In experiments shown in panels A, B, and C, cells were preincubated with the indicated ratios of the antagonist for 1 h. (D) Effect of sequential addition of antagonist on agonist-induced NF-κB activation in J774 cells. Antagonist **8** (5, 10, 20 μg/mL) was added 30 min before (preaddition), together (coaddition), or 30 min after (postaddition) agonist **3** (1 μg/mL). In the experiments shown in panels A−D, nuclear extracts were prepared 1 h after the addition of the agonist and EMSA was carried out as described in the text.

Antagonist Inhibits Activity of Agonist of TLR9 in Vivo in Mice. When administered alone into C57BL/6 mice, **3** (0.25 mg/kg), but not **6** (20 mg/kg), induced several cytokines and chemokines (Figure 4A), suggesting that **3** produces an immune response in mice. To study antagonist activity in vivo, we administered **3** and **6** subcutaneously (sc) to mice in the left and right flanks, respectively, collected blood 2 h later, and measured serum IL-12 levels. Antagonist **6** inhibited the activity of **3** in a dose-dependent fashion (Figure 4B). In these experiments, IL-12 production was completely blocked at a ratio of agonist to antagonist of about 1:4.

We then compared the inhibitory activity of antagonists with control oligonucleotides. As shown in Figure 5A, antagonist **8**, but not control oligonucleotides **10** and **11**, inhibited activity of agonist **3** in mice when administered at the same time as or up to 48 h before agonist. We also compared the inhibitory effects of **6** and two poly dG-based compounds, **12** and **13**, reported as antagonists in the literature.[9,11] Both **12** and **13** inhibited agonist **3**-induced IL-12 production at the 6 h time point (Figure 5B), but produced only 48% and 51% inhibition at 24 h and 6% and 0% inhibition at 48 h, respectively, compared with 83% and 49% inhibition by **6** at the same time points (Figure 5B).

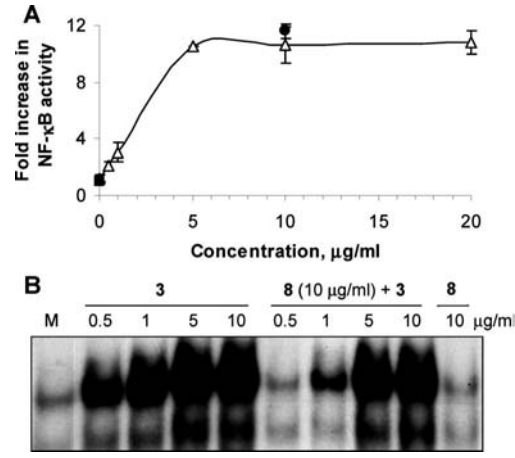


Figure 3. (A) Increasing concentrations of agonist overcomes antagonist inhibition and stimulates NF-κB activation in HEK293 cells expressing TLR9. HEK293 cells were incubated with antagonist **8** at 10 μg/mL in the absence or presence of agonist **3** at increasing concentrations. Filled square indicates stimulation in the absence of any compound. Filled circle indicates stimulation of HEK293 cells with agonist **3** alone at 10 μg/mL. (B) Effect of antagonist on agonist-induced NF-κB activation in J774 cells. J774 cells were stimulated with varying doses of agonist **3** in the absence or presence of 10 μg/mL of antagonist **8** for 1 h. Nuclear extracts were prepared and analyzed by EMSA.

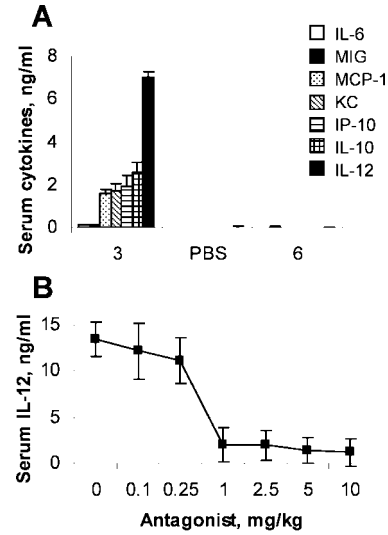


Figure 4. (A) Serum cytokine profiles of agonist **3** (0.25 mg/kg) and antagonist **6** (20 mg/kg) in C57BL/6 mice as determined by luminex multiplex assay. (B) Dose-dependent inhibition of agonist-induced IL-12 production by antagonist in C57BL/6 mice. C57BL/6 mice were injected sc with 0.25 mg/kg agonist **3** in the left flank and various doses of antagonist **6** in the right flank at the same time. The levels of IL-12 were determined by ELISA. In all experiments, $n = 3$ and serum samples were collected 2 h after agonist administration. Data shown are representative of at least three independent experiments.

Specificity of Antagonists for Different TLR Agonists in Vivo. To further study the specificity of antagonist for different TLR agonists in vivo in mice, 10 mg/kg of antagonist **8** was administered sc to C57BL/6 mice and 72 h later an agonist of TLRs 2, 3, 4, 5, 7/8, or 9 was administered in the opposite flank. Blood was collected 2 h after administration of TLR agonist and serum cytokine/chemokine levels were determined using the luminex multiplex assay. The agonists of TLR alone induced significantly higher levels of various cytokines and chemokines than did PBS injected mice. The levels and profiles of cytokines and chemokines induced by each of the TLR

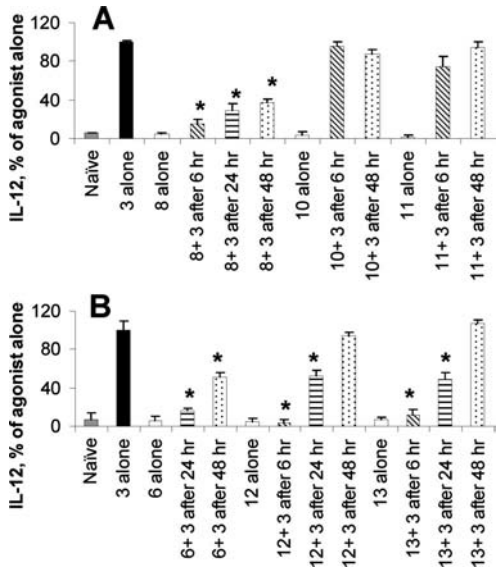


Figure 5. (A) Comparison of inhibition of agonist **3**-induced IL-12 production by compounds **8**, **10**, and **11** in mice. Agonist was administered sc at 0.25 mg/kg, and **8**, **10**, and **11** were administered sc at 10 mg/kg dose. (B) Comparison of inhibition of agonist **3**-induced IL-12 production by antagonist **6**, and poly dG-based compounds **12** and **13** in mice. Agonist was administered sc at 0.25 mg/kg, and **6, 12**, and **13** were administered sc at 10 mg/kg dose in the opposite flank. Data shown are representative of two or three independent experiments. * Indicates statistical significance with $p < 0.05$.

agonists were different as expected (Figure 6). Antagonist **8** inhibited ≥50% production of IL-12, IP-10, IL-10, MCP-1, IL-6, KC, and MIG induced by the TLR9 agonist **3** and RNA-based TLR7/8 agonist **14** (Figure 6). Under the same experimental conditions, antagonist **8** had no significant effect on the cytokines and chemokines induced by the agonists of TLRs 2, 3, 4, and 5 (Figure 6).

Duration of Inhibitory Activity is Dependent on the Dose of Antagonist and Agonist. To study the effect of dose of antagonist on its inhibitory activity, we studied the antagonist **6** at two doses. Six hours after antagonist administration, agonist **3** was administered, and two hours later, serum IL-12 levels were determined. The low and high doses of antagonist inhibited about 50% and 90% of IL-12 production, respectively (Supporting Information Figure 1). We then studied the dose−response of agonist by administering **6** and various doses of **3** after 6 h. Serum IL-12 levels of mice were determined 2 h after agonist administration. These results suggest that inhibitory effect of the antagonist was also dependent on the dose of agonist administered (Supporting Information Figure 2A−C).

To determine the duration of the inhibitory activity of antagonist, we administered **3** to mice at various time intervals after administration of **6** and determined serum IL-12 levels 2 h after agonist administration. Antagonist inhibited production of IL-12 up to 168 h after its administration, with about 50% inhibition of agonist-induced IL-12 production occurring at up to 120 h (Figure 7).

Antagonists Inhibit Agonist-Induced Immune Activation in Human Cells. We further studied antagonists for their ability to inhibit NF-κB activation induced by TLR9 and TLR7/8 agonists in human PBMCs. Both agonists of TLR9 (**5**) and TLR7/8 (**14**) induced greater NF-κB activation compared with medium and antagonist **9** in human PBMCs (Figure 8). Antagonist **9** inhibited NF-κB activation induced by TLR9 (Figure 8A) and TLR7/8 agonists (Figure 8B) when the cells were coincubated with antagonist and agonist together.

We also studied the inhibitory effects of antagonist in cultures of human B cells and PBMCs, using agonist **5** and antagonist **7** or **9**, which contain a human-specific motif. Treatment of human PBMC cultures with TLR9 agonist **5** or TLR7/8 agonist **14**, but not antagonist **9**, resulted in significant production of cytokines and chemokines, including increased levels of IFN-α in culture supernatants (Figure 9A,B). When human PBMCs were incubated with a 1:5 ratio of agonist and antagonist, antagonist **9** significantly inhibited cytokines/chemokines induced by TLR9 agonist **5** and TLR7/8 agonist **14** (Figure 9A,B). Agonists of TLR9 are known to induce human B-cell proliferation. We further studied the ability of antagonists to inhibit TLR9 agonist-induced B cell proliferation. Both antagonists **7** and **9** inhibited B-cell proliferation induced by TLR9 agonist **5** in a dose-dependent manner (Figure 9C).

Inhibitory Activity of Antagonists in MRL/lpr Mouse Spleen Cell Cultures. MRL/lpr mice spontaneously develop systemic lupus erythematosus (SLE), an autoimmune disease.[30] In the present study, purified B cells from the spleens of MRL/lpr mice were cultured with antagonist **8** in the presence or absence of TLR9 agonist **3**. Antagonist **8** alone induced low levels of IL-6 compared with agonist alone. When the cells were cultured with agonist **3** and various concentrations of antagonist **8**, a dose-dependent inhibition of agonist-induced IL-6 production was observed (Figure 10).

Discussion

Synthetic oligodeoxynucleotides containing CpG motifs activate the immune system through TLR9 and induce Th1-type immune responses. Our previous structure−activity relationship studies showed that 2′-*O*-methylribonucleotide modifications incorporated in the first two nucleotide positions adjacent to the CpG dinucleotide on the 5′-side abrogated immune stimulatory activity.[22,27,28] In the present study, we observed that these compounds, referred to as antagonists, did not stimulate immune responses but inhibited the activity of agonists of TLR7/8 and 9 significantly.

In recent reports, certain poly dG-containing oligonucleotides were shown to act as TLR9 antagonists and to be useful in treating autoimmune diseases in animal models.[31−35] However, poly dG-based oligonucleotides form tetraplex and multistranded higher-order structures[19,20] and act as agonists of TLR9 (if a CpG motif is present).[15,16] Certain poly dG compounds have been shown to act as aptamers and inhibit activities of thrombin,[36] HIV,[37−39] RelA,[40] STAT3,[41] and other biological functions due to the formation of various structures. Poly dG-based oligonucleotides lack pharmaceutical attributes due to unpredictable secondary structure formation depending on the experimental conditions, nonsequence-specific protein binding, including immune stimulation.[19,20]

The antagonists described in this paper are distinct in three aspects from the poly dG-based antagonists reported in the literature; (i) no poly dG sequences are present in the antagonist sequences used here, (ii) a CpG or CpR (R = 7-deaza-dG) dinucleotide is required for antagonist activity, and (iii) the two nucleotides adjacent to CpG/CpR on the 5′-side are substituted with 2′-*O*-methylribonucleotides. Therefore, antagonists described herein are structurally different from the reported TLR9 antagonists.

As expected agonists of TLR9 (**1**−**4**) showed TLR9-mediated immune stimulatory activity in HEK293 cells expressing TLR9. On the contrary, antagonists **6**−**8** alone did not induce immune stimulation in HEK293 or mouse macrophage J774 cell cultures compared with oligonucleotides **1**, **2**, and **4** with similar

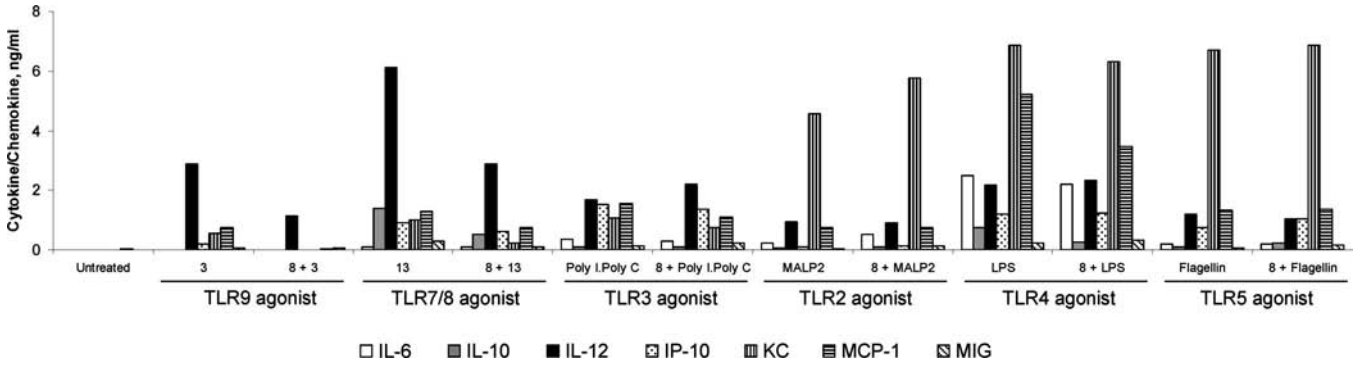


Figure 6. Specificity of inhibition of TLR agonist-induced immune responses by antagonist in mice. C57BL/6 mice were injected sc with antagonist **8** (10 mg/kg) in the right flank and 72 h later, an agonist of TLR2 (MALP2, 0.5 mg/kg), TLR3 (polyI.polyC, 20 mg/kg), TLR4 (LPS, 0.25 mg/kg), TLR5 (flagellin, 0.25 mg/kg), TLR7/8 (**14**, 50 mg/kg), or TLR9 (**3**, 0.25 mgkg) was injected sc in the left flank. Serum cytokine and chemokine levels were determined by luminex multiplex assay. In all experiments, $n = 3$ and serum samples were collected 2 h after agonist administration. Data shown are representative of at least three independent experiments.

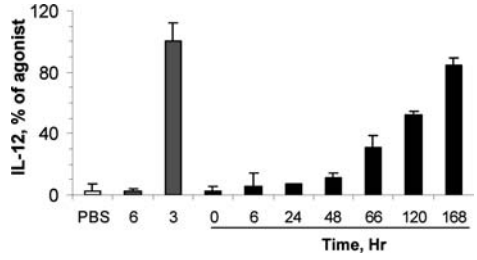


Figure 7. Duration of inhibitory affect of antagonist **6** (20 mg/kg, sc) on TLR9 agonist **3** (0.25 mg/kg, sc)-induced IL-12 in mice. Data shown are one representative experiment of more than six independent experiments.

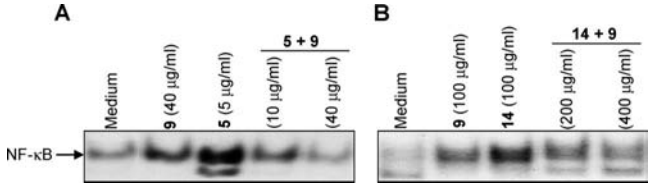


Figure 8. Effect of antagonist on (A) TLR9 and (B) TLR7/8 agonist-induced NF-κB activation in human PBMCs. Human PBMCs were incubated with antagonist at the indicated concentrations for 1 h and then stimulated with the respective agonists for 2 h. Nuclear extracts were isolated and analyzed by EMSA for NF-κB as described in the materials and methods. Data shown are for one representative donor of three studied.

nucleotide sequence and structure. Additionally, antagonists showed a dose-dependent inhibition of TLR9 agonist activity in HEK293 and J774 cells when combined with agonists.

Oligonucleotides containing 2′-*O*-methylribonucleotide substitutions in the 5′-, but not 3′-, flanking sequence adjacent to CpG dinucleotide act as antagonists of TLR9. The combination of TLR9 agonist with oligonucleotide **2** resulted in additive activity, but the combination of agonist with antagonist **6** resulted in the inhibition of agonist-induced NF-κB activation. These results clearly demonstrate that 2′-*O*-methylribonucleotide substitutions in the 5′-flanking sequence adjacent to CpG dinucleotide in antagonists are required for the inhibition of agonist-induced immune stimulatory activity.

In J774-cell culture assays, antagonists inhibited NF-κB activation, suggesting that TLR9 inhibition occurs upstream, but not downstream, of NF-κB activation. Additionally, the ability of antagonists to inhibit cytokines induced by an RNA-based agonist of TLR7/8, but not TLRs 2, 4, and 5, suggests that the inhibition occurs within the endosomal compartment where TLR7 and 9 are expressed. Importantly, the ability of increasing concentrations of TLR9 agonist to overcome the antagonist activity in HEK293 and J774 cell cultures suggests that the effect of antagonists is reversible and is not due to endosomal saturation or toxicity. Antagonists with human-specific motif GTCGTT inhibited IFN-α and other cytokine production in human PBMCs and proliferation of human B cells induced by agonists of TLR9 or TLR7/8 in cell culture assays. Antagonists containing either CpG or CpR motifs had inhibitory activity in these studies.

Antagonists injected subcutaneously at doses of up to 20 mg/kg did not induce cytokine/chemokine production in mice, while TLR9 agonists at doses as low as 0.125 mg/kg (data presented for agonist **3**) induced significant cytokine and chemokine production in mice. Co- or preadministration of antagonists inhibited the activity of TLR9 agonists in mice to a greater degree than did post-administration, suggesting that antagonists block agonist activity by interfering at one of the upstream, but not downstream, signaling events. Antagonists inhibited the cytokine induction by TLR9 agonists when the antagonist was administered together with the agonist or separately at a remote site. The inhibitory effect was dependent on the dose of antagonist as well as agonist, and the inhibitory effect persisted for up to 120 h at a dose of 20 mg/kg. Moreover, compared with poly dG-based oligonucleotides, the novel antagonists described herein showed greater inhibition of immune stimulation by agonists of TLR9 for a longer duration in mice.

TLRs 3, 7, 8, and 9 are expressed intracellularly in endosomal membranes and the other TLRs are expressed on the cell surface. Antagonists studied in the present paper specifically inhibited the activity of agonists of TLR9 and TLR7/8 but not TLR3. Mice lack a functional TLR8. The ability of TLR9 antagonist to inhibit TLR7, but not TLR3, agonist activity suggests that there is a cross-talk between TLR9 and TLR7. Our preliminary studies in TLR7 and TLR9 knockout mice suggest that the presence of TLR9 is required for the inhibition of TLR7 agonist activity by antagonist (unpublished results). Further studies are underway to understand the inhibition of TLR7 agonist activity by antagonist in addition to TLR9 agonist activity. Antagonists did not inhibit activity of agonists of cell surface TLRs 2, 4, and 5.

While activation of TLRs is useful, inappropriate or uncontrolled TLR signaling has been implicated in certain disease processes including various inflammatory and autoimmune diseases.[6,7] The antagonists or inhibitors of TLRs described here might therefore be useful as therapeutic agents in auto-

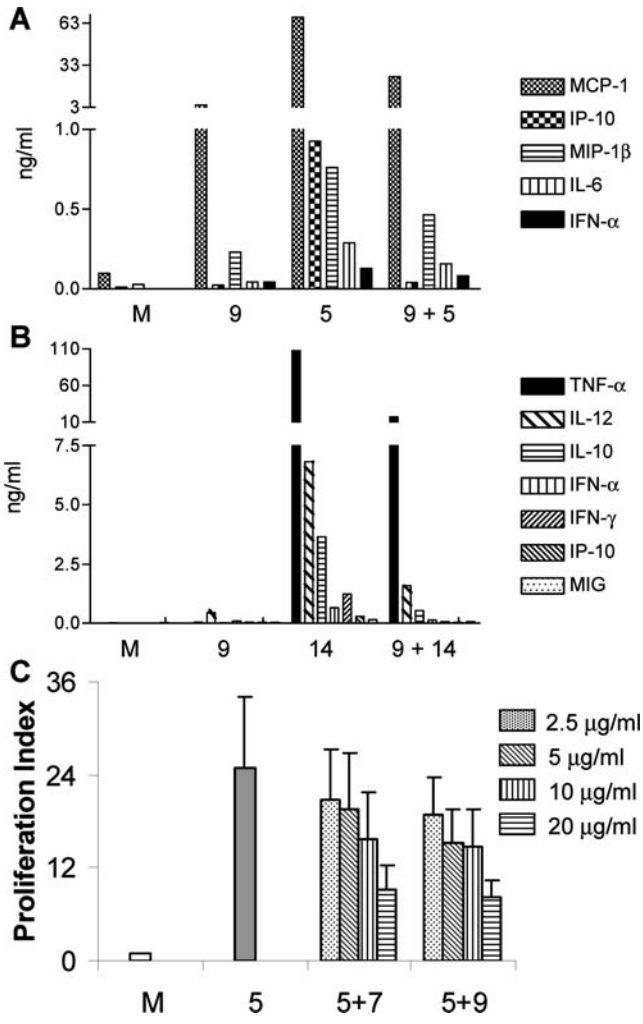


Figure 9. Antagonist inhibits (A) TLR9 and (B) TLR7/8 agonist-induced cytokine secretion by human PBMCs. Human PBMCs from healthy volunteers were isolated and cultured in the presence of agonist and/or antagonist **9** for 24 h at a ratio of 1:4. Supernatants were collected and analyzed by Luminex multiplex assay for cytokine and chemokine levels. The concentration of TLR9 agonist **5** was 5 μg/mL and TLR7/8 agonist **14** was 100 μg/mL. Data shown are for one representative donor of six studied. (C) Antagonists inhibit agonist-induced human B cell proliferation in a dose-dependent manner. Human B ($CD19^+$) cells were isolated from PBMCs and cultured in the presence of compounds for 72 h. Agonist **5** was added at a concentration of 5 μg/mL, and antagonists **7** and **9** were added at 2.5, 5, 10, and 20 μg/mL. Bars represent mean ± SEM of 5 donors.

immune and inflammatory diseases. As an example, our studies showed that antagonists can block TLR9 agonist-induced immune activation in lupus prone MRL/lpr mouse spleen B cells.

The present studies suggest that a CpG or CpR motif and 2′-*O*-methylribonucleotide modifications adjacent to CpG/CpR on the 5′-side are required for antagonist activity. Our previous studies showed that a 5′-accessible end is required for immune stimulatory activity of agonists of TLR9 and suggested that the compounds consisting of two or more 5′-ends, such as **3** and **5**, facilitate TLR9 dimerization and immune stimulation.[42−44] Recent studies have shown that TLR9 exists in dimer form.[45] The binding of CpG oligodeoxynucleotides to the receptor causes conformational changes in the ectodomain of the receptor, thus leading to the recruitment of adapter molecules by the cytoplasmic signaling domain and activation of immune signaling pathways.[45] Non-CpG oligonucleotides also bind to TLR9

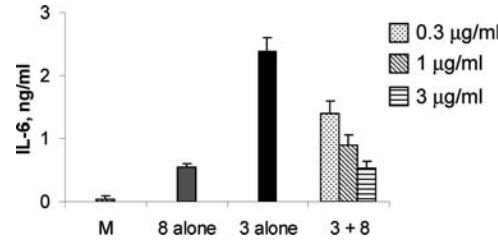


Figure 10. Antagonist inhibits IL-6 secretion by splenic B cells in a dose-dependent manner. Purified spleen B cells from MRL/lpr mice were cultured with 1 μg/mL of agonist **3** alone or in the presence of 0.3, 1, and 3 μg/mL of antagonist **8**, or 3 μg/mL of antagonist **8** alone. After 24 h of culture, supernatants were collected and IL-6 levels were determined by ELISA.

but are not capable of bringing conformational changes in the ectodomain, thus failing to activate immune signaling pathways.[42] Our earlier studies suggested that the activation of TLR9 requires an accessible 5′-end as oligodeoxynucleotides lacking a free 5′-end show no immunostimulatory activity.[43−45] Oligodeoxynuleotides containing CpG motifs (including those containing 2′-*O*-methylribonucleotide substitutions adjacent to CpG on the 3′-side, such as compound **2**) are recognized from the 5′-end, leading to the conformational changes in the ectodomain of the receptor for recruiting adapter molecules and initiating immune signaling pathways. Antagonists **6**−**9** have the same sequences as that of agonists **1** and **4** but with 2′-*O*-methylribonucleotide substitutions 5′ to the CpG dinucleotide. It appears that the presence of 2′-*O*-methylribonucleotide substitutions on the 5′-side adjacent to CpG does not lead to immune activation but can competitively block agonist binding to TLR9. On the contrary, compounds with or without 2′-*O*-methylribonucleotide modifications and lacking CpG dinucleotide (**10** and **11**, respectively) neither act as agonists nor antagonists.

Taken together, CpG or CpR stimulatory motif-containing oligonucleotides with 2′-*O*-methylribonucleotide modifications adjacent to the stimulatory motif on the 5′-side inhibit TLR9-mediated immunostimulatory activity in vitro and in vivo in mice. These novel antagonists may allow specific inhibition of intracellularly expressed TLRs directly, without interfering with other biological factors as is commonly observed with poly dG-based TLR antagonists. Further studies of antagonists in autoimmune disease models are in progress.

Supporting Information Available: Table of purity of compounds as determined by HPLC and CGE, HPLC profiles of all compounds, supporting figure legends, and two figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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