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Synthetic oligoribonucleotides containing arabinonucleotides act as agonists of TLR7 and 8

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ABSTRACT

In continuation of our studies with stabilized immune modulatory RNA (SIMRA) compounds, we have synthesized novel SIMRA compounds incorporating arabinonucleotides to study their effects on TLR7 and TLR8 activation. The SIMRA compounds containing ara-G, ara-C, ara-U or ara-A substitutions activated TLR8 in HEK293 cells. Interestingly, the SIMRA compound containing ara-C also activated TLR7 and stimulated immune responses in vivo in mice. In human PBMC and pDC assays, SIMRA compounds containing arabinonucleotides induced Th1-type cytokine profiles. These results suggest that SIMRA compounds containing arabinonucleotides act as agonists of TLR7 and TLR8.

Single-stranded viral and synthetic RNA (ssRNA) act as agonists of TLR7 and TLR8, which belong to a family of highly conserved receptors called pathogen-associated molecular pattern (PAMP) recognition receptors.[1–3] Both receptors are expressed in the membranes of endosomes in the cells but in different cell types.[4,5] TLR7 is expressed in human B cells and plasmacytoid dendritic cells (pDC) and TLR8 is expressed in myeloid DCs (mDC) and monocytes.[4–6] The immune profiles induced by ssRNA agonists are dependent on the type of cells. In general, TLR7 and TLR8 agonists induce Th1-type immune responses.[1–8]

Although ssRNA is a ligand for TLR7 and TLR8, wide therapeutic application of RNA has been hampered by its susceptibility to nuclease degradation.[9–12] Nuclease degradation of RNA occurs primarily from the 3′-end by exonucleases and sequence-specifically at internal sites by endonucleases. Lipid carriers are commonly used with RNA-based agonists of TLR7/8 for in vitro and in vivo studies to increase nuclease stability.[2,4,13]

We have reported a novel class of synthetic oligoribonucleotides, referred to as stabilized immune modulatory RNA (SIMRA) compounds, in which two short oligoribonucleotides are attached through their 3′-ends. SIMRA compounds are more resistant to nuclease degradation and stimulate immune responses through activation of TLR8. SIMRA compounds that have 7-deaza-guanosine incorporated in place of guanosine activate TLR8 as well as TLR7. SIMRA compounds that act as agonists of TLR8 and dual agonists of TLR7 and TLR8 induce potent immune responses in vivo in mice and non-human primates.[14]

RNA compounds with 2′-modifications such as 2′-deoxy and 2′-methoxy groups have greater nuclease stability but do not induce TLR7- and TLR8-mediated immune stimulatory activity.[15] It is not known whether RNA compounds containing arbinonucleotides act as agonists of TLR7 and TLR8. In the present study, to examine the impact of arabinonucleotides in SIMRA compounds on TLR7 and TLR8 activation, we synthesized SIMRA compounds incorporating arabino-guanosine (ara-G) (**2**), -cytosine (ara-C) (**3**), or -uridine (ara-U) (**4**), in place of G, C, or U residues, respectively, in the parent SIMRA compound **1**, which acts as an agonist of TLR8 (Table 1). Since compound **1** did not have adenosine, we chose SIMRA compound **5**, which contains adenosine and acts as an agonist of TLR8, and substituted adenosine with ara-A (**6**) (Table 1). Compound **7** was used as a negative control.

All compounds were synthesized with phosphorothioate backbone using β-cyanoethylphosphoramidite chemistry on a controlled-pore glass solid support on an automated DNA/RNA synthesizer. All the compounds were purified and characterized as described previously.[14] The MALDI-ToF characterization of all SIMRA compounds are shown in Table 1. The nuclease stability was studied by incubating SIMRA compounds in 1% human serum in PBS for 10 min, followed by analysis by anion-exchange chromatography to determine the percentage of the full-length product. The parent compounds (**1** and **5**) without arabinonucleotides showed about 75% full-length product. The SIMRA compounds with arabinonucleotide substitutions showed 76–86% full-length

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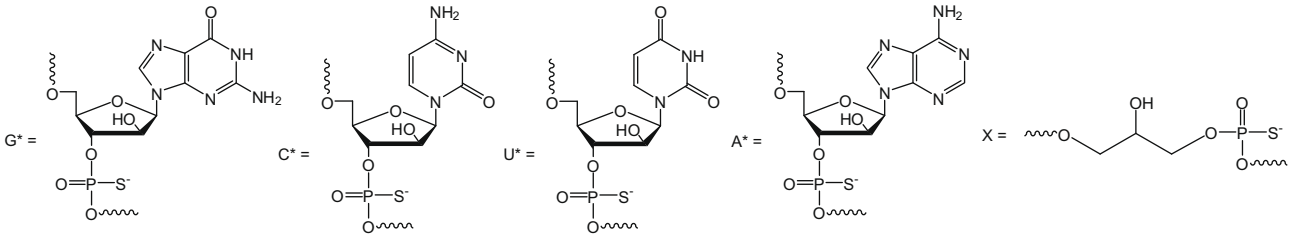
Table 1
Sequences of SIMRA compounds containing arabinonucleotides and their MALDI-ToF mass analysis data

Compound number	Sequence[a]	Molecular weight[b]	
		Calculated	Found
1	5′-UGCUGCUUGUG-X-GUGUUCGUCGU-5′	7486	7487
2	5′-UG*CUG*CUUG*UG*-X-G*UG*UUCG*UCG*U-5′	7486	7485
3	5′-UGC*UGC*UUGUG-X-GUGUUC*GUC*GU-5′	7486	7487
4	5′-U*GCU*GCUUGUG-X-GUGUUCGU*CGU*-5′	7486	7485
5	5′-UGUUGUGUGAC-X-CAGUGUGUUGU-5′	7540	7545
6	5′-UGUUGUGUGA*C-X-CA*GUGUGUUGU-5′	7540	7541
7	5′-AAAAAAAAAAA-X-AAAAAAAAAAA-5′	7690	7688

[a] All compounds have a phosphorothioate backbone; structures of arabinonucleotides (*) and X are shown below.
[b] Molecular weight as calculated and determined (found) by MALDI-ToF Mass Spectrometer.



product depending on the number and nature of arabinonucleotides present. The control compound **7** showed over 90% full-length product under the same conditions.

The ability of SIMRA compounds to act as agonists of TLR8 was studied in HEK293XL cells stably expressing human TLR8.[17] The results are expressed as fold change in NF-κB activity over PBS-treated cells (Fig. 1A). SIMRA compounds **2**–**4** and **6** (containing ara-G, ara-C, ara-U, and ara-A, respectively) activated human TLR8. SIMRA compounds containing ara-C (**3**), ara-U (**4**), and ara-A (**6**) activated TLR8 in a dose-dependent manner at levels similar to those of their parent compounds **1** and **5**. However, SIMRA compound **2** (containing ara-G) produced lower levels of NF-κB activation at lower doses and had similar activity as that of the parent compound **1** at the highest dose. Control compound **7** did not stimulate TLR8.

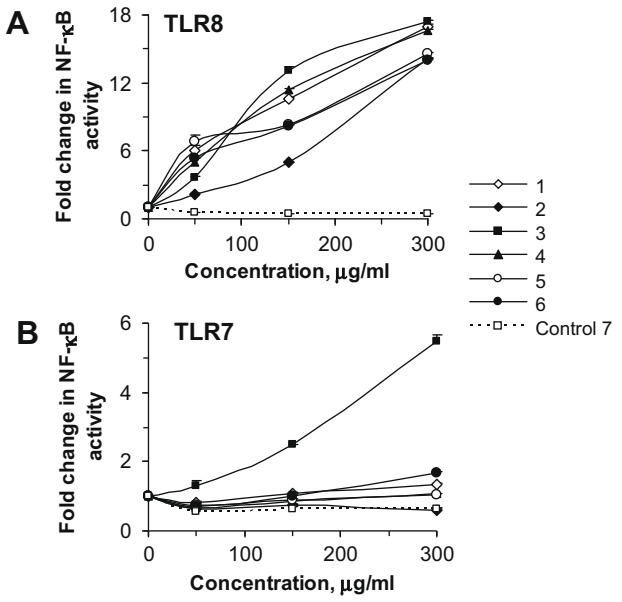


Figure 1. Activation of HEK293XL cells expressing human TLR8 (A) and TLR7 (B) by SIMRA compounds at concentrations of 0, 50, 150, and 300 μg/ml. Data shown are representative of three or more independent experiments.

These results suggest that SIMRA compounds containing arabinonucleotides act as agonists of TLR8.

We then studied the ability of SIMRA compounds containing arabinonucleotides to activate human TLR7 in HEK293XL cells stably expressing TLR7.[17] Both parent compounds **1** and **5** did not activate TLR7. These data are consistent with our earlier report that substitution of G with 7-deaza-G is required for TLR7 activation.[14] Interestingly the SIMRA compound containing ara-C (**3**) showed dose-dependent activation of TLR7 (Fig. 1B). The other SIMRA compounds containing ara-G, ara-A, or ara-U did not activate TLR7 even at the highest dose studied. The control compound **7** had no activity in TLR7 expressing HEK293 cells.

We further studied the ability of SIMRA compounds containing arabinonucleotides and their parent compounds to stimulate immune responses in human PBMC cultures.[17] Parent compounds **1** and **5**, and SIMRA compounds containing arabinonucleotides induced Th1-type cytokine/chemokine profiles in human PBMC cultures (Fig. 2). In general, the levels of cytokines induced by SIMRA compounds containing arabinonucleotides were similar to those induced by the parent compounds. However, SIMRA compound **2** (containing ara-G) induced lower levels of cytokines and chemokines than did parent compound **1**. As expected, SIMRA compound **3**, which activated TLR7 in HEK293 cell assays, induced higher levels of IFN-α than any other SIMRA compound. Control compound **7** produced background levels of cytokines/chemokines in PBMC assays.

We studied the ability of SIMRA compounds **1**–**6** to induce cytokine production by human pDCs.[17] All SIMRA compounds induced cytokine induction in pDCs, but SIMRA compound **3**, which activated both TLR7 and TLR8, induced significantly higher levels of cytokines, especially IFN-α. The other SIMRA compounds (**1**, **2**, **4**–**6**), which activated TLR8 only, induced no or low levels of IFN-α production by human pDCs (Fig. 3). Control compound **7** induced background levels of cytokine induction.

The ability of SIMRA compounds **1**–**6** and control compound **7** to induce immune responses in vivo in C57BL/6 mice was studied.[17] SIMRA compound **3** (containing ara-C), which activated both TLR7 and TLR8 in the cell-based assays, induced significantly higher levels of IL-12, KC, and IP-10 than did the control compound (Fig. 4). As TLR8 is not functional in mice, the other SIMRA compounds, which activated only TLR8, induced background levels of IL-12 in mice. These results suggest that SIMRA compounds are

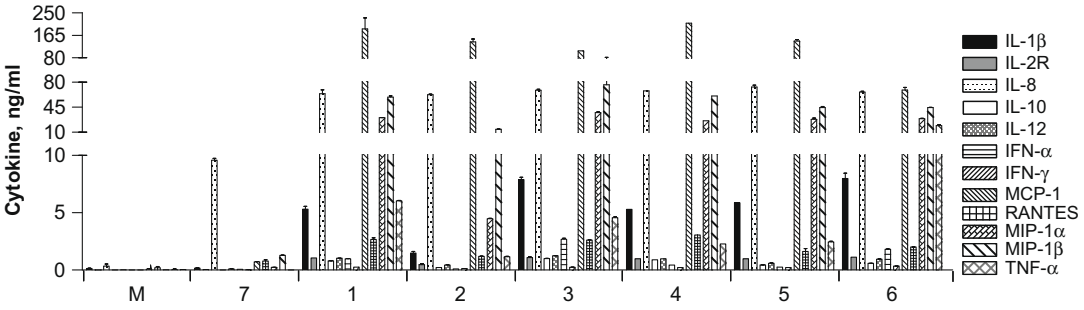


Figure 2. Cytokine and chemokine induction by SIMRA compounds containing arabinonucleotides in human PBMC cultures at 150 μg/ml concentration. M stands for medium control. Data shown are representative of three or more independent experiments.

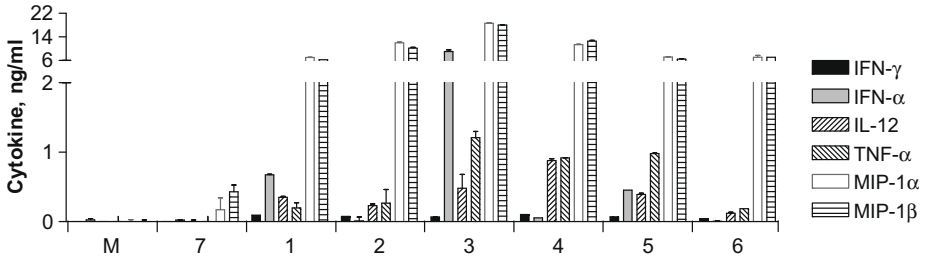


Figure 3. Cytokine and chemokine induction by SIMRA compounds containing arabinonucleotides in human pDC cultures at 150 μg/ml concentration. M stands for medium control. Data shown are representative of three or more independent experiments.

stable in vivo without lipid formulation, and that dual agonists of TLR7 and TLR8 can induce immune responses in mice.

Single-stranded viral and synthetic RNAs are the ligands for TLR7 and TLR8.[1–3] A number of synthetic small molecules, such as guanosine analogs and imidazoquinolines also activate TLR7 and TLR8.[16,18] ssRNA is highly susceptible to nuclease degradation and formulation with lipid carriers has been required to stabilize RNAs against nucleases for in vivo use.[2,9–12] We have previously designed RNAs, referred to as SIMRA compounds, that are linked through their 3′-ends to prevent 3′-exonuclease degradation and which act as TLR8 agonists.[14] In addition, SIMRA compounds in which natural guanosines are replaced by 7-deaza-guanosine activate immune responses through TLR7 in addition to TLR8.[14] In the present study, we studied the ability of TLR8 agonist SIMRA compounds in which ribonucleotides were replaced with arabinonucleotides to act as agonists of TLR7 and TLR8.

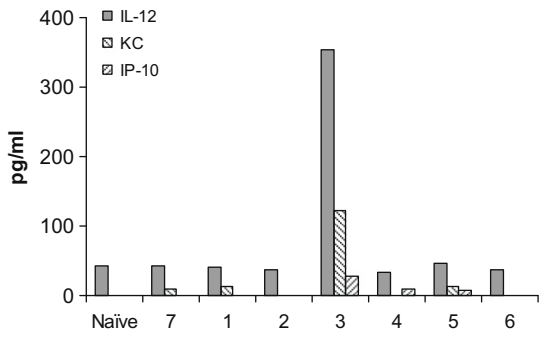


Figure 4. Serum cytokines and chemokines induced by SIMRA compounds containing arabinonucleotides following subcutaneous administration to mice at 50 mg/kg dose. Serum cytokine/chemokine levels were measured by Luminex Multiplex assay. Naïve indicates untreated mice. Data shown are representative of two experiments.

In ribonucleotides, the 2′-OH group of ribose is trans-oriented relative to 1′-heterocyclic base, which is the primary recognition site for ribonucleases and many other RNA enzymes.[19] In contrast, arabinonucleotides have the 2′-OH group oriented cis to the heterocyclic base (Table 1). The cis orientation of 2′-OH group causes the arabinonucleotides to adopt a 2′-endo conformation in contrast to the 3′-endo conformation of ribonucleosides.[20,21] Site-specific substitution of ara-G, ara-C, ara-U, or ara-A in TLR8 stimulating SIMRA compounds showed activation of TLR8 in HEK293XL cells, suggesting that arabinonucleotide substitutions are tolerated by TLR8. In addition, the SIMRA compound that contained ara-C acted as an agonist of both TLR7 and TLR8. Consistent with TLR activation in HEK293 cells, SIMRA compounds containing arabinonucleotides induced Th1-type cytokine production in human PBMCs. SIMRA compound **3** (containing ara-C) induced higher levels of IFN-α than SIMRA compounds containing other arabinonucleotides in both human PBMCs and pDCs, consistent with its ability to activate TLR7, and also stimulated immune responses in vivo in mice. The lower activity of SIMRA compound **2** (containing eight arabinonucleotides) in HEK293 cells and human PBMCs could be a result of extensive conformational changes in the RNA that could affect TLR7/8 recognition and binding. A SIMRA compound with fewer ara-G substitutions showed activity similar to that of the parent compound (data not shown).

In the present Letter, we showed that site-specific substitution of arbinonucletides in TLR8 stimulatory SIMRA compounds does not compromise TLR8 agonist activity. SIMRA compounds containing ara-G, ara-C, ara-U, or ara-A activated TLR8. Previously we have shown that substitution of G with 7-deaza-G in SIMRA compounds leads to activation of both TLR7 and 8. Interestingly, a SIMRA compound containing ara-C substitutions acted as a ligand for both TLR7 and TLR8. The ability to modulate immune responses by incorporating specific arabinonucleotides in SIMRA compounds may prove to be valuable for the design of novel TLR7 and TLR8 agonists as this class of compounds have therapeutic application

in infectious diseases, oncology, asthma, and allergies and as vaccine adjuvants.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.02.021.

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