Interference Separates Immunostimulation from RNA Interference

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Modifications in Small Interfering RNA That Separate Immunoactivation from RNA Interference

Florian Eberle,* Kerstin Gießler,† Christopher Deck,† Klaus Heeg,* Mirjam Peter,* Clemens Richert,† and Alexander H. Dalpke2*

Synthetic small interfering RNA (siRNA) can suppress the expression of endogenous mRNA through RNA interference. It has been reported that siRNA can induce type I IFN production from plasmacytoid dendritic cells, leading to off-target effects. To separate immunomodulation from the desired gene-specific inhibitory activity, we designed RNA strands with chemical modifications at strategic positions of the ribose or nucleobase residues. Substitution of uridine residues by 2'-deoxuryridine or thymidine residues was found to decrease type I IFN production upon in vitro stimulation of human PBMC. Thymidine residues in both strands of a siRNA duplex further decreased immunostimulation. Fortunately, the thymidine residues did not affect gene-silencing activity. In contrast, 2'-O-methyl groups at adenosine and uridine residues reduced both IFN-α secretion and gene-silencing activity. Oligoribonucleotides with 2'-O-methyladenosine residues actively inhibited IFN-α secretion induced by other immunostimulatory RNAs, an effect not observed for strands with 2'-deoxynucleosides. Furthermore, neither 5-methylcytidine nor 7-deaza-2'-deoxyguanosine residues in the stimulatory strands affected IFN-α secretion, suggesting that recognition does not involve sites in the major groove of duplex regions. The activity data, together with structure prediction and exploratory UV-melting analyses, suggest that immunostimulatory sequences adopt folded structures. The results show that immunostimulation can be suppressed by suitable chemical modifications without losing siRNA potency by introducing seemingly minor structural changes. The Journal of Immunology, 2008, 180: 3229–3237.

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2 Address correspondence and reprint requests to Dr. Alexander H. Dalpke, Department of Medical Microbiology and Hygiene, University of Heidelberg, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany. E-mail address: alexander.dalpke@med.uni-heidelberg.de

3 Abbreviations used in this paper: RNAi, RNA interference; DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methysulfate; eGFP, enhanced green fluorescent protein; MFI, mean fluorescence intensity; RISC, RNA-induced silencing complex; siRNA, small interfering RNA; TBDMS, tert-butyldimethylsilyl.

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combination of two assay systems. We studied the effect of sterically demanding 2′-O-methyl groups as well as deletion of the OH function at the 2′-position and nucleobase modifications in a screen involving residue-specific changes in the entirety of the RNA strands. Only thymidine residues replacing uridines gave RNAs with gene-silencing activity comparable to that of unmodified siRNA, but they markedly reduced induction of IFN-α in human peripheral blood cells. We also identified a 2′-O-methyl-ladenosine-containing RNA that led to complete loss of IFN-α induction and suppression of the effect of other stimulatory RNA strands.

Materials and Methods

Reagents for RNA synthesis
5′-[bis(3,5-trifluoromethyl)phenyl]-1H-tetrazole (activator), tetrahydrofuran (THF), pyridine, iodine and water (oxidizer; 0.02 M), acetonitrile, tri-chloroacetic acid in acetonitrile (deblock), tert-butylphenoxyacetyl acetic anhydride (THF), pyridine and N,N-methylimidazole (Cap B), the solid support (cpg), tert-butylmethylsilyl (TBDMs)-protected RNA phosphoramidites, and modified RNA phosphoramidites were purchased from Proligo or ChemGenes. Triethylammonium acetate buffer (0.1 M (pH 7)) was used for RNA purification against a gradient of CH3CN. Hydrofluoric acid in triethylamine (“triethylamine trihydrofluoride”) and methoxytrimethylsilane were from Acros Organics or from Fluka.

Spectrometric characterization
MALDI-TOF mass spectra were acquired on a Bruker Reflex IV spectrometer in negative, linear mode, using a mixture of 2,4,6-trihydroxy-acetophenone (0.3 M in EtOH) and diaminonitromethane citrate (0.1 M in water) at a ratio of 2:1 (v/v) as matrix. UV-Vis spectra were recorded on a NanoDrop ND-1000 spectrophotometer. Purification of the oligonucleotides was performed on a Hitachi HPLC L-6200 system using C18 reversed-phase columns (Machery-Nagel) or C18 Sep-Pak cartridge (Waters).

Media and Abs
RPMI 1640 and sodium pyruvate were obtained from Biochrom. FCS was from Biowest, PBS was obtained from PAA, and DMEM was purchased from Invitrogen.

RNA oligoribonucleotides
The sequences of all RNA strands that were produced are depicted in Figs. 1–3. Unmodified RNA strands, as well as 2′-O-methyluridine-containing strands (imu), were obtained from IBA. All other oligoribonucleotides were custom-synthesized in-house on an Expedite 8909 DNA synthesizer (ABI/PerSepTive Biosystems) on a 1 μmol scale, starting from preloaded controlled pore glass. RNA oligoribonucleotides were deprotected and cleaved from the solid support using concentrated aqueous ammonia (25%) (1 ml) for 35 min at 55°C. The supernatant was collected and the residue was washed with deionized water. Excess ammonia was removed from the combined aqueous solutions with a gentle stream of compressed air directed onto the surface of the solutions.

Phosphoramidites with TBDMs (29) or trisopropylsilyloxymethyl (TOM) groups (30) protecting for the 2′-hydroxy groups similar yields in the chain assembly process. The former are considerably less expensive than the latter, and TBDMs-protected building blocks were therefore used exclusively. A seemingly minor modification to the protocol recommended by the supplier of the phosphoramidites significantly improved yields. The dimethoxytrityl group of the 5′-terminal nucleotide was removed before release from the solid support, and neat triethylamine hydrofluoride was used for the deprotection step, rather than tetrabutylammonium fluoride in tetrahydrofuran (THF), whose tetrabutylammonium ions were found difficult to remove during cartridge purification. The more acidic hydrogen fluoride reagent also minimizes strand cleavage during workup of the deprotection solution, which is caused by fluoride ions. So, after hypholization, triethylamine trihydrofluoride (NEt3, 3 HF) (250 μl) was added. After 16 h, methoxytrimethylsilane (550 μl) was added to precipitate the oligoribonucleotide. The use of trimethylsilyl methyl ether as quenching agent at the end of the deprotection of the 2′-TBDMs groups not only removed residual hydrogen fluoride (a very toxic reagent), but also ensured that the RNA formed an easy-to-harvest pellet upon centrifugation. Therefore, after 20 min, the RNA was spun down by centrifugation on a microcentrifuge for 1 min. The supernatant was aspirated, and the pellet was dissolved in doubly distilled water (150 μl), filtered (0.2 μm pore size), and used for HPLC purification with a gradient from 0 to 30% CH3CN in 30 min, at a flow of 1 ml/min. A single, conventional HPLC step on a C18 reversed-phase column provided the oligoribonucleotides in high yields and purity, as demonstrated by MALDI-TOF mass spectrometry (supporting information can be found online: http://chip.chemie.uni-koeln.de/sup_info/). Control experiments with a sample of RNA 1 synthesized in-house showed the same activity in terms of IFN-α induction as that of the same compound prepared and purified by IBA, confirming that results with samples from either source produce comparable results.

Annealing to duplexes
To generate double-stranded siRNA duplexes, sense (s) and complementary antisense (as) strands of each siRNA were mixed at equimolar concentrations in annealing buffer (20 mM of HEPES, 150 mM of NaCl (pH 7.4)), heated to 90°C for 5 min, followed by cooling-down slowly to room temperature.

Cell isolation
Human PBMCs were isolated from whole blood of young healthy donors. Isolation was performed by standard Ficoll-Hypaque density centrifugation. Blood was diluted 1/2 (v/v) with PBS, underlaid by Ficoll (Biochrom), and centrifuged at 458 × g for 20 min. PBMCs were washed twice with PBS and resuspended in complete medium (RPMI 1640, 2% heat-inactivated autologous serum). PBMCs were plated at 4 × 105 cells/well in a 96-well flat-bottom plate for stimulation assays.

Cell stimulation and IFN detection
All used RNA molecules were encapsulated with DOTAP (N-[(1,2-dioleoyl)propyl]-N,N,N-trimethylammonium methylsulfate) (Roth) in a ratio of 1:2.5 according to the manufacturer’s protocol. Cells were stimulated for 16 h. Cell-free supernatant was removed and analyzed for human IFN-α secretion using a sandwich enzyme-linked immunosorbent assay (Bender MedSystems). Analysis was done in duplicates, and all experiments were performed at least three times unless indicated otherwise. To account for donor heterogeneity, stimulation with 1 μg/ml unmodified RNA oligoribonucleotide 1 was normalized to 100%.

Determination of gene-silencing activity
Human embryonic kidney cells (1.5 × 105 per well of a 24-well culture plate, HEK293) were plated in DMEM containing 10% FCS and no antibiotics. The following day, cells were cotransfected either with 400 ng pSV-biots, the following day, cells were cotransfected either with 400 ng pEGFP-N1 (BD Clontech), encoding enhanced GFP (eGFP), or with 500 ng pSYV-β-galactosidase control vector (Promega), encoding for β-galactosidase together with the respective siRNA in the indicated concentration. Transfection was performed with Lipofectamine 2000 (Invitrogen). After 24 h, cells were washed with PBS, and reporter gene expression was assayed. Expression of eGFP was determined by flow cytometry (FACScCanto, BD Biosciences) in the FITC channel (530/30 nm). Mean fluorescence intensity (MFI) of 20,000 live cells gated according to forward/side-scatter characteristics was detected. β-Galactosidase activity was determined by incubation of the cells for 4 h at 37°C in lysis buffer (100 mM of 2-ME, 9 mM of MgCl2, 0.125% NP40, and 0.15 mM of chlorophenol red-β-galactopyranoside). The reaction was stopped by addition of stopping solution (300 mM of glycine, 15 mM of EDTA), and OD was measured at 570 nm with a 650-nm reference wavelength.

Statistical analysis
Data were analyzed by GraphPad Prism 4.03 program (GraphPad Software). Significant differences were assessed by ANOVA to compare three or more groups followed by Dunnett’s test to compare selected groups. In all figures, ∗ represents p values <0.05.

Results
We assumed that the receptor(s) causing the immunostimulatory side effects of siRNA are structurally more discriminating than is the enzymatic machinery of RNAi. The former have to ensure highly selective differentiation of self from nonself structures, whereas the latter may have been evolved to attack double-stranded RNA from a variety of sources. The choice of modifications to be tested was governed by the following considerations: 1) structural diversity at positions that are likely to be recognized by a high-fidelity receptor of the innate immune system, 2) negligible effects on the base pairing required for siRNA activity (dsRNA), 3)
synthetic accessibility at moderate costs (to facilitate drug development), and 4) precedents in the literature.

The 2'-position was considered critical, as it is the very position where RNA and DNA differ structurally. Two types of modifications at this position were included: those that lack the 2'-oxygen (deoxy derivatives) and those that feature a methyl group at this position (2'-O-methyl derivatives; Fig. 1A). The latter should induce a steric conflict in complexes where the 2'-hydroxy group is located in a specific pocket of a receptor (Fig. 1B). Thymidine as replacement for uridine residues was also included, together with 5-methylcytidine and 7-deazaguanosine residues. In either of these nucleobase-modified residues, a position is altered that affects recognition in the major groove of duplexes. The major groove is the site most frequently involved in sequence-specific recognition of DNA by proteins (31). Duplex-forming potential is known to affect RNAs and the structural characteristics of certain immunostimulatory CpG oligonucleotides (32). Thymidine and 5-methylcytidine are sterically more demanding, and 7-deazaguanosine lacks N7 as a hydrogen bond acceptor (Fig. 1B).

Based on the parent sequence 1 (Fig. 2), which is both immunostimulatory and shows strong interference activity, derivatives were prepared. All ribonucleotides of one given nucleobase were replaced by their 2'-deoxy counterparts (1dA–1dU, Fig. 3A). These were complemented by three base-modified oligoribonucleotides (Fig. 3B), of which 1dT contains residues with structural changes in both the ribose and base. Additionally, 2'-O-methyl derivatives were prepared (1mA–1mU, Fig. 3C). The most promising structural replacement (U/dT, vide infra) was also tested in other sequence contexts (Fig. 3D). All compounds, except 1mU, were prepared via the optimized protocol given in Materials and Methods and were HPLC-purified to homogeneity, as shown by MALDI-TOF mass spectrometry (supporting information can be found online: http://chip.chemie.uni-karlsruhe.de/sup_inf/).

**Analysis of 2'-deoxy-modified RNA oligomers regarding immunostimulation**

First, the 2'-deoxy derivatives (1dA–1dU) of single-stranded (ss) oligoribonucleotide 1 representing the antisense strand of a siRNA directed against eGFP (33) were tested. The RNA strands were formulated with DOTAP (9) and tested for IFN-α secretion from human PBMCs, which is mediated by activation of plasmacytoid dendritic cells. Plasmacytoid dendritic cells had a frequency of ~1.6% (±0.3%) in PBMCs as determined by flow cytometry analysis for expression of BDCA-2 and BDCA-4. Due to donor heterogeneity, IFN-α output was normalized to an internal reference. We observed a dose-dependent increase in IFN-α secretion of unmodified RNA strand 1 over the tested concentration range from 0.01 to 1 μg/ml (Fig. 4A). Individual IFN-α secretion varied between 900 and 1400 pg/ml. Substitution of all uridines in RNA 1 (5 of 22 nucleotides) by their 2'-deoxy counterparts gave 1dU, which showed a significantly decreased immunostimulatory activity. Residual IFN-α secretion was observed when 1dU was added at a concentration of 1 μg/ml. 1dC also diminished IFN-α secretion; however, reduction was far less than observed with 1dU. In contrast, the 2'-deoxyribonucleosides 1dA and 1dG showed no significant inhibition of immunostimulation.

**Immunostimulation with 2'-O-methyl derivatives**

Next, oligonucleotides containing 2'-methoxy groups (Fig. 4B) were analyzed. For the derivatives of 1 with 2'-O-methyl groups at any A or U residue, IFN-α secretion in human PBMCs was completely abrogated. Both modified RNA oligoribonucleotides (1mA or 1mU) showed a complete loss of immunostimulation, even at the highest tested concentration, exceeding the effects of 2'-deoxyuridine (1dU) as replacement. In contrast, incorporation of 2'-O-methyl-cytidine (1mC) did not lead to an alteration in IFN-α induction.
Immunostimulation with base-modified derivatives

Furthermore, nucleobase-modified residues, which should affect recognition processes involving the major groove of duplexes (Fig. 4C), were tested. We observed that the derivative of 1 containing 2'-thymidine residues (1dT) showed significant reduction of IFN-α secretion over all concentrations tested, comparable to that observed with 1dU (Fig. 4A). 5-methylcytidine (1C5Me)-modified RNA strands did not show altered IFN-α secretion as compared with the unmodified parental strand (1), and 7-deazaguanosine (1NG) modified strands were slightly less active at only two concentrations.

Thymidine-containing siRNAs show superior gene-silencing activity

Next, we assessed whether modifications that decrease the immunostimulatory activity of the oligoribonucleotides affect gene-silencing activity of the corresponding siRNA duplexes. The antisense, or leading strands, with their respective modifications (1, 1dT, 1dU, 1mA, or 1mU) were annealed to their unmodified complementary strand (2). By cotransfection of a plasmid encoding eGFP with the duplexes, it was shown that the unmodified siRNA (2/1) efficiently silenced eGFP expression to <5% at all concentrations tested (Fig. 5). Control duplex (8/7) with a different sequence did not even decrease eGFP expression slightly, suggesting that this was a sequence-specific effect. The minor increase observed might be due to an increased transfection efficacy. Among the modified siRNAs, only the one containing the thymidine residues (2/1dT) displayed the same efficiency of silencing to 5% of gene expression as the unmodified counterpart 2/1. Moreover, siRNA 2/1dT was active at all three concentrations tested. In contrast, 2'-deoxyuridine (2/1dU) and 2’-O-methyluridine (2/1mU) as replacements in the siRNAs suppressed eGFP expression much less, with inhibition to 43 and 38% at 1.5 nM of strand concentration. Increasing the siRNA concentration resulted in slightly improved gene silencing, but even with 10-fold higher concentrations, the suppression activity was not as high as that with unmodified (2/1) or thymidine-containing (2/1dT) siRNA. The 2’-

O-methyladenosine-containing 2/1mA was even less effective, showing only a marginal inhibition to 88% of control expression.

Thymidine residues separate immunostimulation from gene silencing

These results indicated that thymidine is a favorable residue for siRNAs, which decreases immunostimulation without affecting gene-silencing activity. To determine whether these observations were specific for the individual siRNA 2/1dT or hold true for siRNA duplexes in general, further siRNAs, targeting GFP at another position (4/3, 4/3dT) or targeting β-galactosidase (6/5, 6/5dT), were designed. Sequences with high GU-content were chosen to allow for immunostimulation. Thymidine residues were again introduced in the leading strands (3dT, 5dT) (Fig. 5). Single-stranded, modified RNA oligoribonucleotides were applied to human PBMCs and IFN-α secretion was measured (Fig. 6, A–C). We observed that all three unmodified RNA strands tested (1, 3, 5) induced IFN-α with rather similar dose-activity profiles, and maximum activity was found in two independent donors. In each case, incorporation of thymidines (1dT, 3dT, 5dT) resulted in a strong reduction of IFN-α secretion, confirming our hypothesis. As observed for RNA strand 1dT, application of >1 μg/ml 5dT resulted
in residual IFN-α secretion, whereas 3dT was completely inactive. Next, siRNA duplexes containing the modified antisense (leading) strand annealed to an unmodified sense (passenger) strand were analyzed for gene-silencing activity (Fig. 6D). Gene expression of both reporter genes (eGFP or galactosidase) in HEK293 cells was suppressed efficiently to levels comparable to those with unmodified siRNAs (2/1 vs 2/1dT, 4/3 vs 4/3dT, 6/5 vs 6/5dT). These results confirm that the thymidine modification in siRNA is separating immunostimulation from gene silencing in more than one sequence context.

**Effect of thymidine residues in both strands of siRNA duplexes**

Having shown that thymidine-modified RNA oligoribonucleotides are able to decrease IFN-α secretion induced by the single-stranded form, we examined to what extent thymidine residues affect immunostimulatory activity induced by double-stranded siRNA. SiRNA against eGFP had been chosen such that both strands, sense (2) and antisense (1), were active in terms of IFN-α induction (see Fig. 8A). We generated siRNA duplexes composed of unmodified and thymidine-modified strands in different orientations to analyze the immunostimulatory capacity of the siRNA duplex as a whole (Fig. 7A). Annealed, unmodified siRNA (2/1) induced IFN-α in a dose-dependent manner. When the leading strand was substituted with a thymidine modified strand (2/1dT), IFN-α secretion was reduced to ~50% as compared with the unmodified siRNA (2/1). We speculated that the residual immunostimulation was due to the presence of the unmodified passenger strand (2). To further decrease immunostimulation, thymidine residues were also introduced in the passenger strand of the siRNA (2dT). This resulted in double-stranded siRNA (2dT/1dT) carrying thymidine residues in either strand. Using this doubly modified siRNA duplex, IFN-α secretion in human PBMCs could be diminished further and was only observed at concentrations >0.3 μg/ml.

In order to achieve sufficient reduction of immunostimulation made it necessary to modify both strands of a siRNA targeting eGFP with thymidines (Fig. 7A). Therefore, it was necessary to verify that incorporation of these modifications in both strands did not affect gene silencing (Fig. 7B). In fact, we observed that even a siRNA consisting of two modified single strands (2dT/1dT) was still as efficient in gene silencing as the unmodified counterpart (2/1). The concentration necessary for gene silencing (0.02–0.1 μg/ml) was far below the concentration for which residual stimulation was observed.

**Antagonistic effects on IFN-α secretion**

It had been reported that 2’-O-methyluridine-, 2’-O-guanosine-, and 2’-O-adenosine-modified RNA oligoribonucleotides act as TLR7 antagonists (26). From the results presented above (Fig. 7A), it seemed unlikely that thymidine-containing RNA strands act as dominant antagonists. To address this issue, we repeated experiments from above including siRNA duplexes composed of an unmodified sense strand and 2’-O-methyladenosine residues (2/1mA) or 2’-O-methyluridine residues (2/1mU). It was observed that both single strands of the siRNA against eGFP (1, 2) induced IFN-α, with RNA strand 2 being slightly more active (Fig. 8A). The annealed, unmodified siRNA 2/1 was as effective as the RNA
when applied alone. In contrast to the siRNA with thymidines (2/1dT), a siRNA composed of 2'-O-methyladenosines in the antisense strand (2/1mA) was completely unable to induce IFN-α secretion. This indicates that the adenosine-modified antisense strand 1mA suppressed the otherwise stimulatory sense strand 2 in trans. This dominant inhibitory effect was not observed with the strand containing 2'-O-methyluridines (2/1mU).

To ascertain whether 2'-O-methyladenosines differ in their effect from thymidines in terms of their antagonistic activity, human

FIGURE 6. Thymidine-modified siRNAs separate gene-silencing activity from immunostimulation. A–C, Human PBMCs were stimulated with single-stranded, antisense RNA oligoribonucleotides 1 (A), 3 (B), or 5 (C) and compared with the thymidine-modified counterparts (1dT, 3dT, 5dT). IFN-α secretion from PBMCs of two individual donors (D1, D2) is shown. D, HEK293 cells were transfected with the indicated reporter plasmids encoding eGFP or β-galactosidase (gray bars). Annealed siRNA duplexes composed of an unmodified sense and thymidine-modified antisense strand and directed against eGFP (EGFP1, EGFP2) (2/1, 2/1dT, 4/3, 4/3dT) or β-galactosidase (BG) (6/5, 6/5dT) were tested for gene-silencing activity (n = 2) (black bars).

FIGURE 7. Effect of thymidine in the leading vs the passenger strand on gene-silencing activity and IFN-α secretion. A, Human PBMCs were stimulated with single-stranded RNA oligoribonucleotides 1, 2, or the siRNA duplex thereof (EGFP), which was composed of unmodified strands (2/1), thymidine (2/1dT), 2'-deoxyuridine (2/1dU), 2'-O-methyluridine (2/1mU), or 2'-O-methyladenosine (2/1mA) modification in the antisense (leading) strand. IFN-α secretion was measured (n = 3, mean ± SD), *(p < 0.05 as compared with control siRNA 2/1). B, The annealed siRNA duplexes were tested for gene-silencing activity (black bars) on HEK293 cells transfected to express eGFP (gray bars) (n = 3, mean ± SD) at two concentrations of 1.5 or 7.5 nM.

FIGURE 8. 2'-O-Methyladenosine-modified, but not thymidine-modified, RNA strands possess antagonistic activity. A, Human PBMCs were stimulated with single-stranded RNA oligoribonucleotides 1, 2, or the siRNA duplex thereof (EGFP), which was composed of unmodified strands (2/1), thymidine (2/1dT), 2'-deoxyuridine (2/1dU), 2'-O-methyluridine (2/1mU), or 2'-O-methyladenosine (2/1mA) modification in the antisense (leading) strand. IFN-α secretion was measured (n = 3, mean ± SD), *(p < 0.05 as compared with control siRNA duplex 2/1). B, Human PBMCs were stimulated with antisense RNA oligoribonucleotide 1. The indicated modified RNA oligoribonucleotides also in antisense orientation were added in equal concentrations. IFN-α secretion was measured (n = 3, mean ± SD), *(p < 0.05 as compared with control with addition of unmodified RNA 1.
PBMCs were stimulated by simultaneous addition of modified and unmodified strands, with both being in the antisense orientation (Fig. 8B). The result confirmed that 2′-O-methyladenosine is a residue that completely suppresses the immunostimulatory activity of the unmodified strand (1+1mA), acting as a dominant antagonist. In contrast, thymidine-containing RNA strands affected stimulation by the unmodified strand (1+1dT) only slightly, behaving as an immunological more “silent” agent. Again, for 2′-O-methyl uridine-modified RNA (1+1mU), only a weak antagonistic activity was observed.

**UV-melting curves reveal structure formation**

Our data suggest that uridine residues are critical for immunostimulatory activity, as do data from the literature (9, 12, 34). Uridine residues also play pivotal roles in the formation of defined three-dimensional structures. For example, they can form two hydrogen bond base pairs with both adenine and guanine (35). We therefore asked whether the immunostimulatory sequence 1 shows signs of forming base pairs. A solution of sequence 1 in PBS buffer was subjected to UV-monitored thermal denaturation, which yielded the melting curve shown in Fig. 9A. This curve exhibits two sigmoidal transitions, one just below room temperature, and one at ~50°C. This is in agreement with the predicted structures for intramolecular folding and dimerization (Fig. 10), both of which feature two duplex regions, either of which contains uridine residues. Although the region of lower stability may no longer be predominant at body temperature, it is energetically close enough to be relevant for molecular recognition events. Thymidine-containing 1dT does not show a significant low temperature transition (Fig. 9A), while the high temperature transition is essentially unchanged. This may be the consequence of the steric effect of the methyl groups of the thymine residues.

We then performed UV-melting analyses for EGFP3s, a sequence that is not immunostimulatory and lacks uridine residues. A much broader transition with significantly reduced overall hyperchromicity was obtained (Fig. 9B). Structure prediction algorithms do not detect a significant folding or dimerization propensity for EGFP3s, with no more than two nucleotides engaging in forming base pairs (supporting information can be found online: http://chip.chemie.uni-karlsruhe.de/sup_inf/). The appearance of the absorbance vs temperature plot of this nonstimulatory sequence is fairly similar to that of poly(C), a homopolymer that does not have the propensity to fold into well-defined structures at neutral pH (Fig. 9B). Exploratory assay on the immunostimulatory effect of eight other RNA sequences, combined with structure predictions, showed that all sequences with immunostimulatory activity have the propensity to form uridine-containing duplex/stem regions with predicted melting points above body temperature (37°C) (supporting information can be found online: http://chip.chemie.uni-karlsruhe.de/sup_inf/). Although they are not the focus of our current study, these results may stimulate additional experiments on the structure-forming propensity of RNAi agents.

**Discussion**

The discovery that certain synthetic siRNA duplexes activate the RNAi machinery without stimulating a rigorous IFN response in mammalian cells (2) was considered a breakthrough for the development of RNAi-based therapeutics (36). Closer scrutiny revealed that although injection of naked siRNA does not necessarily result in systemic IFN induction in vivo (17), a combination with lipoid or polycation-based delivery systems does produce side effects. Certain siRNAs induce a prominent IFN response (18–20, 28), which seems to be due to activation of plasmacytoid dendritic cells via TLRs (12, 16, 20, 37). Therefore, therapeutic siRNAs should be chemically modified for clinical use to minimize immune activation (23); otherwise, siRNAs could share the fate of antisense therapeutics, for which immunostimulation via TLR9 was recognized only during clinical trials as a complicating factor (5).
Recognition of siRNAs through TLR7/8 (12), TLR3 (14), or dsRNA-dependent protein kinase (19) are molecular recognition processes that should be distinct (and spatially separated) from RNA interference. It is clear that siRNAs induce immunostimulation in a sequence-dependent manner. Although the exact recognition mechanisms of TLR7/8 have not been identified, it has been suggested that GU-rich sequences are stimulatory (9, 12, 16). At least for some cell types, oligomers of uridylic acids alone are particularly active (34), but these are not useful as siRNA agents. It is assumed that better defined “stimulatory motifs” exist, but so far it is not possible to predict the immunostimulatory potential merely from the sequence. Oligoribonucleotides, such as strand 1, contain no more than roughly the statistically expected number of G and U residues (12 of 22 strands).

Our results with RNA strands containing 2'-deoxy and 2'-O-methyl residues identified the 2'-deoxy versions of uridine (including thymidine), but none of the other residues (including 2'-deoxyguanosine), as structural alternatives that significantly reduced IFN-α secretion. This suggests that the 2'-position in uridine is indeed critical for recognition. In agreement with this hypothesis, we observed that 2'-O-methyladenosine and 2'-O-methyluridine in RNA strand 1 abolished immunostimulation. From the literature it is also known that 2'-O-methylguanosine reduces immunostimulation (25–27). The methyl group abolishes hydrogen bonding of the 2'-OH group as donor, and it much reduces the likelihood that it acts as an H-bond acceptor. Neither 5-methylcytidine nor 7-deazaguanosine modified RNA-affected IFN-α secretion much. Both modifications affect the surface of the nucleobases located in the major groove of a duplex. Therefore, any obstacle in the major groove, such as the methyl group of 5-methylcytidine, is not sufficient to suppress immunorecognition.

The combination of 2'-deoxyoxygenation and methyl group at the 5-position of pyrimidine, as realized in thymidine residues, is quite effective in diminishing immunostimulation. The tests with thymidines in two additional stimulatory RNA sequences, supported by results from other groups with 2'-deoxyuridine-containing RNA strands (24, 25, 27), confirm that uridine is a universal target for decreasing immunostimulatory activity.

Whereas 2'-deoxyuridine- and thymidine-containing strands still showed some activity when applied at higher concentrations, 2'-O-methyluridine and 2'-O-methyladenosine were completely inactive, and 2'-O-methyladenosine even inhibited IFN-α secretion as an antagonist. Others have observed that 2'-O-methyluridines make RNA an antagonist (25, 26). The lack of an effect with 2'-O-methylcytidines might indicate that the stimulatory sequence motif (at least in the oligoribonucleotides tested) does not contain cytidine residues. Although we did not test for positional effects, others reported that substituting only two uridines with 2'-O-methyluridines completely abrogates immunostimulation (27).

The differences between 2'-O-methyladenosine and thymidine in terms of antagonistic vs silent behavior became even more apparent when analyzing siRNA duplexes. Modifying both strands with thymidine resulted in a strong decrease in immunostimulatory far it is not possible to predict the immunostimulatory potential merely from the sequence. Oligoribonucleotides, such as strand 1, contain no more than roughly the statistically expected number of G and U residues (12 of 22 strands).

Our results with RNA strands containing 2'-deoxy and 2'-O-methyl residues identified the 2'-deoxy versions of uridine (including thymidine), but none of the other residues (including 2'-deoxyguanosine), as structural alternatives that significantly reduced IFN-α secretion. This suggests that the 2'-position in uridine is indeed critical for recognition. In agreement with this hypothesis, we observed that 2'-O-methyladenosine and 2'-O-methyluridine in RNA strand 1 abolished immunostimulation. From the literature it is also known that 2'-O-methylguanosine reduces immunostimulation (25–27). The methyl group abolishes hydrogen bonding of the 2'-OH group as donor, and it much reduces the likelihood that it acts as an H-bond acceptor. Neither 5-methylcytidine nor 7-deazaguanosine modified RNA-affected IFN-α secretion much. Both modifications affect the surface of the nucleobases located in the major groove of a duplex. Therefore, any obstacle in the major groove, such as the methyl group of 5-methylcytidine, is not sufficient to suppress immunorecognition.

The combination of 2'-deoxyoxygenation and methyl group at the 5-position of pyrimidine, as realized in thymidine residues, is quite effective in diminishing immunostimulation. The tests with thymidines in two additional stimulatory RNA sequences, supported by results from other groups with 2'-deoxyuridine-containing RNA strands (24, 25, 27), confirm that uridine is a universal target for decreasing immunostimulatory activity.

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The differences between 2'-O-methyladenosine and thymidine in terms of antagonistic vs silent behavior became even more apparent when analyzing siRNA duplexes. Modifying both strands with thymidine resulted in a strong decrease in immunostimulatory activity. Importantly, the range of concentration that was necessary for gene silencing was below what induced residual IFN-α release. In contrast, the 2'-O-methyladenosine completely inhibited siRNA-mediated immunostimulation. Additionally, it was reported that 2'-O-methyluridine also inhibits TLR9 activation by type A CpG oligodeoxynucleotides in humans and mice, thus lacking TLR7/8 specificity (26). Taken together, the results here show that thymidines are immunologically silent modifications that do not interfere with the RNA-recognizing receptor(s). Clinically, this finding may be significant, as a functional TLR7 may be necessary for defense against some viruses (8).

The lack of specificity of the RNAi machinery deserves comment. It has been reported previously that the 2'-OH in the ribose backbone is not absolutely necessary for RNAi (22). Certain modifications in the leading antisense strand of a siRNA seem to be tolerated, with 2'-fluoro derivatives performing better than their 2'-O-methyl counterparts (38). However, it was shown that modifications had to be restricted to certain regions of a siRNA, yet the exact requirements differ for various modifications (39, 40). In general, it is thought that modifications in the middle of the duplex are not well tolerated, as this part is important for RISC activity (41). For example, 2'-O-methyluridines in the leading (antisense) strand of a siRNA are not tolerated, while the same modification in the passenger strand is less problematic (27). Our results confirm that 2'-O-methyladenosine and 2'-O-methyluridine modifications in the leading strand of a siRNA, while not entirely detrimental, lead to lowered gene-silencing activity. In contrast, the thymidine-containing siRNAs are fully inhibitory, even when both strands are modified. This conclusion held true for two additional siRNA sequences, suggesting a general principle. We observed that the thymidine-containing siRNAs are consistently more inhibitory than are their 2'-deoxyuridine counterparts, possibly because their methyl group slightly increased duplex stability.

Although these findings are valuable for the design of future RNAi agents, they do not necessarily resolve the issue as to what are the molecular details of the molecular recognition event(s) leading to IFN-α secretion. It is too early to speculate on the structural basis of the sequence-specific molecular recognition event(s) leading to the immunostimulatory activity of sequences such as 1, and the lack thereof for compounds like 1dT. The following can be stated without any doubts, however: 1) compound 1 does show signs of structure formation in its UV-melting curve, 2) the low temperature transition in this curve is significantly reduced for 1dT, and 3) uridine residues are involved in forming base pairs in all sequences evaluated with structure prediction algorithms thus far. Because defined three-dimensional structures are advantageous for selective molecular recognition (32), this finding may not be accidental.

In summary, we have identified thymidine as a modification that can be introduced into siRNA without affecting gene-silencing activity while inhibiting IFN-α secretion from cells of human blood. An oligoribonucleotide with 2'-O-methyladenosines that lacks TLR7 antagonistic activity was found to be an active inhibitor of immunostimulation.

Disclosures

The authors have no financial conflicts of interest.

References


