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Synthetic oligoribonucleotides-containing secondary structures act as agonists of Toll-like receptors 7 and 8

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ABSTRACT

Single-stranded RNAs act as ligands of Toll-like receptors (TLRs) 7 and 8 and induce immune responses. In the present study, we have designed and synthesized phosphorothioate oligoribonucleotides (ORNs) with self-complementary sequences that form duplex structures with either 3′- or 5′-overhanging sequences. We studied the new ORNs for their duplex formation, nuclease stability, and ability to induce immune-stimulatory activate through TLR7 and TLR8 in TLR-transfected cell lines, human PBMCs, human pDCs, and *in vivo* in mice. Thermal melting and gel electrophoresis studies showed that all ORNs formed secondary structures and that the thermal stability of the duplex is depended on the length and GC composition of the duplex. Nuclease stability of ORNs increased with increasing thermal stability of the duplex formed. All ORN showed TLR8 activity in HEK293 cells, and induced cytokine and chemokine production in human PBMC cultures. In addition to TLR8 activity, two ORNs containing a 'CUGAAUU' motif in the duplex-forming region induced immune stimulation through TLR7 in HEK293 cells, human PBMC and pDC cultures, and *in vivo* in mice. These results suggest that secondary structures in ORN provide nuclease stability and lead to stimulation of immune responses through TLR8 as well as TLR7 depending on the presence of specific nucleotide motifs.

Introduction

Innate immunity is the first line of defense against pathogen invasion. Toll-like receptors (TLRs) are a family of innate immune receptors that recognize specific pathogen-associated molecular patterns (PAMPs) [1–3]. Of the 10 known TLRs in humans, TLR7 and TLR8 recognize viral and synthetic single-stranded RNA (ssRNA). Both the receptors are expressed in endosomal compartments, but in different cell types [4–9]. TLR7 is predominately expressed in human plasmacytoid dendritic cells (pDCs) and B cells and TLR8 is expressed in monocytes and myeloid DCs (mDCs) [10]. Activation of TLR7 and TLR8 by their ligands leads to induction of Th1-type of cytokines/chemokines and initiation of adaptive immunity [4–9,11–13].

We previously reported a novel RNA structure, referred to as stabilized immuno-modulatory RNA (SIMRA), in which two short phosphorothioate oligoribonucleotides (ORNs) were linked through their 3′-ends via a linker [14]. These novel structures were more stable against 3′-*exo* and *endo* nuclease digestion in human serum than were unmodified ORNs [14]. SIMRA compounds showed immune-stimulatory activity *in vitro* in TLR8-transfected cell lines, human primary cells and *in vivo* in non-human primates [14]. When chemical modifications, such as 7-deaza-guanosine or arabino-cytidine, were incorporated into certain sequences to replace guanosine or cytidine, respectively, the resulting SIMRA compounds activated both TLR7 and TLR8 in cell-based assays and *in vivo* in mice and in non-human primates [14,15].

Formation of secondary structures by RNA can provide stability against single strand-specific *exo*- and *endo*-nucleases [15–17]. Natural RNA folds on itself and forms secondary structures through base-pairing interactions. However, whether the presence of secondary structures in RNA affects recognition by TLR7 and TLR8, which are ssRNA receptors, remains largely unknown. In this paper we designed and synthesized ORN containing secondary structures and studied their ability to act as agonists of TLR7 and TLR8 and induce immune responses.

Materials and methods

Synthesis of ORNs. ORNs were synthesized on a BioAutomation (Plano, TX) Mermade 6 DNA/RNA synthesizer using DMT-nucleoside or diDMT-glycerol linked CPG-solid support and ribonucleoside phosphoramidites obtained from ChemGenes (Wilmington, MA). Beaucage reagent was used as an oxidizing agent to obtain

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phosphorothioate internucleotide linkages [18]. All ORNs were deprotected, cleaved from solid support, and then purified using an anion-exchange HPLC. The purified ORNs were desalted, lyophilized, and characterized by HPLC, capillary gel electrophoresis, and MALDI-TOF mass spectroscopy.

UV thermal melting studies of ORNs. ORNs at 2-μM concentration in 1 mL of 10-mM sodium phosphate buffer, pH 7.2, containing 150-mM NaCl were heated for 5 min at 95 °C and allowed to return to room temperature slowly. The solutions were stored at 4 °C overnight before thermal melting temperature (T_m) was measured. The thermal melting experiments were carried out on a Perkin–Elmer Lambda 20 UV/VIS Spectrophotometer equipped with a Peltier temperature controller and a multi cell holder. Data were collected at each degree by heating or cooling the samples at a rate of 0.5 °C/min. The data were collected and analyzed using Templab software on a personal computer attached to the instrument. Each experiment was carried out at least two times.

Non-denaturing polyacrylamide gel electrophoresis of ORNs. ORNs (0.35 OD) were dissolved in 150-mM NaCl, 10-mM sodium phosphate, pH 7.2 buffer and heated at 95 °C for 5 min and cooled down to ambient temperature slowly. The agonists were loaded onto a 15% non-denaturing polyacrylamide gel with glycerol. The PAGE was run at 5 °C and visualized by UV shadowing at 254 nm.

Nuclease stability study of ORNs. Approximately 0.5 OD of ORNs were added to 1% human serum (Sigma–Aldrich, St. Louis, MO) in PBS. Then the mixture was incubated at 37 °C. The samples were removed after 10 min and analyzed by anion-exchange HPLC and the percentage of intact full-length ORNs remaining was determined.

Human TLR7- and TLR8-transfected HEK293XL cell assays. HEK293 cells stably expressing human TLR7 or TLR8 (Invivogen, San Diego, CA) were cultured and transiently transfected with SEAP (secreted form of human embryonic alkaline phosphatase) reporter plasmid (pNifty2-Seap) (Invivogen) as described previously [15]. After transfection, medium was replaced with fresh culture medium, ORNs were added to the cultures, and the cultures were continued for 18 h. At the end of ORN treatment, culture supernatants were harvested and used for measuring SEAP activity by the QuantiBlue method following the manufacturer's protocol (Invivogen). The data are shown as fold increase in NF-κB activity over PBS control.

Human PBMC and pDC isolation. PBMCs from freshly drawn healthy human volunteer blood (Research Blood Components, Brighton, MA) were isolated by Ficoll density gradient centrifugation (Histopaque-1077; Sigma). Plasmacytoid dendritic cells (pDCs) were isolated from PBMCs by positive selection using the BDCA4 cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions.

Human PBMC and pDC assays. Human PBMCs (5×10^6 cells/ml) and pDCs (1×10^6 cells/ml) were plated in 96-well plates. ORNs dissolved in PBS were added to the cell cultures at various concentrations. The cells were then incubated at 37 °C for 24 h. The levels of cytokines and chemokines in the culture supernatants were measured by using a human multiplex kit on the Applied Cytometry Systems Luminex 100/200 instruments, and the data were analyzed by using StarStation software, version 2.0. The required reagents were purchased from Invitrogen.

Mouse in vivo cytokine assay. ORNs were administered subcutaneously at 50 mg/kg dose to female C57BL/6 mice (5–6 weeks old; Charles River Labs; Wilmington; MA; $n = 3$). Two hours latter, blood was collected by retro-orbital bleeding, and serum cytokine and chemokine levels were determined by Luminex Multiplex assay. All of the animal experimental procedures were performed in accordance with the approved protocols and guidelines of the Institutional Animal Care and Use Committee of Idera Pharmaceuticals.

Results

Design of ORN sequences

We designed six ORNs containing self-complementary sequences, which facilitate formation of inter-molecular secondary structures (Table 1). All ORNs contain phosphorothioate internucleotide linkages. Purified ORNs were desalted, lyophilized, and characterized by HPLC, capillary gel electrophoresis, and MALDI-TOF mass spectrometry (Supplementary Table 1). ORNs **1** and **2** were 22-mers with a 14-nucleotide self-complementary sequence at the 3′-end, allowing them to form 14-base pair (bp) duplexes with 8-mer overhangs at the 5′-ends. ORNs **1** and **2** were designed with different GC content in the duplex-forming region to vary thermal stability of the resulting duplexes and their effect on TLR7/8 activation. ORNs **3** and **4** were designed based on ORN **1** and **2**, respectively, but with a 5′-duplex structure and 3′-overhanging sequences (Table 1). ORN **5** was a three-branched molecule with a 10-mer self-complementary sequence, allowing it to form an inter-molecular duplex structure (Table 1). ORN **6** was designed to have a 10-mer self-complementary sequence adjacent to glycerol linker, allowing it to form an inter-molecular, but not intra-molecular, overlapping duplex structure that could lead to a multimeric structure (Table 1). ORNs **7** and **8** are used as positive controls, which were previously characterized as TLR8 and dual TLR7 and TLR8 agonists, respectively [15]. ORN **9** was a non-immune-stimulatory compound that served as a negative control.

Formation of secondary structures by ORNs

UV thermal melting experiments were carried out to confirm the formation of secondary structures by ORNs. In UV thermal melting studies, ORNs **1**–**6** showed dissociation of secondary structure (melting) as a function of temperature (Fig. 1A). The melting temperature (T_m) values measured from melting curves for each compound ranged from 30 to 61 °C. ORNs **1** and **3**, which had eight GC base pairs in the duplex-forming sequence, showed higher T_m values than did ORNs **2** and **6** with four GC base pairs (Supplementary Table 1).

The formation of inter-molecular secondary structures by ORNs **1**–**6** was also studied using a non-denaturing polyacrylamide gel electrophoresis (Fig. 1B). Control ORN **9** had faster mobility on the gel, suggesting no secondary structure formation. ORNs **1**–**4** and **6** (with similar nucleotide lengths) had slower moving bands than did ORN **9**, suggesting formation of secondary structures. ORN **6** moved much slower than did the other structures, suggesting the formation of overlapping multimeric structure as shown in Table 1. ORN **5**, which was a 34-mer, had slightly slower mobility than ORNs **1**–**4**.

Nuclease stability of ORNs

Serum stability of the secondary structure-forming ORNs **1**–**6** was studied. After 10 min of incubation in 1% human serum, ORNs **1**–**6** had 42–85% full-length products remaining, which was comparable to ORNs **7** and **8** (Supplementary Table 1). ORNs that had higher T_ms had greater stability against nucleases than did those with lower T_ms (**1** vs **2** and **3** vs **4**). Control ORN **9** had over 90% full-length product under the same conditions, due to its polyA sequence.

Activity of ORNs in HEK293XL cells expressing human TLR8

Activation of TLR8 by secondary structure-containing ORNs was evaluated in HEK293XL cells stably expressing human TLR8. All