Unique O-Methoxyethyl Ribose-DNA Chimeric Oligonucleotide Induces an Atypical Melanoma Differentiation-Associated Gene 5-Dependent Induction of Type I Interferon Response


ISIS Pharmaceuticals, Carlsbad, California (S.A.B., T.M., F.L.R., P.C., S.G., A.S., W.A.G., G.H., R.P., S.M.F., S.P.H.); and Department of Genetics and Molecular Biology, Institute for Virus Research, Kyoto University, Kyoto, Japan (H.K.)

Received March 5, 2012; accepted April 12, 2012

ABSTRACT

Antisense oligonucleotides (ASO) containing 2′-O-methoxyethyl ribose (2′-MOE) modifications have been shown to possess both excellent pharmacokinetic properties and robust pharmacological activity in several animal models of human disease. 2′-MOE ASOs are generally well tolerated, displaying minimal to mild proinflammatory effect at doses far exceeding therapeutic doses. Although the vast majority of 2′-MOE ASOs are safe and well tolerated, a small subset of ASOs inducing acute inflammation in mice has been identified. The mechanism for these findings is not clear at this point, but the effects are clearly sequence-specific. One of those ASOs, ISIS 147420, causes a severe inflammatory response atypical of this class of oligonucleotides characterized by induction in interferon-β (IFN-β) at 48 h followed by acute transaminitis and extensive hepatocyte apoptosis and necrosis at 72 h. A large number of interferon-stimulated genes were significantly up-regulated in liver as early as 24 h.

We speculated that a specific sequence motif might cause ISIS 147420 to be mistaken for viral RNA or DNA, thus triggering an acute innate immune response. ISIS 147420 toxicity was independent of Toll-like receptors, because there was no decrease in IFN-β in Toll/interleukin-1 receptor-domain-containing adapter-inducing IFN-β or Myd88-deficient mice. The involvement of cytosolic retinoic acid-inducible gene (RIG)-I-like pattern recognition receptors was also investigated. Pretreatment of mice with melanoma differentiation-associated gene 5 (MDA5) and IFN-β promoter stimulator-1 ASOs, but not RIG-I or laboratory of genetics and physiology 2 (LGP2) ASOs, prevented the increase in IFN-β and alanine aminotransferase induced by ISIS 147420. These results revealed a novel mechanism of oligonucleotide-mediated toxicity requiring both MDA5 and IPS-1 and resulting in the activation of the innate immune response.

INTRODUCTION

Antisense oligonucleotides (ASOs) are short molecules, typically 18 to 22 nucleotides long, designed to hybridize with a target mRNA and modulate gene expression and protein levels in a sequence-dependent manner in animals and humans (Yacyshyn et al., 1999; Bennett, 2007). The most advanced ASOs are chimeric phosphorothioate (PS)-modified oligonucleotides, which have a central DNA region of 8 to 16 nucleotides, flanked on the 5′ and 3′ ends with two to five 2′-O-methoxyethyl ribose (2′-MOE) residues (McKay et al., 1999; Bennett, 2007). These 2′-MOE ASOs possess excellent pharmacokinetic properties (Geary et al., 2003, 2009; Yu et al., 2004) and exhibited robust pharmacological activity in several animal models of human disease when administered systemically with clinically relevant routes and schedules of administration (Graff et al., 2007; Merki et al., 2008; Mullick et al., 2011). Pharmacologic activity has also been demonstrated in clinical trials (Kastelein et al., 2006).

ABBREVIATIONS: ASO, antisense oligonucleotide; PS, phosphorothioate; MOE, O-methoxyethyl ribose; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PRR, pattern recognition receptor; TLR, Toll-like receptor; FDR, false discovery rate; MDA5, melanoma differentiation-associated gene 5; RIG, retinoic acid-inducible gene; RLR, RIG-I-like receptor; IFN, interferon; IPS-1, IFN-β promoter stimulator-1; TRIF, Toll/interleukin-1 receptor-domain-containing adapter-inducing IFN-β; MYD88, myeloid differentiation primary response gene 88; KO, knockout; IPA, Ingenuity Pathway Analysis; TBST, Tris-buffered saline/Tween 20; ANOVA, analysis of variance; SDM, standard deviation of mean; STAT1, signal transducer and activator of transcription 1; HMG Box, high-mobility group box; LGP2, laboratory of genetics and physiology 2.
As ASOs progress through development for therapeutic indications, their safety and tolerability profiles are being extensively studied. As a class, PS ASOs have the potential to elicit proinflammatory effects, and the potency for this effect is highly sequence-specific (Krieg et al., 1996; Monteith et al., 1997; Roberts et al., 2005). These proinflammatory effects tend to occur at a greater degree in rodents. 2'-MOE ASOs are generally well tolerated, displaying reduced proinflammatory effects caused by the release of cytokines via the activation of monocyte and/or dendritic cells compared with PS ASOs (Henry et al., 2000, 2007; Senn et al., 2005; Younis et al., 2011). In addition, 2'-MOE ASOs avoid the incorporation of CpG dinucleotide motifs capable of eliciting severe proinflammatory effects through the activation of Toll-like receptor (TLR) 9. Some mild residual proinflammatory effects of the 2'-MOE oligonucleotides are still observed in mice at doses exceeding expected therapeutic doses, and the degree of the effect is sequence-dependent. Typically, these effects are characterized by progressive multiorgan lymphohistiocytic infiltration and increased production of chemokines such as monocyte chemotactic protein-1. At higher doses examined in toxicology studies in rodents it is not uncommon to see a mild increase in serum transaminases [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] associated with the proinflammatory effects (Henry et al., 1997). Although most 2'-MOE ASOs fall into this category, in screening of large numbers of oligonucleotides a fraction of sequences examined tended to produce an unusual level of proinflammatory effect (Henry et al., 2007). Such a 2'-MOE ASO compound, ISIS 147420, was identified in mice to cause acute inflammatory responses and associated hepatic damage characterized by rapid and severe transaminitis after a single dose. Although such compounds will not enter clinical development, a clear understanding of the mechanism leading to these effects is warranted by the need to develop antisense oligonucleotide therapeutics that are safe and well tolerated in patients.

The best-characterized example of a sequence-specific motif that determines the potency of proinflammatory effects is the case of TLR9 ligands in which oligonucleotides possess optimal CpG sequence motifs (Hemmi et al., 2000; Chuang et al., 2002; Vollmer et al., 2004). In recent years, more pattern recognition receptors (PRRs) have been shown to recognize foreign nucleic acids pathogen-associated molecular patterns representing a central pillar of the innate immune response against bacteria, viruses, and other pathogens (Kawai and Akira, 2009; Iwasaki and Medzhitov, 2010; Kumagai and Akira, 2010; Takeuchi and Akira, 2010). Several PRR families are capable of distinguishing unique molecules present in bacteria, viruses, and other pathogens, including foreign nucleic acids. One such family includes the cell surface and endosome-associated TLRs of which TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids (Hemmi et al., 2000; Chuang et al., 2002; Matsushima et al., 2004; Sioud, 2006; Marshall-Clarke et al., 2007; Thompson and Locarnini, 2007; Ablasser et al., 2009). Another family, RIG-I-like receptors (RLRs), comprise cytosolic viral ribonucleic acid sensors MDA5, RIG-I, and LGP2 (Yoneyama et al., 2004, 2005; Hornung et al., 2006; Pichlmair et al., 2006; Venkataraman et al., 2007; Barral et al., 2009; Satoh et al., 2010). We hypothesized that ISIS 147420 might be eliciting an acute inflammatory response through the activation of one of those PRRs.

In the present study, the mechanism underlying the marked proinflammatory effect and transaminitis induced by ISIS 147420 was examined. Whole-genome profiling was performed to identify biological pathways induced before the onset of transaminitis. Knockout mice and ASO-targeting genes implicated in those pathways were used to further elucidate those pathways.

### Materials and Methods

#### Animal Treatment

Animal experiments were approved by the Animal Welfare Committee and conducted according to the guidelines of the American Association for the Accreditation of Laboratory Animal Care. Male BALB/c, C57BL6, and MYD88(−/−) mice were obtained from Charles River Breeding Laboratories (Portage, MI) (Yamamoto et al., 2003). Male STAT1(−/−) mice (Meraz et al., 1996) and their congenic wild-type control (129SvEv) mice were obtained from Taconic Farms (Germantown, NY). Male TRIP(−/−) mice (C57BL/6J-Ticam1tm1Jaa/J) (Yamamoto et al., 2003) were obtained from The Jackson Laboratory (Bar Harbor, ME).

Male IFNAR1(−/−) and IFNGR1(−/−) (129SvEv) mice (Hoshino et al., 2002) were obtained from B&K Universal Limited (Hull, UK). MDA5(−/−) mice have been described previously (Kato et al., 2006).

The experimental design relating to the use of knockout mice is detailed in Table 1. All animals were housed in temperature-controlled conditions under a light/dark cycle with food and water supplied ad libitum. Animal weights were monitored before dosing throughout the live phase of the study. Compounds were dissolved in phosphate-buffered saline, filter-sterilized, and administered by subcutaneous injection in a volume corresponding to 10 mg/kg animal weight. Immediately before sacrifice, mice were anesthetized with isoflurane, and terminal bleed was performed by cardiac puncture. Plasma was isolated from whole blood and analyzed for clinical chemistries and cytokine levels. ALT and AST levels were determined by using an automated analyzer.

#### Experimental design for knockout mice

<table>
<thead>
<tr>
<th>Background Strain</th>
<th>KO Model</th>
<th>Treatment</th>
<th>Duration</th>
<th>Group Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>129SvEv</td>
<td>WT</td>
<td>Saline</td>
<td>72</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ISIS 147420</td>
<td>72</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ISIS 147420</td>
<td>96</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>141923</td>
<td>72</td>
<td>4</td>
</tr>
<tr>
<td>STAT1</td>
<td>Saline</td>
<td>72</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>ISIS 147420</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ISIS 147420</td>
<td>96</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>141923</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>IFNAR1</td>
<td>Saline</td>
<td>72</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>ISIS 147420</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ISIS 147420</td>
<td>96</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>141923</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>IFNGR1</td>
<td>Saline</td>
<td>72</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>ISIS 147420</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ISIS 147420</td>
<td>96</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>141923</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>C57BL6</td>
<td>WT</td>
<td>Saline</td>
<td>72</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>ISIS 147420</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ISIS 147420</td>
<td>96</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>141923</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>TRIF</td>
<td>Saline</td>
<td>72</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>ISIS 147420</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ISIS 147420</td>
<td>96</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>141923</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>MYD88</td>
<td>Saline</td>
<td>72</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>ISIS 147420</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ISIS 147420</td>
<td>96</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>141923</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>129/B6</td>
<td>WT</td>
<td>Saline</td>
<td>72</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>ISIS 147420</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ISIS 147420</td>
<td>96</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>141923</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 1**

Experimental design for knockout mice
Oligonucleotide Design and Synthesis. To identify mouse ASO inhibitors, rapid throughput screens were performed in vitro as described previously (Watts et al., 2005). From those initial screens, lead antisense inhibitors were chosen. In brief, 78 oligonucleotides complementary to each mRNA target were tested in primary mouse hepatocytes to determine the potency of each ASO (data not shown). The top three ASOs for each target were selected on the basis of in vitro IC_{50} values and were further validated by assessing the reduction of target mRNAs in BALB/c mice dosed with the candidate ASO at 50 mg/kg for 3 consecutive days followed by analysis of mRNA levels in the liver on the fifth day (Supplemental Table 1). The first five bases and last five bases of c江mric ASOs have a 2′-O-(2-methoxy)-ethyl modification, and the ASOs also have a phosphorothioate backbone. This chimeric design has been shown to provide both increased nuclease resistance and mRNA affinity, while maintaining the robust RNase H-terminating mechanism used by these types of ASOs (McKay et al., 1999; Bennett, 2007).

RNA Analysis. RNA was extracted from the liver samples by using RNeasy columns (QIAGEN, Valencia, CA) according to the manufacturer’s protocol. RNA samples were analyzed by fluorescence-based quantitative real-time polymerase chain reaction using an Applied Biosystems (Foster City, CA) 7700 sequence detector. Target RNA levels were normalized to the total RNA concentration determined by using ribogreen. Primers and probes for analysis of the expression of different genes were designed by using Primer Express Software (Applied Biosciences, Carlsbad, CA) (Supplemental Table 2). For the analysis, 100 ng of total RNA was used.

Hematoxylin and Eosin Staining. Liver samples were fixed in formalin and embedded in paraffin, and four-micron sections were mounted on positive-charged glass slides then stained for hematoxylin and eosin.

RNA Preparation and Microarray Experiments. The MouseWG-6 v1.1 Expression BeadChip (Illumina, San Diego, CA) was processed in accordance with the manufacturer’s instructions. Total RNA (200 ng) was used for cRNA in vitro transcription and labeling with the TotalPrep RNA Labeling Kit by using Biotinylated-UTP (Ambion, Austin, TX). Hybridization was carried out in accordance with the Illumina Hybridization System manual. The raw data matrix extracted from Illumina Beadstudio was uploaded into GeneSpring GX 7.3.1 software (Agilent Technologies, Santa Clara, CA) for downstream analysis.

Microarray Analysis. Microarray data were imported into GeneSpring GX 7.3.1 for analysis. The standard normalization procedures recommended for the GeneSpring GX 7.3.1 software for one-color array data were followed (Fig. 2). In brief, data transformation was corrected for a low signal, with values recorded at a 1.5-fold differential expression based on a corrected multiple testing correction false discovery rate (FDR) <0.05, and transcripts were further filtered by selecting transcripts modulated by ISIS 147420 at 1.5-fold in relation to ISIS 141923 at the various time points. We reasoned that the most biologically significant findings would relate to genes differentially expressed between mice treated with ISIS 147420 for 24 h (n = 4) and mice that received ISIS 141923 (n = 4) (Table 2). The Benjamini-Hochberg FDR was used to decrease the possibility of false positives caused by multiple comparisons (Benjamini and Hochberg, 1995).

### Results

**ISIS 147420 Induces Sequence-Specific Proinflammatory Effect.** ISIS 147420 produces a spectrum of splenomegaly, inflammatory cell infiltrate, and liver injury that is not typical of most 2′-MOE ASOs. Mice were treated twice per week with 50 mg/kg of either ISIS 147420 or ISIS 141923 up to 3 weeks. Treatment with 50 mg/kg ISIS 147420 caused a significant increase in both liver and spleen weight compared with both saline- and ISIS 141923-treated animals (Fig. 1A). In addition, ISIS 147420 markedly increased the level of transaminases in the plasma 1 week after the first administration (Fig. 1B). Another 2′-MOE ASO that is representative of the class, ISIS 104838, administered twice per week at 50 mg/kg for 13 weeks, produced no remarkable change in transaminase level or organ weights (data not shown). Histopathological observations in livers of mice treated with ISIS 147420 included signs of apoptosis, profound eosinophilic cytoplasmic degeneration with glycogen

<table>
<thead>
<tr>
<th>Time Point</th>
<th>p &lt; 0.05</th>
<th>FDR &lt; 0.05</th>
<th>p &lt; 0.05 and Fold</th>
<th>FDR &lt; 0.05 and Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 h</td>
<td>1398</td>
<td>0</td>
<td>213</td>
<td>0</td>
</tr>
<tr>
<td>24 h</td>
<td>2069</td>
<td>16</td>
<td>652</td>
<td>15</td>
</tr>
<tr>
<td>48 h</td>
<td>6985</td>
<td>1890</td>
<td>3863</td>
<td>1561</td>
</tr>
<tr>
<td>72 h</td>
<td>10581</td>
<td>6632</td>
<td>8311</td>
<td>5945</td>
</tr>
</tbody>
</table>

**Pathway Analysis.** A total of 652 transcripts that had a fold change more than 1.5 and significant change (p < 0.05) were used for the pathway analysis. Illumina transcript identifiers were imported into Ingenuity Pathway Analysis (IPA) 9 software. Among the probesets, 557 transcripts were unique and annotated. Of these transcripts, 391 were mapped to the IPA database. The Core Analysis function included in IPA was used to interpret the mouse data in the context of canonical pathways. Both up- and down-regulated identifiers were defined as value parameters for the analysis. Significance of the canonical pathways was tested by the Fisher’s exact test p value (Table 3).

**Western Blot Analysis.** Liver samples were homogenized in phosphate-buffered saline containing protease inhibitor cocktail (Calbiochem, San Diego, CA). Protein samples (30 μg) were separated on a Tris-glycine 4 to 20% gel (Invitrogen, Carlsbad, CA) and subsequently transferred to a polyvinylidene difluoride membrane (Invitrogen). Membranes were incubated at room temperature in blocking buffer consisting of 5% nonfat dry milk in TBST for 1 h, then incubated with primary antibodies (1:1000) against RIG-I, IPS-1 (Cell Signaling Technology, Danvers, MA), and MDA5 (Lifespan Biosciences, Seattle, WA). After thorough washing with TBST, horseradish peroxidase-conjugated secondary antibodies (1: 10,000 dilution in TBST; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) were applied, and the blots were developed by using ECL-plus reagent (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

**Enzyme-Linked Immunosorbent Assay.** Plasma was collected by cardiac puncture. IFN-α, IFN-β, and IFN-γ levels in the plasma were analyzed by using enzyme-linked immunosorbent assay according to the manufacturer’s protocol (PBL, Piscataway, NJ).

**Statistical Analysis.** The data of in vivo studies are expressed as the mean ± SDM. Comparisons of in vivo data between two groups were analyzed with an unpaired Student’s t test. Multiple comparisons were evaluated with one-way analysis of variance (ANOVA), followed by a post hoc Dunnett’s test with JMP version 9 (SAS Institute, Cary, NC). p <0.05 was considered significant.
The hepatotoxicity of ISIS 147420 could be elicited by a single dose. Increasing the dose to 100 mg/kg resulted in a cumulative necrosis surrounded with lymphohistiocytic infiltrates (Fig. 1C).

Atypical MDA5 Activation by Short Oligonucleotide 153

The hepatotoxicity of ISIS 147420 could be elicited by a single dose. Increasing the dose to 100 mg/kg resulted in a faster onset of transaminitis in animals treated with ISIS 147420, whereas animals treated with ISIS 141923 remained unaffected (Fig. 1D). Daily monitoring of plasma transaminase levels revealed normal ALT levels through 48 h, followed by a marked increase in ALT and AST levels 72 h after initiation of treatment only with ISIS 147420.

**ISIS 147420 Induces a Type I Interferon Response in Expression Profile Before the Onset of Toxicity.** RNA expression analysis was performed in liver at 8, 24, 48, and 72 h after a single dose of saline, 100 mg/kg ISIS 147420, or 100 mg/kg ISIS 141923. To identify changes in global gene expression in liver, we performed a multistep analysis of the microarray gene expression data. Whereas, ISIS 141923 produced no significant modulation of gene expression, the earliest significant modulation of gene expression induced by ISIS 147420 was seen 24 h after treatment (Fig. 2). ISIS 147420 treatment at 24 h perturbed 652 probe sets with significantly altered expression levels (p < 0.05; 1.5 × fold change). Among the probesets, 557 were unique, and annotated transcripts were submitted to IPA to determine the most significantly overrepresented canonical biological pathway annotation. The top canonical biological pathways curated by IPA significantly perturbed by ISIS 147420 24 h after treatment were related to the recognition of pathogens by cytosolic PRR and interferon response, suggesting ISIS 147420 induced an early innate immune response 48 h before the elevation of plasma transaminases (Table 3). To further investigate the link between interferon signaling and ISIS 147420, we determined whether the probesets modulated by ISIS 147420 resulted in the modulation of 40 of those probesets at 24 h, which represents a much higher enrich-
ment of those terms than expected by chance alone (Fisher's exact test, \( p = 0.00001 \)) (Fig. 3).

The potential role of the IFN pathway was further evaluated by the measurement of plasma levels of type I IFN (\( \alpha \) and \( \beta \)) and type II IFN-\( \gamma \) (Fig. 4). IFN-\( \alpha \) level was significantly elevated 48 h after treatment with 50 mg/kg ISIS 147420. Treatment with 100 mg/kg ISIS 147420 resulted in increased production of both IFN-\( \alpha \) and IFN-\( \beta \) 48 h after treatment. In contrast, treatment with 300 mg/kg control ASO, ISIS 104838 failed to cause any significant increase in either IFN-\( \alpha \) or IFN-\( \beta \) compared with saline-treated animals at all time points examined. The level of IFN-\( \gamma \) was significantly (\( p < 0.05 \)) but only moderately increased by treatment with 100 mg/kg ISIS 147420 48 h after dosing compared with type I IFN.

Interfering with Type I Interferon Signaling Abrogates ISIS 147420-Mediated Hepatotoxicity. Mapping of genes modulated by ISIS 147420 24 h after treatment on the enriched canonical “regulation of innate immune response by cytosolic RNA sensing protein” pathway, revealed a number of key regulatory nodes (STAT1, IFNAR1) putatively respon-
sible for mediating the type I IFNs induced by ISIS 147420 (Fig. 5). To validate the involvement of those genes, ISIS 147420 was administered to STAT1-, IFNAR1-, and IFNGR1-deficient (KO) mice. The background strain of the STAT1, IFNAR1, and IFNGR1 KO mice (129SvEv) is less sensitive to the effects of ISIS 147420 than BALB/c mice. To induce an increase in plasma transaminase levels 72 h after a single-dose administration, 129SvEv wild-type mice had to be dosed with 300 mg/kg ISIS 147420. Unlike wild-type animal controls, STAT1 and IFNAR1 KO animals treated with 300 mg/kg ISIS 147420 failed to show any increase in either IFN-β levels or transaminases 96 h after treatment (Fig. 6, A and B). In contrast, the increase in either IFN-β levels or transaminases in IFNGR1 KO mice was similar to the strain control, confirming the specific involvement of type I, not type II, IFN response. These results suggested the propagation of a type I IFN response might be triggered by the sequence-specific activation of any of several pattern recognition receptors (e.g., TLR3, TRL7, TRL9, RIG-I, or MDA5) by ISIS 147420 as suggested by the enrichment of several canonical pathways involving those receptors (Table 3).

**ISIS 147420 Effects Are Mediated Independently of MYD88 or TRIF Pathways.** Consideration was given to the possibility that the chemical and sequence nature of ISIS 147420 might be a ligand for one of the TLR-recognizing nucleic acids. However, ISIS 147420 does not contain the CpG motif; therefore, it is unlikely that TLR9 is the target receptor. TLR7 and TLR3 seemed also unlikely, because those receptors have previously been shown to recognize single- or double-stranded RNA but not DNA (Diebold et al., 2004; Karikó et al., 2004; Kulka et al., 2004). Nonetheless, the potential involvement of those TLRs was assessed. ISIS 147420 was administered to TRIF (adaptor for TLR3)- and MYD88 (adaptor for all other TLRs)-deficient mice (Fig. 6C). The background strain of those KO mice (C57BL/6) is less sensitive to the effects of ISIS 147420 than that of BALB/c mice. To induce an increase in plasma transaminase levels 72 h after a single-dose administration, C57BL/6 wild-type mice were dosed with 300 mg/kg ISIS 147420. However, administration of ISIS 147420 to TRIF- or MYD88-deficient mice did not alter the toxicity, suggesting the toxicity was mediated independently of any TLR.

**Antisense-Mediated Knockdown of IPS-1 and MDA5 Prevents ISIS 147420 Interferon Response and Hepatotoxicity.** Cytosolic receptors capable of recognizing foreign RNA, RLRs (RIG-I, MDA5, and LGP2), were also investi-
gated for their potential role in the toxicity induced by ISIS 147420. A potential role for this pathway was implicated by increases in mRNA levels for RIG-I, MDA5, and LGP2 in mice livers treated with ISIS 147420 to 2.2-, 1.7-, and 2.3-fold above saline level, respectively (Fig. 3). In contrast to RIG-I and MDA5, IPS-1 mRNA levels were unchanged by ISIS 147420 treatment. Antisense oligonucleotides targeting RIG-I, MDA5, IPS-1 (adaptor protein for RIG-I and MDA-5), or LGP2 were designed and characterized. The most potent ASOs targeting either RIG-I (ISIS 413867), MDA5 (ISIS 147420 induces IFN-α and IFN-β production in BALB/c mice 48 h after a single dose of ISIS 147420. Plasma levels of IFN-α, IFN-β, and IFN-γ from mice treated with ISIS 147420 or ISIS 104838 at the indicated time points and concentration after oligonucleotide treatment were determined by enzyme-linked immunosorbent assay. *, p < 0.05; **, p < 0.01; ***p < 0.001 using ANOVA followed by Dunnett’s multiple comparison t test based on saline group for a given duration. Data are expressed as mean ± SDM (IFN-α and IFN-β, n = 5; IFN-γ, n = 3).
Fig. 5. Canonical pathway underlines genes involved in the regulation of innate immune response by cytosolic RNA-sensing proteins. Nodes in red represent up-regulated genes at 24 h after ISIS 147420 treatment. Nodes in gray represent genes present on the microarray but not significantly modulated. Histograms next to each node represent the fold change at 8, 24, 48, and 72 h after ISIS 147420 treatment.
433392), IPS-1 (ISIS 413732), or LGP2 (ISIS 499269) decreased their mRNA target to levels that were 30, 7, 32, and 15% of the saline-treated group, respectively (data not shown). To ascertain the involvement of these cytosolic RNA receptors in the mechanism of toxicity, BALB/c mice were administered with 50 mg/kg anti-RIG-I, anti-MDA5, anti-IPS-1, anti-LGP2 ASO, or a control oligonucleotide (ISIS 104838) for 3 consecutive days. On the fifth day, the animals were treated with 100 mg/kg ISIS 147420. Serial blood samples were collected for measurement of ALT, and the animals were sacrificed 4 days after administration of ISIS 147420.

Pretreatment with control ASO, ISIS 104838, had no effect on the onset of hepatotoxicity induced by ISIS 147420. Under these conditions, ISIS 147420 caused a marked increase in ALT and IFN-β protein levels in the plasma (Fig. 7A). The level of the interferon-stimulated gene IFIT2 was also increased in liver to the same degree as saline control. Anti-IPS-1 ASO (ISIS 413732) pretreatment reduced both IPS-1 mRNA down to 6% of the saline-treated group level (Fig. 7A) and protein level (Fig. 7C). Pretreatment with anti-IPS-1 ASO (94% reduction in IPS-1 mRNA) abrogated the transaminitis and IFN-β production caused by ISIS 147420, thereby maintaining transaminases and IFN-β at levels similar to those of the saline-treated group. In addition, anti-IPS-1 and anti-MDA5 ASO pretreatments prevented the induction of interferon-stimulated genes such as RIG-I and IFIT2, suggesting a broad impact of those ASOs on the induction of type I IFN response. Abrogation of the ISIS 147420-mediated inflammatory response by anti-IPS-1 or anti-MDA5 ASO was further confirmed by the normal liver morphology in contrast to animals pretreated with either ISIS 104838 control ASO or anti-RIG ASO (ISIS 413867), which showed signs of apoptosis, profound eosinophilic cytoplasmic degeneration with glycogen depletion and hyperchromatic nuclei, and centrilobular coagulative necrosis surrounded with lymphohistiocytic infiltrates in the liver (Fig. 8).

To independently confirm MDA5 involvement, MDA5-deficient (KO) mice were administered with 100 mg/kg ISIS 147420. Unlike wild-type animal controls, MDA5 KO animals treated with ISIS 147420 did not show any evidence of liver injury as illustrated by the absence of transaminases increase 96 h after treatment (Fig. 7D).

**Single-Nucleotide Change Abrogates ISIS 147420 Inflammatory Properties.** ISIS 147420, ISIS 141923, and
ISIS 104838 have a similar chemical backbone and modification, arguing that the chemical structure was not solely responsible for the unique properties of ISIS 147420. Although ISIS 147420 does not possess any of the known optimal sequence motifs for inflammation, these properties are known to be highly sequence-dependent. Thus we sought to better understand the relationship between sequence and inflammatory properties of ISIS 147420. To that end, ISIS 361476 and ISIS 361481, MOE gapmer oligonucleotides containing a single-base substitution of ISIS 147420 were designed (Fig. 9A). Mice treated with ISIS 147420 displayed elevated levels of IFN-β and transaminases 96 h after administration (Fig. 9, B and C). It is noteworthy that the single-base substitutions in ISIS 361476 and ISIS 361481 treatment completely mitigated the elevated levels of IFN-β or ALT. Thus, the inflammatory properties of ISIS 147420 are highly sequence-dependent.

Discussion

Antisense oligonucleotides within a specific structural chemical class of molecules are unique from one to the next based primarily on their sequence, but otherwise they tend to have very consistent physicochemical properties within a class with similar length, solubility, and charge-to-mass properties (Bennett and Swayze, 2010). Because of this, phosphorothioate oligonucleotides tend to have pharmacokinetic, tissue uptake, and even many tolerability properties that are independent of sequence (Geary et al., 2007; Henry et al., 2007; Geary, 2009). However, there is also the potential for effects to be highly sequence-dependent, especially in regard to the potential to induce proinflammatory properties. There have been several publications and reviews that describe these so-called class toxicities (Henry et al., 2001, 2007; Levin et al., 2001; Jason et al., 2004). However, with the screening and testing of an ever greater number of oligonucleotides, there is consistently a small fraction of oligonucleotides that tend to have either an unusually severe manifestation of the common class effects or occasionally a sequence that produces a unique toxicity. In these cases, there is the possibility that the unique behavior is attributed to the antisense pharmacology, but in most cases, the reason for these unusual properties is not known.

The most well studied examples of unique sequence-specific, hybridization-independent proinflammatory behavior are the CpG dinucleotide-containing oligonucleotides (Hart-
mann and Krieg, 2000; Hartmann et al., 2000; Vollmer et al., 2004; Younis et al., 2006). We have identified a novel phenotype and mechanism of activation of the innate immune response by an artificial chimeric oligonucleotide that is independent of the so-called CpG motif. The effects of this oligonucleotide (ISIS 147420) are characterized by a pattern of type I IFN production and transaminitis that has been observed only with this specific 2′-MOE oligonucleotide to date. Production of type I IFN by a non-CpG 2′-MOE ASO is very unique (Fig. 4). An exaggerated pharmacologic effect based on the intended target (cpt1-α mRNA) was ruled out by examining other cpt1-α-targeting ASOs in the same chemical class but of differing sequence, which showed no evidence of IFN production or toxicity while maintaining their ability to reduce target mRNA (data not shown).

Until recently, RIG-I like proteins were thought to specifically mediate the innate response to foreign RNA not DNA, but evidence from two independent groups (Ablasser et al., 2009a; Chiu et al., 2009) has shown that the innate immune response could be induced by B-form double-stranded DNA through the activation of the RIG-I like pathways. They reported that the induction was mediated by RNA polymerase III for transcribing B-DNA templates into double-stranded RNA containing a 5′-triphosphate moiety. RIG-I, in turn, mediates a type I IFN response through the recognition of the newly synthesized RNA. In contrast, Choi et al. (2009) proposed that both RIG-I and MDA5 contributed to the activation of type I IFN response upon B-DNA stimulation. Using an antisense-mediated knockdown approach and MDA5-deficient mice, we have demonstrated that the IFN production mediated by ISIS 147420 and the transaminitis that ensued depended on the expression of both MDA5 and IPS-1, but not RIG-I.

Satoh et al. (2010) have reported that under certain conditions LGP2 could be required to mediate the recognition of some RNA viruses but not synthetic RNA compounds such as poly(I:C) by MDA5. Using an antisense knockdown, we could detect only partial and variable dependence of ISIS 147420-mediated type I IFN response upon LGP2, suggesting LGP2, unlike MDA5, is not essential to ISIS 147420 properties. They also investigated the involvement of HMGB1 in ISIS 147420 response. HMGBs were also reported to act as universal promiscuous sensors for foreign nucleic acids channeling.

![Fig. 8. Morphology of livers treated with ISIS 147420 after ASO pretreatment with anti-IPS-1, anti-RIG-I, anti-MDA5, or control ASO. A to C, treatment with saline (A), ISIS 147420 (100 mg) for 96 h after pretreatment with either anti-IPS-1 ASO (ISIS 413732) (B) or anti-MDA5 ASO (ISIS 433392) (C) resulted in normal liver morphology. D to F, treatment with ISIS 147420 (100 mg/kg) for 96 h alone (D) or after pretreatment with control ASO (ISIS 104838) (E) or anti-RIG-I ASO (ISIS 413867) (F) resulted in severe liver necrosis.]

![Fig. 9. A, oligonucleotides with a single-nucleotide change (underlined) to ISIS 147420 sequence were designed. B and C, IFN-β levels (B) and plasma ALT levels (C) from mice treated with a single dose (100 mg/kg) of ISIS 147420, ISIS 361476, or ISIS 361481 for 96 h were measured. *** p < 0.001 using ANOVA followed by Dunnett’s multiple comparison t test based on the saline group. Data are expressed as mean ± SDM.]
them to either TLRs or RLRs (Yanai et al., 2009). Antisense-mediated knockdown of HMGB1 did not affect ISIS 147420 transaminitis, suggesting that ISIS 147420 does not mediate the activation of the MDA5 pathway through an interaction with HMGB1 (Yanai et al., 2009; data not shown).

In our present study, we demonstrate that a short single-stranded 2′-MOE-modified phosphorothioate DNA (20 bases) can activate the MDA5 pathway in a sequence-specific manner. At this point, it remains unclear whether the activation of the MDA5 pathway is mediated by a direct interaction between ISIS 147240 and MDA5 or through some more complex indirect mechanism, possibly involving RNA polymerase III (Ablasser et al., 2009a; Chiu et al., 2009). Although RNA intermediates have been implicated in the stimulation of the RIG-I pathway by DNA (Ablasser et al., 2009a; Chiu et al., 2009), the evidence for an intermediate MDA5 substrate generated by a mechanism similar to the indirect activation mediated by RNA polymerase III has not been clearly established.

Stable RNAi H degradation products generated during the course of antisense-mediated mRNA target knockdown have been described previously (Condon and Bennett, 1996; Thoma et al., 2001). We hypothesize that an aberrant stable RNA product resulting from the hybridization between ISIS and the target mRNA might act as a substrate for MDA5, thereby triggering a type I interferon response. In summary, our present study reveals an unexpected, but essential, role for the cytosolic RNA sensor, MDA5, in the activation of innate immune response by a non-CpG 2′-MOE phosphorothioate oligonucleotide. To our knowledge this finding is unique, and efforts to identify other 2′-MOE oligonucleotides (including other hepatoxic oligonucleotides) sharing the MDA5/IPS-1 dependence have been unsuccessful to date. Additional work that depends on the development of a cell-based system will be required to understand the nature of the interaction with MDA5. This finding provides useful information to both our understanding of innate immune activation and our efforts for designing and developing safe ASOs. ISIS 147420 may provide a novel tool for improving our understanding of the role of MDA5 in the mediation of innate immune response and may lead to the development of novel antiviral therapeutic strategies.

Acknowledgments

Breeders of MYD88(−/−) mice were graciously donated by Dr. H. Hemmi (Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). Authorization to use the TRIF(−/−) mice came from Dr Bruce Beutler (The Scripps Research Institute, La Jolla, CA).

Authorship Contributions

Participated in research design: Burel, Gaarde, Hung, Freier, and Henry.

Conducted experiments: Burel, Machemer, Ragone, Kato, Cauntay, Greenlee, Salim, Gaarde, and Peralta.

Performed data analysis: Burel, Machemer, Ragone, Kato, and Hung.

Wrote or contributed to the writing of the manuscript: Burel and Henry.

References


Krieg AM, Matson J, and Fisher E (1996) Oligodeoxynucleotide modifications deter-
mine the magnitude of B cell stimulation by CpG motifs. *Antisense Nucleic Acid Drug Dev* 6:133–139.


Address correspondence to: Dr. Sebastien Burel, ISIS Pharmaceuticals, Carlsbad, CA 92010. E-mail: sburel@isiph.com