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Nucleobase and Ribose Modifications Control Immunostimulation by a MicroRNA-122-mimetic RNA

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Supporting Information

ABSTRACT: Immune stimulation is a significant hurdle in the development of effective and safe RNA interference therapeutics. Here, we address this problem in the context of a mimic of microRNA-122 by employing novel nucleobase and known 2′-ribose modifications. The nucleobase modifications are analogues of adenosine and guanosine that contain cyclopentyl and propyl minor-groove projections. Via a site-by-site chemical modification analysis, we identify several immunostimulatory 'hot spots' within the miRNA guide strand at which single base modifications significantly reduce immune stimulation. A duplex containing one base modification on each strand proved to be most effective in preventing immune stimulation.

MicroRNAs (miRNAs) are endogenous ~22 nucleotide RNAs that regulate gene expression by inhibiting the translation of mRNAs (mRNAs) via the RNA interference (RNAi) pathway. MicroRNA-122 (miR-122) is of particular therapeutic interest, being specifically and abundantly expressed in healthy liver cells but substantially down-regulated in hepatocellular carcinomas (HCC).[1] Mimics of miR-122 represent an attractive therapy for treating HCC, as evidenced by recent reports indicating that restoration of miR-122 expression in non-miR122 expressing HCC cells reverses their tumorigenic properties[2,3] and that small molecule activation of miR-122 selectively induces apoptosis in an HCC cell line.[4] In addition, the biodistribution of miR-122 is complementary to the delivery profile of current liposomal delivery vehicles for nucleic acids, which deliver the majority of the dose to the liver.[5,6]

We created a miR-122 mimic by synthesizing a 22 nt duplex RNA (Figure 1a). Such a microRNA mimic is functionally equivalent to the native miRNA. When we administered the miR-122 mimic in a lipid-nanoparticle formulation to peripheral blood mononuclear cells (PBMCs), we observed production of the cytokine tumor necrosis factor-α (TNF-α), indicative of immune stimulation (Figure 2). Immunostimulatory activity is a significant and undesirable off-target effect of RNA therapeutics due to the toxicity of induced cytokine production. The miR122 guide strand contains two occurrences of an alternating U/G motif, and such motifs have been implicated in contributing to immune stimulation.[7] The guide strand of a mature miRNA confers a unique spectrum of activity across a network of genes

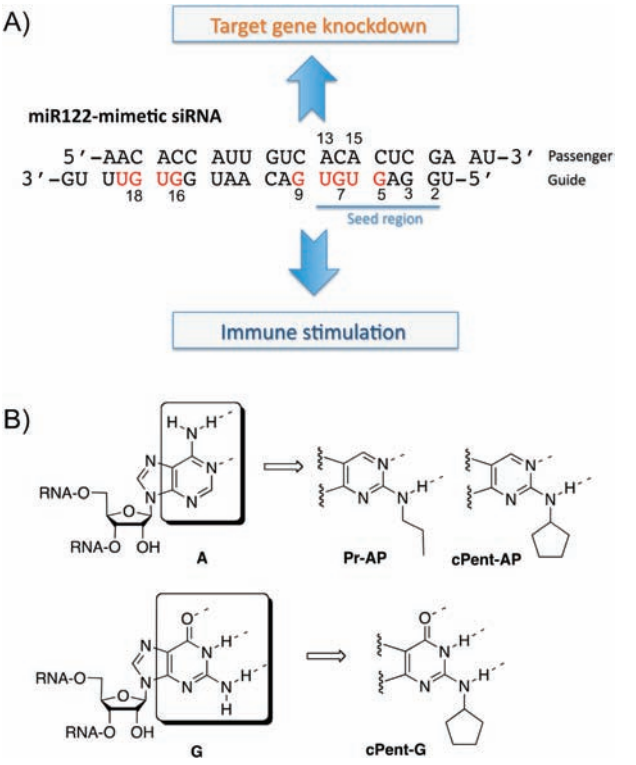

Figure 1. (A) Structure of the microRNA-122-mimetic RNA used in this study. Modification sites are indicated by numbered nucleotides, and putative immunostimulatory regions are highlighted in red. (B) Base modifications with minor groove-directed substituents replace selected adenosines and guanosines.

and must be conserved. Consequently, immunostimulatory properties cannot be tempered by sequence changes but, rather, must be addressed by chemical modification.

It is known that modification of the ribose 2′-position can inhibit the immunostimulatory properties of single-stranded RNAs and short duplex RNAs;[8−11] however, little is known about the effect of base modifications or the position dependence of modifications on immune stimulation. MiRNA mimics, like other short duplex RNAs, cause immunostimulation via pattern

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recognition receptors of the innate immune system, primarily the toll-like receptors (TLRs) 3,[12,13] 7, and 8.[14,15] The recognition of U and UG-rich motifs is consistent with the involvement of TLRs 7 and 8, whose activation is sequence-specific.[16−18] The mechanism of TLR 7/8 activation is poorly characterized; however, it stands to reason that since the oligonucleotide sequence is a criterion for activation of the innate immune system, the nucleobases (in addition to riboses) are being recognized. Therefore, we hypothesized that creating structural modifications to the nucleobases that alter their shape while maintaining their ability to engage in Watson/Crick base pairing could destroy interactions between the microRNA mimic and immune receptors.

It has previously been shown that certain modifications in the major groove of a short interfering RNA (siRNA) are ineffective at blocking immune stimulation;[9] thus, we decided to investigate the immunosuppressant effect of minor groove-directed base modifications. In addition, minor groove modifications block double-stranded RNA binding motif (dsRBM) proteins implicated in sequence-independent RNAi off-target effects (such as the RNA-dependent protein kinase (PKR) and adenosine deaminase that acts on RNA 1 (ADAR1)).[19] Such modifications could potentially shield microRNA mimics from both TLR-mediated immunostimulatory effects and dsRBM-mediated off-target effects.

We synthesized a novel guanosine analogue containing an N^2-cyclopentyl group as an O^6-*p*-nitrophenethyl, 5′-DMTrO, 2′-TBDMSO, 3′-β-cyanoethyl phosphoramidite (cPent-G, Figure 1b) and incorporated it at selected positions, replacing guanosines, in the guide strand of the miR-122 mimic. We made substitutions within putative immunostimulatory motifs (5′-GUGU-3′ sections) and within the seed region (nucleotides 2−8). Thermal melting experiments indicated that cPent-G is well tolerated in a base pair with C (see Supporting Information). Within the passenger strand, we incorporated adenosine replacements at two positions opposite the 5′-most putative immunostimulatory motif of the guide strand. We used 2-aminopurines containing N^2-propyl and N^2-cyclopentyl substituents[20] (Pr-AP and cPent-AP, respectively, Figure 1b), which have previously been shown to be excellent analogues of A in base pairs with U.[20]

To test RNAi activity, we measured the knockdown of two native miR-122 targets[21,22] (Necap2, Slc7a1) after 24 h at 10 nM in Hepa1−6 cell culture (Figures 2, 3). The guide strand modification analysis revealed position-dependent effects on miR-122 activity as a result of cPent-G incorporation. The base modification was tolerated at positions 3 and 5, both of which are within the seed region, and at positions 9, 16, and 18. However, it was not tolerated at positions 2 and 7, also within the seed region (Figure 2a). The observation of a position-sensitive minor groove modification in the guide strand is consistent with our previous work,[19] which also demonstrated that position 2 is sensitive to minor-groove modification. The cause of this position dependence is most likely variation in the distance between protein components of the RNA-induced silencing complex (RISC) and the minor-groove edge of the loaded guide strand. For instance, modeling of the Ago crystal structure and a loaded oligonucleotide highlights a steric hindrance for the nucleotide base at position 2 relative to several amino acid residues.[19,23] RNAi activity was retained with multiple guide strand modifications, such as the incorporation of cPent-G at positions 9 and 16.

Within the passenger strand, base modifications (Pr-AP and cPent-AP) were tolerated at positions 13 and 15 (Figure 3), both

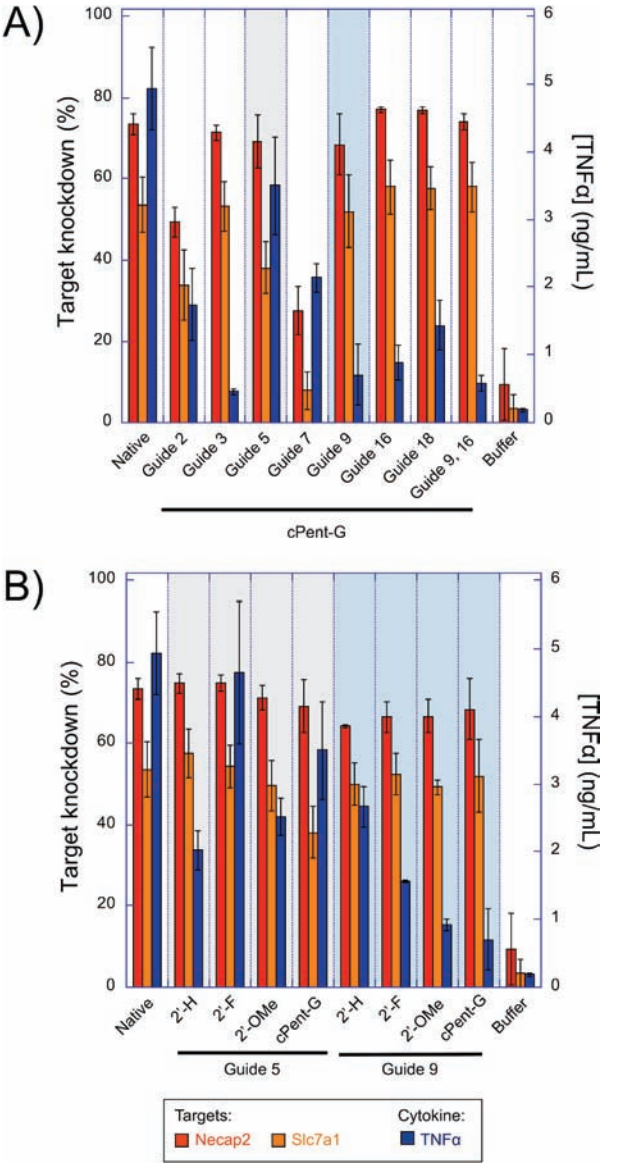

Figure 2. Target knockdown and cytokine levels for chemical modifications made to the guide strand of the microRNA mimic. (A) N^2-Cyclopentyl-G (cPent-G) modification at selected positions. Background shading indicates the positions that are investigated in second half of graph. (B) N^2-Cyclopentyl-G (cPent-G) and 2′-ribose modifications at positions 5 and 9.

individually and simultaneously. We also prepared a duplex containing a single cyclopentyl modification in both strands (cPent-G at guide 9 and cPent-AP at passenger 13), for reasons addressed below, and this retained native RNAi activity. Finally, to allow for the comparison of base and ribose modifications, we prepared RNA duplexes containing 2′-methoxy (2′-OMe), 2′-fluoro (2′-F), and 2′-deoxy (2′-H) modifications (at either A or G, as appropriate) in a few of the modification patterns. Consistent with the base modifications, the ribose modifications retained activity at positions 5 and 9 of the guide strand (Figure 2b) and at positions 13 and 15 of the passenger strand (see Supporting Information).

Next, we determined the immunostimulatory properties of the modified miRNA mimics by analyzing TNF-α production in

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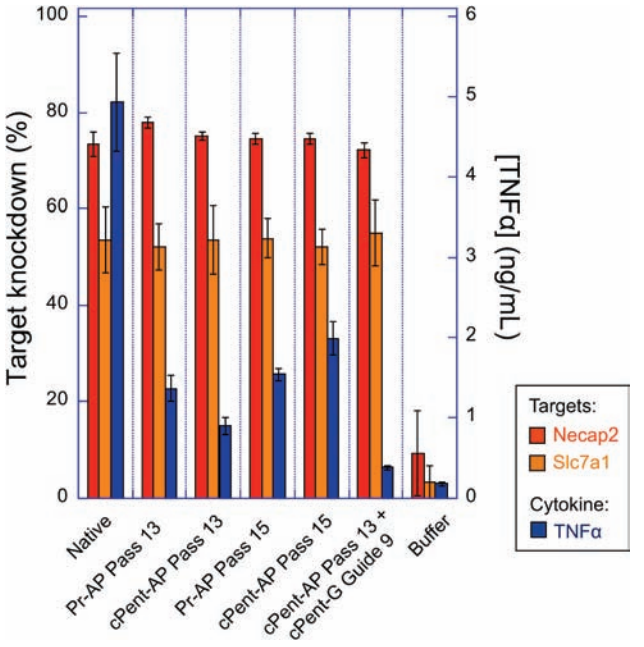

Figure 3. Target knockdown and cytokine levels for chemical modifications made to the passenger strand and to both passenger and guide strands.

PBMCs (Figures 2, 3) 16−20 h post-transfection. The effect of base modifications on TNF-α stimulation was highly position-specific and did not exclusively correlate with the locations of the putative immunostimulatory domains outlined above. cPent-G incorporation at positions 3, 9, and 16 on the guide strand was most effective at preventing immune stimulation (Figure 2a). All of these modified miR-122 mimics displayed TNF-α levels that were at least 5-fold lower than those of the unmodified duplex. Both positions 9 and 16 are within a 5′-GUGUG-3′ motif; however, position 3 is outside of a putative immunostimulatory region. In contrast, cPent-G modification at positions 5 and 7 were minimally effective at reducing TNF-α levels, and both of these sites are within a putative immunostimulatory region. These observations query the validity of our initial assumption that the UG-rich motifs define the immunostimulatory regions. Rather, the site-by-site analysis identified several positions that are immunostimulatory 'hot spots', at which single base modifications inhibit the recognition of the siRNA, or siRNA-lipid complex, by the immune receptors.

We then investigated the generality of the position-specific nature of TNF-α stimulation by testing ribose modifications at positions 5 and 9 of the guide strand (Figure 2b). These two sites were chosen based on their contrasting effects on TNF-α production with cPent-G modifications. We found that the effect of 2′-F and 2′-OMe modifications mirrors the effect of the cPent-G modifications, in that both of these ribose modifications were ineffective at blocking immune stimulation at position 5 but effective at position 9. 2′-H modifications were not highly effective at either of these sites. Interestingly, at position 9 the effectiveness of immunosuppression correlates with the size of the modification at the 2′-position. Previous reports indicate that as few as two 2′-OMe-G residues in the guide strand of an immunostimulatory siRNA are required to inhibit cytokine induction;[24] however no information exists on the position dependence of modifications throughout a microRNA guide strand and the occurrence of several immunostimulatory sites, such as those identified here.

For the passenger strand, modification at either position 13 or 15 with either Pr-AP or cPent-AP reduced immune stimulation. cPent-AP modification at position 13 reduced TNF-α levels 5-fold and was as effective as the most effective ribose modification at this site (2′-F; see Supporting Information). Based on the results of the guide and passenger strand modifications, we investigated the effect of miR-122 containing dual modifications. When two effective modification sites were combined in either the guide or passenger strands, the resulting duplex did not further decrease TNF-α levels (i.e., dual cPent-G modifications at positions 9 and 16 of the guide strand and dual cPent-AP modifications and positions 13 and 15 of the passenger strand) (Figures 2, 3).

Since additional modification within the same strand was ineffective, we tried combining two singly modified strands. Modifications at position 13 of the passenger strand and position 9 of the guide strand are both highly effective at suppressing cytokine levels, equaling or improving upon the corresponding ribose-modified RNAs, and were thus combined to form a doubly modified duplex. These two sites are located adjacent to each other and within opposite strands of the duplex. The resulting miRNA mimic reduced TNF-α below the levels recorded for singly modified duplexes containing each of the component strands (Figure 3). This modification strategy was the most effective out of the base-modification strategies evaluated in this work. At this time we cannot predict the generality of the effect of this modification pattern. However, the occurrence of localized 'hot spots' within the miR-122 sequence where modification has a greater effect than at other sites prompts the investigation of additional immunostimulatory sequences incorporating these base modifications.

In summary, we present a new approach for blocking immune stimulation in miRNA mimics by means of minor-groove localized base projections. Incorporation of single modifications at sites throughout the guide strand of a miR-122 mimic revealed several positions to be immunostimulatory 'hot spots', critical to cytokine stimulation. The position-dependent nature of immune stimulation was mirrored by 2′-ribose modifications at two sites. A duplex containing a single base modification on each strand was superior in inhibiting immune stimulation while retaining RNAi activity. This work shows that minor-groove base modifications inhibit immune stimulation in short duplex RNAs and thereby provides an additional route to generating nonimmunostimulatory RNAi therapeutics.

■ ASSOCIATED CONTENT

Supporting Information. Procedure for the synthesis of N^2-cyclopentyl guanosine phosphoramidite and details of RNA synthesis and purification. Procedures for the RNAi activity and cytokine assays, and additional assay data. This material is available free of charge via the Internet at http://pubs.acs.org.

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