

2'-O-methyl-modified RNAs Act as TLR7 Antagonists

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RNA molecules such as single-stranded RNA (ssRNA) and small interfering RNA (siRNA) duplexes induce Toll-like receptor (TLR)-mediated immune stimulation after intracellular delivery. We have previously shown that selective incorporation of 2'-O-methyl (2'OMe) residues into siRNA abrogates cytokine production without reduction of gene silencing activity. Here we show that 2'OMe-modified RNA acts as a potent inhibitor of RNA-mediated cytokine induction in both human and murine systems. This activity does not require the direct incorporation of 2'OMe nucleotides into the immunostimulatory RNA or that the 2'OMe nucleotide-containing RNA be annealed as a complementary strand to form a duplex. Our results indicate that 2'OMe RNA acts as a potent antagonist of immunostimulatory RNA. We further show that 2'OMe RNA is able significantly to reduce both interferon- α (IFN- α) and interleukin-6 (IL-6) induction by the small-molecule TLR7 agonist loxoribine in human peripheral blood mononuclear cells (human PBMCs), in murine Flt3L dendritic cells (Flt3L DCs), and *in vivo* in mice. These results indicate that 2'OMe-modified RNA may have utility as an inhibitor of TLR7 with potential applications in the treatment of inflammatory and autoimmune diseases that involve TLR7-mediated immune stimulation.

Received 1 May 2007; accepted 28 May 2007; published online 19 June 2007. doi:10.1038/sj.mt.6300240

INTRODUCTION

The mammalian innate immune system has evolved mechanisms for recognizing a number of nucleic acid species as signatures of potential pathogens. Specific Toll-like receptors (TLRs)¹ have now been identified that recognize double-stranded RNA (TLR3),² single-stranded RNA (ssRNA) (TLR7 and TLR8),³⁻⁵ and CpG DNA (TLR9)⁶ in both humans and mice. Common features of these nucleic acid-sensing TLRs are their intracellular localization and induction of Type I interferons such as IFN- α and inflammatory cytokines upon activation. TLR7 is typically expressed by plasmacytoid dendritic cells (plasmacytoid DCs) and B cells and is activated by ssRNA viruses, synthetic ssRNA rich in guanosine and uridine, and nucleoside analogs such as

loxoribine and imidazoquinoline amines.^{3-5,7} TLR8 is phylogenetically close to TLR7. Although these receptors recognize structurally similar RNA and nucleoside analogues,⁸ TLR7 and 8 are typically expressed on distinct immune cell types. Murine TLR8 does not respond to conventional TLR7/8 ligands and, until recently,⁹ was thought to be non-functional in mice.⁴ RNA can also generate interferon responses through TLR- and dsRNA-dependent protein kinase (PKR)-independent mechanisms, including the cytoplasmic RNA receptors RIG-1 and Mda-5.¹⁰

Activation of the immune system through TLRs causes the rapid production of pro-inflammatory cytokines and interferons that orchestrate the developing innate and adaptive immune response to infection. Aberrant or excessive stimulation of these pathways is thought to underlie many inflammatory and autoimmune disorders. For example, RNA- and DNA-associated autoantigens in systemic lupus erythematosus have been shown to drive pathologic autoantibody and interferon production through TLR7 and TLR9 activation.¹¹⁻¹⁵ Inhibitors or antagonists of TLR signaling are therefore considered to have significant therapeutic potential.

We and others have shown that siRNA and short hairpin RNA duplexes that mediate RNA interference can cause potent activation of innate immune cells, particularly when intracellular delivery is facilitated with lipidic or polycation vehicles.¹⁶⁻¹⁸ siRNA and its ssRNA components trigger IFN- α production from plasmacytoid DCs and inflammatory cytokine production from myeloid DCs in a sequence-dependent manner.¹⁶⁻¹⁸ This response is primarily mediated through TLR7 in mice¹⁷ and TLR7/8 in humans.⁴ We have previously found that the incorporation of 2'-O-methyl (2'OMe)-uridine or 2'OMe-guanosine residues into an siRNA duplex or its ssRNA components eliminates their immunostimulatory activity both *in vitro* and *in vivo*,¹⁹ and these observations have subsequently been confirmed by gene expression analysis.²⁰ It has also been reported that chemical modification of nucleotides in mammalian RNA, including 2'OMe modifications, suppresses their ability to activate DCs.²¹ Strikingly, suppression of immunostimulatory activity requires only a small percentage of nucleotides (<10%) in the RNA species to be modified, suggesting that TLR7/8-mediated immune stimulation by RNA is potently inhibited by the presence of 2'OMe nucleotides.¹⁹

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To extend these observations and to determine how 2'-OMe-modified ssRNA (2'-OMe RNA) may exert this potent inhibitory effect we tested the ability of 2'-OMe RNA to antagonize TLR7-mediated immune stimulation. Here we report that 2'-OMe RNA directly inhibits cytokine induction by unrelated immunostimulatory RNA and by the small-molecule TLR7 agonist loxoribine in both murine and human systems. This inhibitory activity of 2'-OMe RNA is also manifest *in vivo*. On the basis of these data we suggest that 2'-OMe RNA acts as an antagonist of TLR7-mediated immune stimulation.

RESULTS

2'-OMe RNA inhibits cytokine induction by non-complementary, immunostimulatory RNA

We have previously shown that the introduction of as few as two 2'-OMe-uridine or 2'-OMe-guanosine nucleotides into siRNA duplexes effectively abrogates their immunostimulatory activity.¹⁹ These inhibitory effects did not require the stimulatory strand within the duplex to be directly modified, suggesting that immune recognition of the intact RNA duplex is effectively inhibited by 2'-OMe nucleotides incorporated within the molecule. To examine whether this so-called *trans*-inhibitory effect requires the modified RNA to be annealed to the immunostimulatory RNA species, we encapsulated non-complementary 2'-OMe RNA and immunostimulatory native RNA into lipid particles and tested their ability to stimulate cytokine responses from human peripheral blood mononuclear cells (PBMCs). For initial experiments, two previously described ssRNAs that contain 2'-OMe-uridines (Luc-mU and NP-mU) were evaluated.¹⁹ In their modified form, these ssRNAs do not induce measurable IFN- α production from human PBMCs (Figure 1a and ref. 19).

We encapsulated 2'-OMe RNA into lipid nanoparticles at a 1:1 molar ratio with immunostimulatory single-stranded (β gal-AS, ApoB-AS) or duplex (ApoB1 siRNA) RNA. Lack of duplex formation or dimerization between the 2'-OMe RNA and the other RNA species was confirmed by non-denaturing PAGE analysis of the RNA (data not shown). Each of the immunostimulatory RNAs induced high levels of IFN- α when applied to PBMC cultures alone at RNA doses ranging from 0.1 to 3 μ g/ml. This immune response was completely abrogated when these native immunostimulatory RNAs were administered with either of the non-complementary 2'-OMe RNAs (Figure 1a-c). This inhibitory effect appeared robust because 2'-OMe RNA effectively antagonized the IFN- α induction associated with a fourfold molar excess of the native immunostimulatory RNA. Formulation of immunostimulatory RNA with an inherently non-stimulatory native ssRNA had no effect on cytokine induction (data not shown).

To test whether other 2'-OMe nucleotides possessed similar inhibitory capacity, modified RNAs were synthesized incorporating either 2'-OMe-guanosine, 2'-OMe-adenosine, or 2'-OMe-cytidine residues. 2'-OMe-G- and 2'-OMe-A- but not 2'-OMe-C-modified RNA inhibited cytokine production after formulation with immunostimulatory RNA (Figure 1d and e). These findings complement previous reports demonstrating that incorporation of 2'-OMe-C residues is ineffective at directly abrogating the immunostimulatory activity of ssRNA or siRNA.^{19,21}

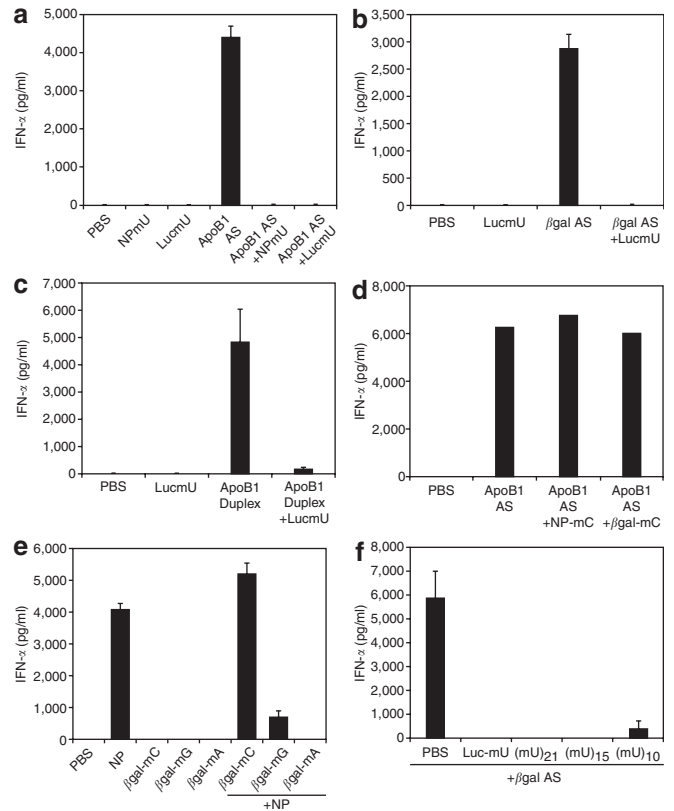


Figure 1 2'-O-methyl (2'-OMe) RNA inhibits RNA-mediated interferon- α (IFN- α) production from human peripheral blood mononuclear cells (PBMCs). IFN- α responses from human PBMCs after treatment with immunostimulatory RNA either alone or formulated with 2'-OMe RNA. Robust IFN- α induction by (a) 1 μ g/ml ApoB1 AS or (b) 0.15 μ g/ml β gal AS single-stranded RNA (ssRNA) and (c) 1.5 μ g/ml ApoB1 duplex RNA is abrogated by administration of the 2'-OMe-uridine RNAs NPmU or LucmU at equimolar concentrations. (d) The 2'-OMe-cytidine RNAs NP-mC or β gal-mC 2'-OMe-cytidine RNAs do not inhibit IFN- α induction by 1 μ g/ml ApoB1 AS ssRNA. (e) 2'-OMe-guanosine (β gal-mG)- and 2'-OMe-adenosine (β gal-mA)-modified RNA but not 2'-OMe-cytidine (β gal-mC)-modified RNA inhibits IFN- α induction by 0.5 μ g/ml NP ssRNA. (f) 2'-OMe-uridine homopolymers (mU)₂₁, (mU)₁₅, and (mU)₁₀ of 21-, 15-, and 10-nt length, respectively, inhibit IFN- α induction by 0.15 μ g/ml β gal AS ssRNA. In each experiment RNAs were administered either alone or at a 1:1 molar ratio of immunostimulatory RNA to 2'-OMe RNA. Inhibition of cytokine induction was observed at all RNA doses tested from 0.1 to 3 μ g/ml. Data represent mean IFN- α in supernatants after 24 hours' culture + SD of triplicate wells and are representative of at least two separate experiments. PBS, phosphate-buffered saline.

To determine whether the inhibitory effects of 2'-OMe RNA required the modified nucleotides to be presented in a particular sequence or positional context, we tested the inhibitory activity of 2'-OMe-uridine homopolymers of 21, 15, or 10 nt in length. 2'-OMe-uridine 21-mers [(mU)₂₁] and 15-mers [(mU)₁₅] were equally effective at inhibiting cytokine production from human PBMCs when formulated with immunostimulatory ssRNA. 2'-OMe-uridine 10-mers [(mU)₁₀] also significantly reduced cytokine induction, although inhibition with these shorter oligonucleotides was not absolute (Figure 1f).

The above experiments conducted in human PBMC cultures were repeated using murine Flt3L-derived DCs (Flt3L DCs). Culturing murine bone marrow cells with Flt3L generates a

mixed culture of myeloid DCs and plasmacytoid DC-like cells that are responsive to TLR7 ligands including ssRNA.⁴ The results using murine Flt3L DCs were similar to those obtained in human PBMC cultures. Administration of (mU)₂₁ with immunostimulatory ssRNA completely abrogated measurable IFN- α and interleukin-6 (IL-6) production on Flt3L DCs (**Supplementary Figure S1a and b**). Taken together, these results demonstrate that 2'OMe RNA potently inhibits immune stimulation mediated by short RNA molecules. Inhibition of this pathway in both mice and humans is achieved by the incorporation of 2'OMe-U, 2'OMe-G, or 2'OMe-A nucleotides with no apparent positional or sequence-dependent requirements within the modified RNA.

2'OMe RNA does not antagonize cytokine induction by type B and C CpG DNA

To determine whether 2'OMe RNA also inhibited immune stimulation by TLR9 agonists, various CpG DNA oligonucleotides (ODNs) were formulated with either (mU)₂₁ RNA or Luc-mU at a 1:1 molar ratio and applied to Flt3L-DCs. ODNs 6295, 1826, and M362 were selected as representative of CpG Type A, B and C ODNs, respectively, in the murine system. Each TLR9 agonist was synthesized with phosphodiester backbones and shown to be highly immunostimulatory in murine Flt3L cultures when formulated in lipid nanoparticles. Formulation with (mU)₂₁ RNA had no inhibitory effect on the level of IFN- α induction by Type B (1826) or Type C (M362) ODNs, indicating that 2'OMe RNA does not inherently antagonize TLR9 activation (**Figure 2a and b**). However, formulation of 2'OMe RNA was found to cause significant, but not absolute, inhibition of the IFN- α response to a Type A ODN (6295) (**Figure 2c**). Experiments were repeated with a range of ODN concentrations from 0.1 to 5 μ g/ml with similar effect. To examine this differential effect of 2'OMe RNAs on TLR9 agonists further, these experiments were repeated in human PBMC cultures. As in the mouse system, formulation of type C (M362) ODN with 2'OMe RNA had little or no effect on IFN- α induction by human PBMCs, whereas the response to a human Type A ODN (2216) was effectively abolished (**Figure 2d and e**). In both mouse and human models, analysis of inflammatory cytokines such as IL-6 and tumor necrosis factor- α mirrored the results for IFN- α (data not shown). Furthermore, 2'OMe RNA had no effect on the cytokine response elicited by polyI:C (**Figure 2f**), a long double-stranded RNA homolog that activates mammalian cells through both TLR3 and PKR. Taken together, these findings suggest that 2'OMe RNAs specifically inhibit TLR7/8-mediated activation by immunostimulatory RNA but do not directly antagonize the other nucleic acid sensing TLRs. The basis for the differential inhibition of Type A ODNs by 2'OMe RNA is unclear at present. Although immune stimulation by this ODN class requires TLR9,²² unlike other ODNs, stimulatory activity is dependent on its oligomerization through G-quartets in the ODN sequence. Formulation with 2'OMe RNA did not disrupt oligomer formation by the Type A ODN.

2'OMe RNAs inhibit immune activation by the TLR7 agonist loxoribine *in vitro*

2'OMe RNAs were tested for their ability to inhibit cytokine production by the defined TLR7 agonist loxoribine (7-allyl-8-oxoguanosine, Lox), a guanosine analog that preferentially activates

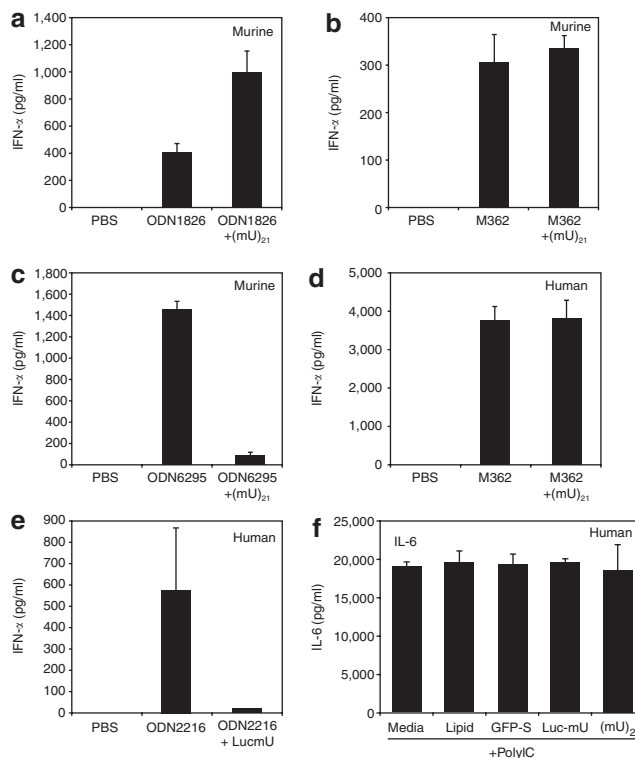


Figure 2 2'-O-methyl (2'OMe) RNA does not inhibit cytokine production by Type B or C DNA oligonucleotides (ODN) or PolyI:C *in vitro*. Interferon- α (IFN- α) responses from (**a–c**) murine Flt3L dendritic cells and (**d–e**) human peripheral blood mononuclear cells (PBMCs) treated with either lipid-formulated CpG ODN alone or in combination with the 2'OMe RNAs (mU)₂₁ or Luc-mU. CpG ODNs tested were (**a**) Type B ODN 1826, (**b, d**) Type C ODN M362, and (**c, e**) Type A ODN 6295 or 2216, respectively. Cells were treated with either 0.5 μ g/ml ODN alone or with an equimolar amount of the indicated 2'OMe RNA. 2'OMe RNA did not inhibit IFN- α induction by Type B or C ODNs; however, responses to Type A ODNs were significantly reduced. (**f**) Interleukin-6 (IL-6) induction by human PBMCs treated with soluble PolyI:C (10 μ g/ml) plus either native GFP-S single-stranded RNA, Luc-mU or (mU)₂₁ 2'OMe RNA (1.4 μ g/ml), or lipid vehicle alone. In each experiment data are mean pg/ml cytokines + SD of triplicate cultures 24 hours after treatment and are representative of at least two independent experiments. GFP, green fluorescent protein; PBS, phosphate-buffered saline.

human and mouse TLR7.²³ When it was added as free drug to human PBMC cultures, 300 μ mol/l Lox induced robust IFN- α and IL-6 production (**Figure 3a and b**). Addition of formulated 2'OMe RNA (Luc-mU) at 1.5 μ g/ml (~0.2 μ mol/l) reduced-Lox induced IFN- α by 55 \pm 16% and IL-6 by 62 \pm 10% in replicate experiments. Furthermore, (mU)₂₁ provided even more potent inhibition of the response to Lox, with IFN- α and IL-6 levels reduced 72 \pm 5% and 80 \pm 1%, respectively, in replicate experiments.

Potent cytokine induction in murine Flt3L DC cultures was achieved with a tenfold lower concentration of Lox (30 μ mol/l) than in human PBMC culture. Under these conditions in which Lox is still in a 140-fold molar excess, 0.2 μ mol/l (mU)₂₁ inhibited Lox-mediated IFN- α induction by 87 \pm 11% and IL-6 by 69 \pm 1% (**Figure 3c and d**). In both human and murine systems, non-stimulatory native RNA or the lipid vehicle alone had no effect on cytokine induction, indicating that inhibition of the response to Lox was specific to the 2'OMe RNA. These results support

the conclusion that 2'OMe RNAs act as an antagonist to TLR7-mediated immune stimulation.

2'OMe RNA inhibits cytokine production by TLR7 agonists *in vivo*

To determine whether our observations could be extended *in vivo*, mice were treated with immunostimulatory ssRNA (β gal AS) and (mU)₂₁ formulated into lipid particles. As expected, administration of immunostimulatory ssRNA alone induced significant elevations in plasma IFN- α and IL-6, whereas (mU)₂₁ alone induced no measurable cytokine response. As observed *in vitro*, formulation of (mU)₂₁ with the ssRNA agonist eliminated measurable IFN- α and IL-6 induction, indicating that the inhibitory effects of 2'OMe RNA still manifest *in vivo* (Figure 4a and b).

Preliminary studies indicated that plasma cytokine levels peaked approximately 2 hours after intravenous injection of 1 mg aqueous solution of Lox into mice (data not shown). To determine whether 2'OMe RNA is able to inhibit Lox-mediated immune stimulation *in vivo*, 100 μ g of lipid-formulated (mU)₂₁, native non-stimulatory ssRNA (GFP-S), or phosphate-buffered saline (PBS) control was administered intravenously 2 hours before treating mice with 1 mg Lox. Plasma cytokine levels were then determined 2 hours after Lox administration. Control mice pre-treated with non-stimulatory native ssRNA mounted a robust response to loxoribine as assessed by plasma IFN- α and IL-6 levels (Figure 4e and f). Pre-treatment with (mU)₂₁ RNA significantly reduced loxoribine-mediated cytokine induction relative to both the GFP-S- and the PBS-treated mice. Plasma IFN- α and IL-6 levels in (mU)₂₁ RNA-treated mice were significantly reduced, by 79 \pm 5% and 72 \pm 8%, respectively, compared with those in PBS-pre-treated mice (Figure 4c and d) and by 92 \pm 2% and 96 \pm 1%, respectively, compared with those in mice treated with GFP-S ssRNA (Figure 4e and f).

Taken together, these results show that 2'OMe RNA acts as an antagonist of TLR7-mediated immune stimulation both *in vitro* and *in vivo*. This feature of chemically modified RNA may have utility in developing novel therapeutics for use in inflammatory and autoimmune diseases that are driven by TLR7-mediated immune activation.^{14,15}

DISCUSSION

Immune stimulation by short RNA species is effectively blocked by the introduction of 2'OMe nucleotides.^{19,21,24} Our current studies extend these observations to demonstrate that 2'OMe RNAs act to antagonize the immune recognition of unrelated native RNA species as well as small-molecule TLR7 ligands such as Lox. Our studies initially utilized a variety of 2'OMe-modified 21-mer ssRNA sequences to test their inhibitory effects on RNA-mediated immune stimulation. Each of these modified RNAs proved to be an effective inhibitor, suggesting that the antagonistic effect is not sequence dependent. This is supported by subsequent results demonstrating potent antagonism with 2'OMe-U homopolymers as short as 10 nt in length. One exception to these general effects was our observation that RNA containing 2'OMe-C was ineffective at antagonizing RNA-mediated cytokine induction, a finding that is consistent with previous studies that incorporated 2'OMe-C directly into immunostimulatory

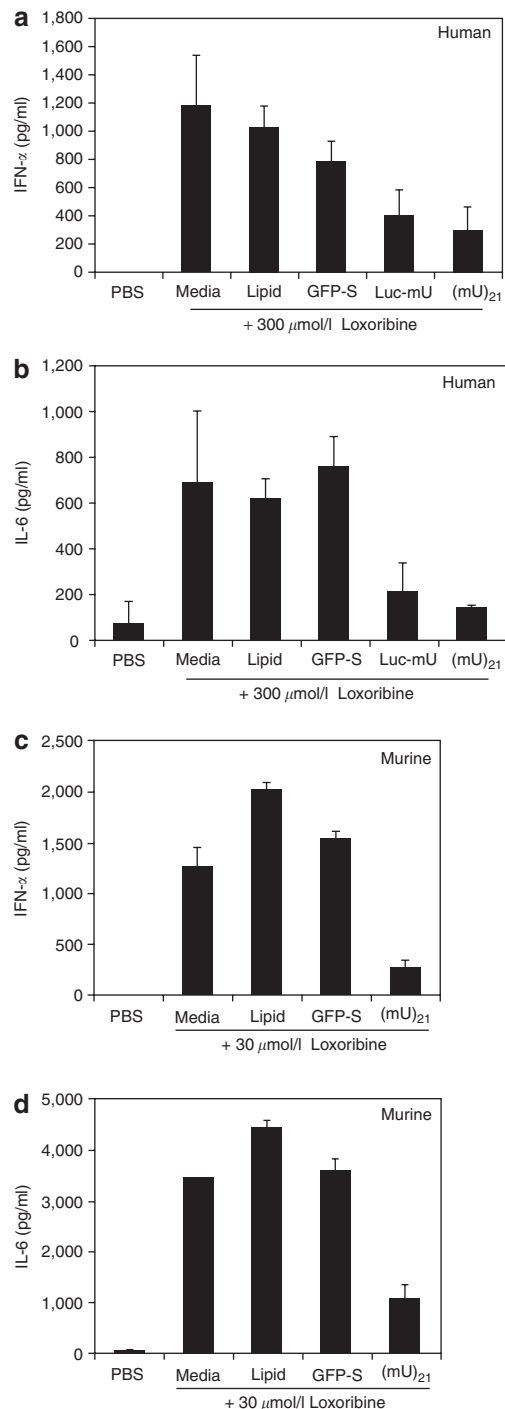


Figure 3 2'-O-methyl (2'OMe) single-stranded RNA (ssRNA) inhibits loxoribine-mediated interferon- α (IFN- α) and interleukin-6 (IL-6) production in both human and murine systems *in vitro*. Cytokine responses from (a, b) human peripheral blood mononuclear cells or (c, d) murine Flt3L dendritic cells treated with the TLR7 agonist loxoribine at 300 μ mol/l or 30 μ mol/l, respectively. Cells were treated simultaneously with soluble loxoribine plus either medium alone, lipid vehicle (lipid), or lipid-formulated native ssRNA (GFP-S) or 2'OMe RNAs [Luc-mU or (mU)₂₁] for 24 hours before secreted (a, c) IFN- α and (b, d) IL-6 were assayed. Control cultures received phosphate-buffered saline (PBS) vehicle only; RNA was added at 0.2 μ mol/l (~1.4 μ g/ml) final concentration. Data reflect mean cytokine levels + SD of triplicate cultures and are representative of at least two independent experiments. GFP, green fluorescent protein.

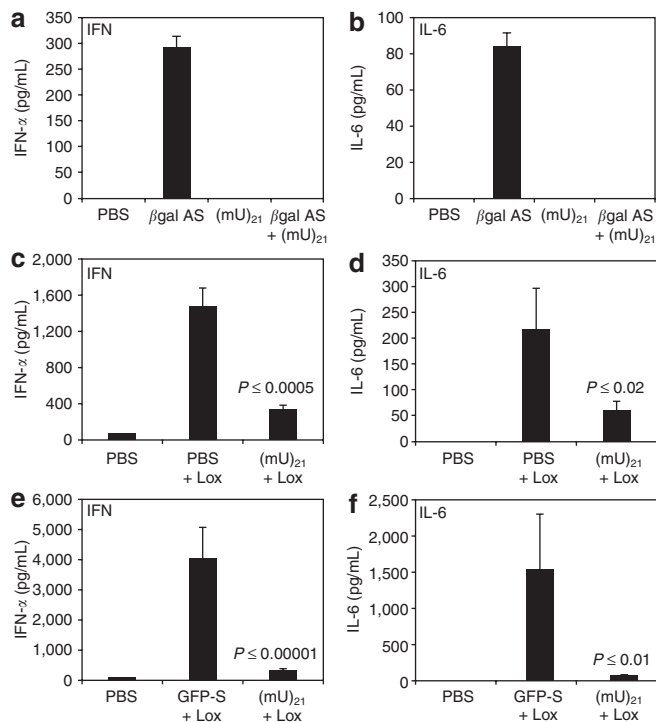


Figure 4 2'-O-methyl (2'OMe) RNA inhibits single-stranded RNA (ssRNA) and loxoribine-mediated cytokine production *in vivo*. **(a)** Interferon- α (IFN- α) and **(b)** interleukin-6 (IL-6) induction in mice treated with either immunostimulatory ssRNA (β gal AS), 2'OMe RNA [(mU)₂₁] or β gal AS + (mU)₂₁ formulated at a 1:1 molar ratio in lipid particles. Plasma cytokines were measured 6 hours after intravenous administration of formulations containing 40 μ g β gal AS RNA. **(c-f)** Treatment of mice with 2'OMe RNA inhibits **(c, e)** IFN- α and **(d, f)** IL-6 induction by loxoribine (Lox). Mice received 100 μ g formulated (mU)₂₁ 2 hours before the administration of 1 mg soluble Lox in phosphate-buffered saline (PBS). Control groups were pre-treated with either **(c, d)** PBS or **(e, f)** 100 μ g formulated GFP-S RNA, a native ssRNA with negligible immunostimulatory activity. Plasma IFN- α and IL-6 levels 2 hours after intravenous Lox administration were significantly reduced in (mU)₂₁-treated mice compared with mice receiving either PBS or formulated control RNA. Data are mean + SD of $n = 4$ mice per group and are representative of two separate experiments. GFP, green fluorescent protein

RNA.^{19,21} This implies that the mechanism underlying the immunosuppressive effects of 2'OMe RNA distinguishes O-methyl substitutions at the 2' ribose position in a base-dependent context. The precise mechanism by which 2'OMe RNA inhibits TLR7-mediated immune stimulation is not understood. One possibility is that 2'OMe RNA acts as a competitive antagonist by binding directly to TLR7 or some other adaptor protein. However, 2'OMe RNA was able to antagonize a fourfold molar excess of immunostimulatory RNA, suggesting that the antagonist RNA would have significantly higher receptor affinity or slower dissociation kinetics assuming a mechanism of direct competition for ligand binding. Alternative possibilities include 2'OMe RNA-induced receptor internalization or the transduction of inhibitory signals. It is interesting to note that recent work utilizing micro-arrays indicates that 2'OMe RNA does not induce significant changes in gene expression when transfected into TLR7-expressing immune cells,²⁰ and this is consistent with our own analysis of selected genes (data not shown).

The antagonistic effects of 2'OMe RNA did not result in the inhibition of related TLR signaling pathways. 2'OMe RNA had no inhibitory effect on cytokine induction by the TLR9 agonists CpG Type B and C ODNs or by the TLR3 agonist polyI:C (**Figure 2**). These findings indicate that 2'OMe RNAs do not globally disrupt MYD88- or TRIF-dependent pathways utilized by nucleic acid-sensing TLRs. TLR8 is phylogenetically close to TLR7 and is also activated by ssRNA in humans.⁴ The expression patterns, however, are distinct, with B cells and plasmacytoid DCs typically expressing TLR7, whereas myeloid DCs and monocytes constitutively express TLR8. These differences likely account for the respective bias toward predominantly IFN- α (TLR7) or pro-inflammatory cytokine (TLR8) induction profiles.²⁵ Because 2'OMe RNA abolishes both these cytokine responses to ssRNA in human PBMCs that contain TLR7- and TLR8-expressing cell types, these findings imply that 2'OMe RNA can antagonize both TLR7 and TLR8 in human cells.

Given that 2'OMe RNAs did not inhibit responses towards CpG Type B and Type C ODNs, it was surprising to find that these modified RNAs were able to antagonize human and mouse CpG Type A ODNs (**Figure 2c** and **e**). This may suggest that ssRNA TLR7/8 agonists and Type A ODNs have a common receptor or adaptor in their signaling pathways that is inhibited by 2'OMe RNAs. We and others have shown that neither of these classes of TLR agonists induces strong activation of NF- κ B reporter constructs in TLR-expressing HEK293 cells, suggesting that these TLR ligands may require an adaptor or co-receptor that is absent from the TLR-HEK293 cell system (M.R. and A.J., unpublished data).^{16,22}

We believe that this report is the first to detail inhibition of TLR7 activation by an antagonistic RNA. Naturally occurring inhibitory ligands of TLR4²⁶ and TLR9 signaling²⁷ have been described previously. CpG motifs in DNA can be immune stimulatory (CpG-S) or immune neutralizing (CpG-N), depending on the motifs flanking sequences.^{27,28} Repetitive telomeric DNA sequences²⁹ and other phosphorothioate ODNs (PS-ODNs) containing G-quartets³⁰ also display potent TLR9 inhibition. At present it is unclear whether the effects of these inhibitory DNAs on TLR9 activity are the result of competitive antagonism for TLR9 binding or occur via an indirect effect on the TLR9 signaling pathway.

Certain PS-ODNs have been shown to inhibit TLR7 but not TLR9 activation, and it was suggested that this selective inhibition was sequence dependent.³¹ Other groups, however, have reported that PS-ODNs can inhibit TLR7 in a sequence-independent manner and can also have differential effects on TLR8 activation.^{32,33} Interestingly, certain TLR7-inhibitory ODNs, including poly dT, caused significant enhancement of TLR8 activation.^{32,33} This skewing of the response from TLR7 toward TLR8 appears so strong that the poly dT ODN somehow facilitates stimulation of TLR8 by Lox³² and reveals previously unknown functionality in murine TLR8 when administered with a human TLR8-specific agonist.⁹ We have not observed this phenomenon of skewing toward TLR8 activation in our studies. 2'OMe RNA in combination with either ssRNA or Lox inhibited both IFN- α and inflammatory cytokines, including IL-6 and tumor necrosis factor- α , in human PBMCs, murine Flt3L DCs, and *in vivo*, thereby demonstrating the absence of significant TLR8-mediated immune stimulation.

Dysregulated activation of the immune system through TLR pathways is believed to drive many inflammatory and autoimmune disorders. TLR7 has recently been shown to play a major role in the activation of autoreactive B cells^{14,15} and subsequent development of systemic autoimmune disease such as systemic lupus erythematosus.^{11–13} The production of both pathogenic autoantibodies and Type I interferons that are hallmarks of systemic lupus erythematosus pathogenesis³⁴ can be driven by RNA-associated autoantigens and immune complexes through TLR7 activation.^{14,15,35} Antagonism of the TLR7 pathway therefore provides a potential therapeutic option that targets several key components of this disease. Our results suggest that 2'OMe RNA may represent a novel therapeutic candidate for this application.

MATERIALS AND METHODS

siRNA. The siRNAs used in these studies were synthesized at the University of Calgary (Calgary, AB Canada) or at Dharmacon (Lafayette, CO) and received as desalted, de-protected ribonucleotides. Duplexes were annealed as described previously.¹⁹ All native and 2'OMe-modified sequences are listed in **Supplementary Table S1**. ODNs used in these studies were synthesized by Integrated DNA Technologies (Coralville, IA). All ODN sequences are listed in **Supplementary Table S2**.

Lipid encapsulation of RNAs and ODNs. RNAs and ODNs were encapsulated in lipid particles by a process of spontaneous vesicle formation as described previously.^{19,36}

Cell isolation and culture. Human PBMCs were isolated from whole blood of healthy donors by a standard Ficoll-Hypaque density centrifugation. Blood was diluted 1:1 with PBS, layered onto Ficoll-Paque Plus (GE Healthcare, Waukesha, WI), and centrifuged at 1,600 rpm for 30 minutes. PBMCs were washed in PBS twice, followed by re-suspension in complete medium (Roswell Park Memorial Institute 1640 medium, 10% heat-inactivated fetal bovine serum, 2 mmol/l L-glutamine, and 1% penicillin/streptomycin). PBMCs were plated at 2.5×10^5 cells/well in 96-well plates for cytokine induction assays.

Flt3L DCs were generated as described by Gilliet *et al.*³⁷ using 100 ng/ml murine Flt3 ligand (PeproTech, Rocky Hill, NJ)–supplemented media. Femurs and tibias of female Balb/C mice were isolated and rinsed in sterile PBS. The ends of bones were cut and marrow was harvested in complete medium (RPMI 1640 medium, 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 25 mmol/l HEPES, 50 μ mol/l 2-mercaptoethanol). Bone marrow cells were passed through a 70- μ mol/l strainer and centrifuged at 1,000 rpm for 7 minutes and re-suspended in complete medium supplemented with 100 ng/mm² murine Flt3Ls to 2×10^6 cells/ml. Subsequently, 2 ml of cells were seeded in 6-well plates and 1 ml fresh complete medium was added every 2 or 3 days. On day 9 of culture non-adherent cells were washed in complete medium and plated into 96-well plates at concentrations ranging from 0.5 to 2.5×10^5 cells/well.

Lox (InvivoGen, San Diego, CA), polyIC (Sigma, St. Louis, MO), and formulated nucleic acids were diluted in PBS and added to either human PBMC or Flt3L DC cultures. Cells were incubated for 24 hours at 37°C before supernatants were assayed for cytokines by enzyme-linked immunosorbent assay.

Cytokine enzyme-linked immunosorbent assay. All cytokines were quantified using sandwich enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. These were mouse and human IFN- α (PBL Biomedical, Piscataway, NJ) and IL-6 (eBioscience, San Diego, CA).

In vivo cytokine induction. Animal studies were performed at Protiva Biotherapeutics in accordance with Canadian Council on Animal Care

guidelines and following protocol approval by the local Animal Care and Use Committee. Female Balb/C mice were subjected to a 2-week quarantine and acclimation period before use at 6–8 weeks of age. RNA formulations were administered by standard injection into the lateral tail vein in 0.2 ml PBS. Six hours after injection, blood was collected by cardiac puncture and processed as plasma for cytokine analysis. In experiments studying inhibition of cytokine induction by loxoribine, formulated RNA (100 μ g) was administered intravenously 2 hours before intravenous administration of 1 mg loxoribine in PBS. Blood was collected by cardiac puncture 2 hours after administration of loxoribine and processed as plasma for cytokine analysis.

ACKNOWLEDGMENTS

The authors would like to acknowledge the receipt of a Natural Sciences and Engineering Research Council Industrial Fellowship (M.A.R.). The authors would like to thank Heidi Jantzen (Protiva Biotherapeutics) for technical expertise. All authors are employees of Protiva Biotherapeutics.

SUPPLEMENTARY MATERIAL

Figure S1. 2'OMe RNA inhibits RNA-mediated IFN- α and IL-6 production from murine Flt3L DC.

Table S1. RNA sequences used in this study.

Table S2. DNA sequences used in this study.

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