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Nucleoside Optimization for RNAi: A High-Throughput Platform

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Supporting Information

ABSTRACT: The RNA induced silencing complex (RISC) contains at its core the endonuclease Argonaute (Ago) that allows for guide strand (GS)-mediated sequence-specific cleavage of the target mRNA. Functionalization of the sugar/phosphodiester backbone of the GS, which is in direct contact with Ago, presents a logical opportunity to affect RISC's activity. A systematic evaluation of modified nucleosides requires the synthesis of phosphoramidites corresponding to all four canonical bases (A, U, C, and G) and their sequential evaluation at each position along the 21-nucleotide-long GS. With the use of a platform approach, the sequential replacement of canonical bases with inosine greatly simplifies the problem and defines a new activity baseline toward which the corresponding sugar-modified inosines are compared. This approach was validated using 2′-*O*-benzyl modification, which demonstrated that positions 5, 8, 15, and 19 can accommodate this large group. Application of this high-throughput methodology now allows for hypothesis-driven rational design of highly potent, immunologically silent and stable siRNAs suitable for therapeutic applications.

RNA interference (RNAi) is a mechanism of post-translational gene silencing where double-stranded RNAs (dsRNAs), such as short interfering RNAs (siRNAs), induce degradation of sequence-specific homologous mRNA.[1−3] siRNAs are RNA duplexes containing 19−21 nucleotides that are widely used for functional genetic or cellular screens.[4] RNAi-based therapy presents an attractive opportunity to engage targets not accessible through conventional small molecules.[5−7] To realize the immense therapeutic potential of RNAi, many properties of siRNAs, including potency,[8] stability,[9] immunogenicity,[10,11] and delivery,[12−14] must be optimized.

The RNA-induced silencing complex (RISC) is associated with an RNAase H-type endonuclease, Argonaute (Ago), that allows for guide strand (GS)-mediated sequence-specific cleavage of the target mRNA.[15] A crystal structure of *Thermus thermophilus* Argonaute 2 (Ago2) containing a chemically modified GS revealed that nucleotides 2−8 of the GS are preassembled in an A-form helix and that the GS makes contact with the surface of Ago2 through its sugar/phosphodiester backbone.[16,17] This repetitive structure of the RNA scaffold bodes well for the highly conserved primary roles of RNA, transcription[18] and translation,[19] both of which require rapid ratcheting movement along the sugar/phosphodiester backbone. In contrast, the static, cofactor-like interaction of unsubstituted RNA with the potential binding pockets on the Ago2 protein surface is not optimal and could account for RISC's overall slow performance.[20,21]

Chemical modifications of the sugar/phosphodiester backbone present a logical opportunity to optimize the performance of this complex. Improved siRNA−Ago2 interaction should result in increased binding selectivity; more effective strand selection; and improved catalytic turnover, siRNA−Ago2 complex stability, and product release. Moreover, chemically modified siRNA duplexes are expected to be more resistant to RNAase-mediated cleavage and have decreased affinity to toll-like receptors and dsRNA-dependent protein kinase, resulting in decreased immunogenicity.[22,23]

Most of the chemically modified nucleosides used today in RNAi were synthesized to convey enzymatic stability to RNA oligomers, and their design was not guided by siRNA−Ago2 binding considerations.[24−26] Although the development of structure−activity relationships (SARs) for novel nucleosides at distinct positions on the GS would be highly beneficial, it is hindered by the complexity of the associated chemistry. Such an effort would require independent SAR data collection for each nucleotide along the siRNA GS, as the local Ago2 protein surface adjacent to each particular position is different. Furthermore, access to sugar-modified nucleosides containing all four canonical bases would be necessary, since a systematic investigation would utilize a test siRNA with a predetermined sequence of adenosine (A), guanosine (G), cytidine (C), and uridine (U) bases.

In theory, synthesis of 21-siRNA oligomers containing one instance of the modified nucleoside (positions 1−21, "walkthrough") would be necessary. Evaluation of a single modification in a 96-well-plate format (500nM loading/well) would typically require ∼1 g of each of the four canonical sugar-modified nucleoside phosphoramidites. Depending on the character of the modification, an estimated 40 synthetic steps would be needed to secure these building blocks. This logistical complexity renders the interrogation of such a huge chemical space intractable.

In this communication, we report a platform solution for high-throughput evaluation of chemically modified nucleosides. According to this, the natural bases present in a canonical test oligomer (ApoB 10162, Figure 1A, X = cytosine, "Unmodified") are sequentially replaced by a suitable universal base.[27−29] We selected hypoxanthine[30] for its nearly isoenergetic Watson−Crick

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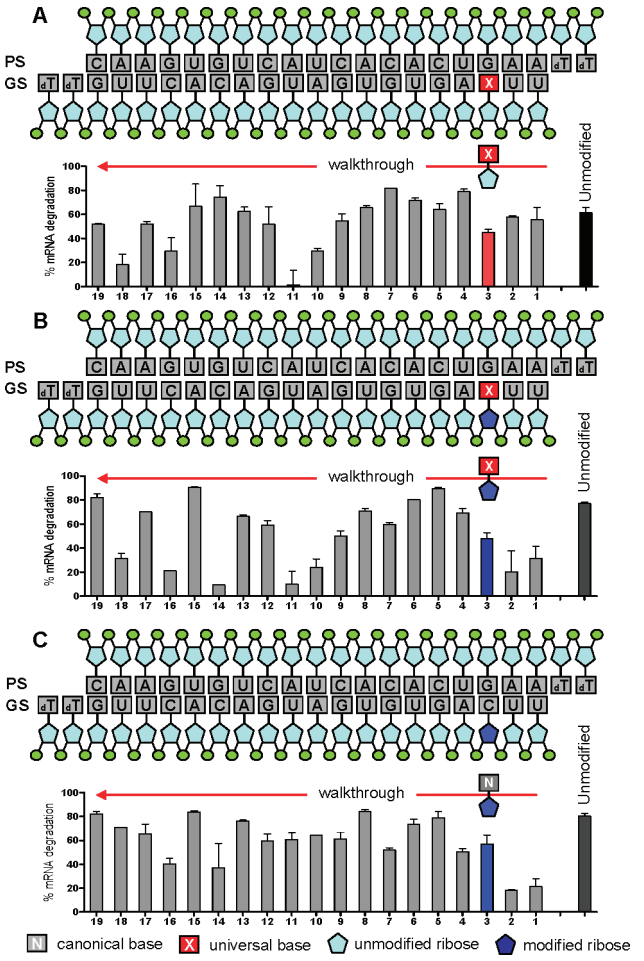
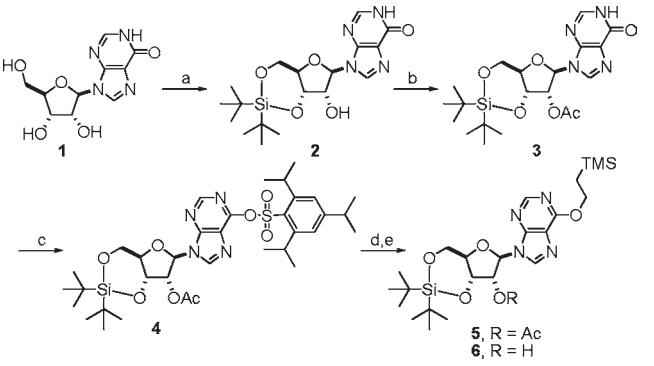


Figure 1. Position-dependent effect of (A) the ribose-unmodified universal base (e.g., inosine), (B) the ribose-modified universal base (e.g., substituted inosine), and (C) the ribose-modified canonical base on mRNA degradation. The numbers along the *x* axis indicate the position along the guide strand (GS) of the siRNA duplex, with position 1 being the 5′ end. Walkthrough connotes that the ribose-modified nucleoside was systematically substituted at each position along the GS and analyzed for mRNA target degradation (*y* axis). "Unmodified" represents the level of target mRNA inhibition determined for the all-ribose duplex.

interaction with cytosine, uracil, adenine,[31] and to a lesser extent with guanine.[32] The cell-based performance of this universal-nucleoside-containing RNA set establishes a new activity baseline (Figure 1A, X = hypoxanthine) toward which the analogous sugar-modified oligomers are compared (Figure 1B, X = hypoxanthine, modified ribose, i.e., 2′-*O*-benzyl). If the activity of the sugar-modified inosine surpasses that of the unmodified inosine placed at the same position along the siRNA oligomer, this relationship should be also reflected in the improved performance of the sugar-modified full canonical nucleoside. Similarly, if the effect of the sugar-modified inosine is detrimental to activity, we would expect this also to be paralleled by the modified canonical nucleoside. The four chemically modified canonical nucleosides (Figure 1C, N = canonical base, modified ribose, i.e. 2′-*O*-benzyl) are then synthesized only when the modification has demonstrated a benefit using this simplified platform.

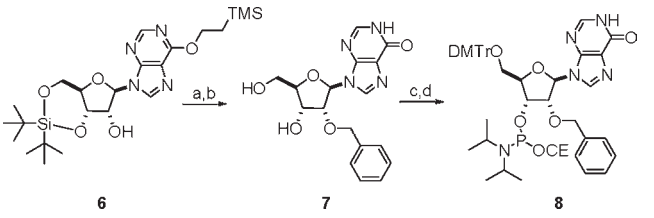
Any sugar modification capable of projecting a group toward the Ago2 surface can in principle be used to optimize the

Scheme 1[a]



[a] Conditions: (a) $^{t}Bu_2Si(OTf)_2$, imidazole, DMF; (b) Ac_2O, TEA, DMAP, DMF; (c)[33] $ArSO_2Cl$, TEA, DMAP, DCM; (d) trimethylsilyl ethanol, DABCO, DBU, dioxane; (e) MeONa, 0 °C, MeOH.

Scheme 2[a]



[a] Conditions: (a) benzyl bromide, BEMP, MeCN; (b) TBAF, KF, 55 °C, THF; (c) DMTCl, pyridine; (d) 2-cyanoethyl diisopropylchlorophosphoramidite, 1-methyl imidazole, DIEA, DCM. DMTr = 4,4′-dimethoxytrityl; CE = cyanoethyl.

performance of the RISC complex. Substituents placed at position 2′-*O*, 1′-*C*, or 4′-*C* are all capable of affecting this interaction, and the design of more sophisticated nonribose alternatives is definitely possible. Many of the chemical modifications that are tolerated in siRNAs reside at the 2′ position of the ribose ring,[34,35] and we demonstrate the utility of our high-throughput approach by evaluating a simple 2′-*O*-benzyl modification.

Replacement of the four canonical heterocycles, corresponding to A, U, C, and G, with a universal base allows for the design of a downstream relay such as **6**, greatly simplifying the associated chemistry (Scheme 1). With this intermediate in hand, the final 2′-*O*-benzyl-modified phosphoramidites became accessible in as few as four chemical steps (Scheme 2). To facilitate high-throughput parallel synthesis of analogous phosphoramidites containing modified benzyl groups, the first two and last two steps in Scheme 2 could be combined into single operations.

As a test sequence, we used a synthetic double-stranded 21-mer corresponding to positions 10162 to 10182 of the mRNA from the human *ApoB* gene. The siRNAs were tested at a subsaturating concentration of 1 nM that is capable of target-specific mRNA degradation and can be detected by quantitative polymerase chain reaction in cell-based assays (Figure 1A, X = cytosine, "Unmodified"). First, the ability of siRNAs containing sugar-unmodified universal nucleotides to degrade target mRNA was assessed (Figure 2A1, black bar, first three positions shown). This established a new baseline toward which the sugar-modified inosine-containing siRNAs were compared (Figure 2A1, blue bar). To account for variations between measurements, the raw

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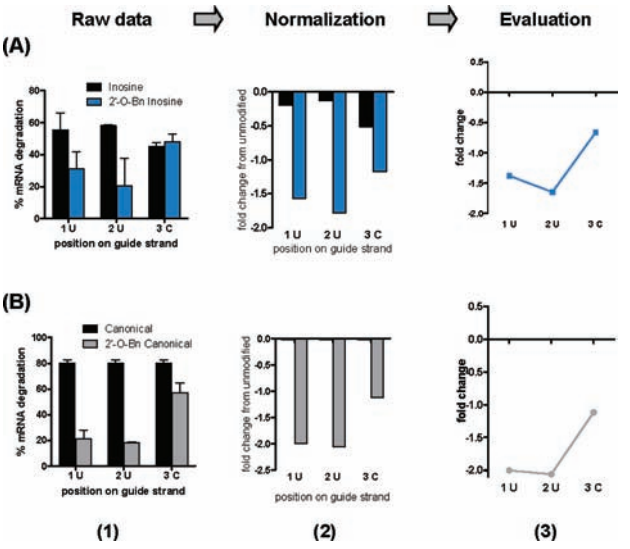

Figure 2. Schematic representation of the workflow used to evaluate 2′-*O*-benzyl modification: (A) inosine and (B) canonical background. Only positions 1−3 are shown.

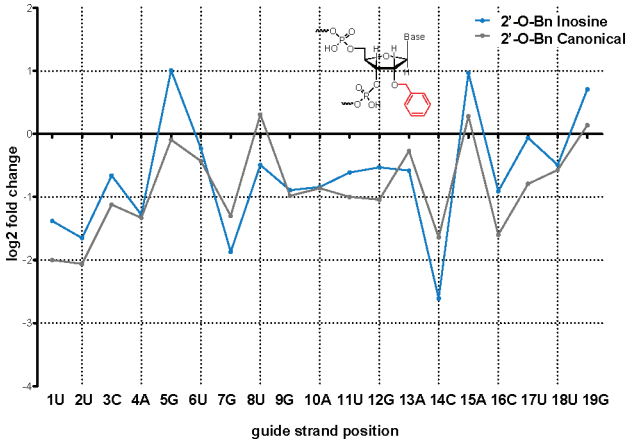

Figure 3. Position-dependent effect of 2′-*O*-benzyl modification on target mRNA degradation evaluated on the inosine (blue) and canonical (black) siRNA (r^2 = 0.64, p < 0.0001; ApoB10162).

data were normalized to unmodified canonical siRNA (Figure 2A2) and compared with the inosine baseline (Figure 2A3; Figure 3, blue).

The canonical siRNA (Figure 1A, X = cytosine, "Unmodified") at 1 nM concentration exhibited 60% mRNA levels. With the exception of positions 11 (opposing the mRNA cleavage site) and 18, replacement of any canonical heterocycle with hypoxanthine (Figure 1A, X = hypoxanthine) resulted in the loss of no more than 50% of its activity. The sugar-modified siRNAs containing 2′-*O*-benzylinosines at positions 1−4, 12, 14, and 18 further decreased the mRNA degradation in comparison with the sugar-unmodified inosine baseline. Therefore, we concluded that a 2′-*O*-benzyl modification would be detrimental if placed here as part of a canonical nucleoside. On the other hand, the performance of 2′-*O*-benzyl-modified inosine at positions 5, 8, 15, and 19 was similar or better than the sugar-unmodified inosine baseline, so we concluded that a 2′-*O*-benzyl-modified nucleoside would be well-tolerated with respect to activity when placed at positions 5, 8, 15, or 19 of the siRNA (Figure 3, blue).

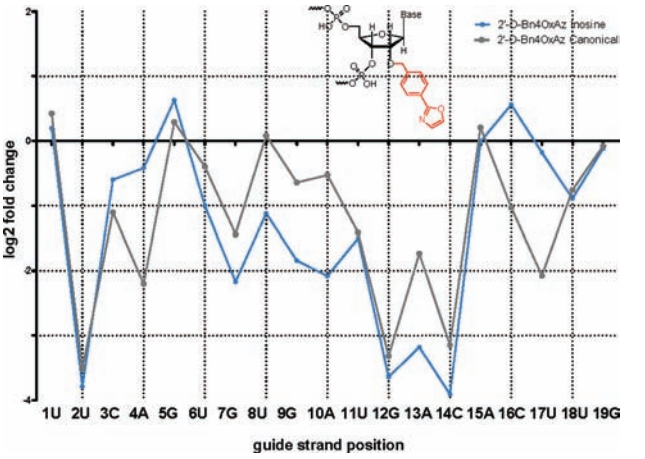

Figure 4. Position-dependent effect of 2′-*O*-(4-oxazol-2-yl)benzyl modification on target mRNA degradation evaluated on the inosine (blue) and canonical (black)siRNA (r^2 = 0.50, p < 0.0007; ApoB10162).

To verify these predictions, 2′-*O*-benzyl-modified canonical nucleoside amidites (corresponding to A, U, C, and G) were synthesized following proprietary or published procedures,[36] incorporated into the GS of the test siRNA (Figure 1C, modified ribose, N = canonical base), and evaluated (Figure 2B1−3). As predicted by the inosine platform, positions 5, 8, 15, and 19 are the most accommodating of 2′-*O*-benzyl modifications, and these siRNAs have the same or better activity as the unmodified oligomers (Figure 3, black). Potency data obtained for canonical sequences carrying the 2′-*O*-benzyl modifications confirmed these observations (Figure S2 and Table S3 in the Supporting Information). To the best of our knowledge, this finding represents the first report of relatively large benzyl group being tolerated along the GS, including in the seed region.

The mRNA target selection of an siRNA-primed RISC complex is controlled by the base sequence of the GS anchored by the Ago2. As a consequence, sugar modification is largely sequence-independent, enabling a fragment screening approach to optimization of the siRNA−protein surface interaction. Evaluation of the 2′-*O*-benzylribose using an siRNA targeting the human propyl hydroxylase domain 2 (PHD2) gene, which lacks homology with the ApoB sequence, confirmed this assertion and highlights the potential of the platform approach to siRNA optimization (Figure S4).

To exemplify the potential for SAR data collection, the spatially more demanding 2′-*O*-(*p*-oxazol-2-yl)benzyl group was examined. The results suggested marginal tolerability at positions 5, 15, and 16 (Figure 4, blue) and intolerability at positions 2 and 11−14. These observations lead to the prediction that the modification would not be beneficial. Synthesis of the canonical 2′-*O*-(*p*-oxazol-2-yl)benzyl phosphoramidites and the corresponding siRNA (Figure 4, black) verified this prediction experimentally.

A platform comparison of the 2′-*O*-benzyl group in its natural (*ribo*) and unnatural (*arabino*) configurations suggested that the RNAi performance of a modifying group is not simply a consequence of its size. The 2′-*O*-*ara*-benzyl group was less tolerated, with notable difference at positions 7 and 15 (Figure S5).

As expected, sequential inclusion of inosine caused a position-dependent decrease in the melting temperature (T_m) that was not substantially changed by 2′-*O*-benzylation (Figure S6). Similarly,

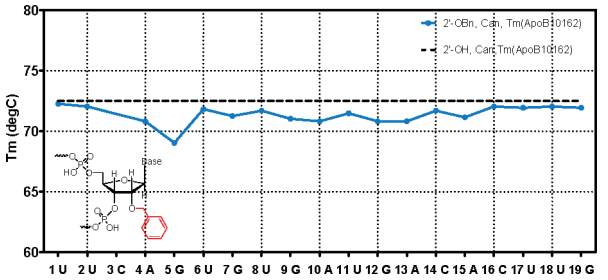

Figure 5. Position-dependent effect of 2′-*O*-benzyl modification on the melting temperature (T_m) of the duplex: modified siRNA (blue) and unmodified siRNA (black) (ApoB10162).

a walkthrough of 2′-*O*-benzyl-modified canonical nucleosides did not cause a substantial deviation of T_m values either, and no apparent correlation to the observed positional effect on target mRNA levels was observed (Figure 5).

In this communication, we have described a novel high-throughput approach for evaluation of chemically modified nucleosides for RNAi. The method was validated using inosine as a universal nucleoside and exemplified using 2′-*O*-benzyl and 2′-*O*-(*p*-oxazol-2-yl)benzyl modifications in multiple sequences. The 2′-*O*-benzyl-modified nucleosides exhibited an excellent correlation with the SAR data collected using the universal base. Application of this high-throughput methodology now enables hypothesis-driven design in analogy to small-molecule fragment screening. This platform approach enhances the value of innovative chemistry in the discovery of highly potent, immunologically silent, and stable siRNAs suitable for therapeutic applications.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures, four figures and one table mentioned in the text, and complete ref 25. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Fire, A. Z. *Angew. Chem., Int. Ed.* **2007**, *46*, 6967.
(2) Mello, C. C. *Angew. Chem., Int. Ed.* **2007**, *46*, 6985.
(3) Grimm, D. *Adv. Drug Delivery Rev.* **2009**, *61*, 672.
(4) Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. *Nature* **2001**, *411*, 494.
(5) de Fougerolles, A.; Vornlocher, H. P.; Maraganore, J.; Lieberman J. *Nat. Rev. Drug Discovery* **2007**, *6*, 443.
(6) Castanotto, D.; Rossi, J. J. *Nature* **2009**, *457*, 426.
(7) Siomi, M. C. *Adv. Drug Delivery Rev.* **2009**, *61*, 668.
(8) Koller, E.; Propp, S.; Murray, H.; Lima, W.; Bhat, B.; Prakash, T. P.; Allerson, C. R.; Swayze, E. E.; Marcusson, E. G.; Dean, N. M. *Nucleic Acids Res.* **2006**, *34*, 4467.
(9) Watts, J. K.; Deleavey, G. F.; Damha, M. J. *Drug Discovery Today* **2008**, *13*, 842.
(10) Sioud, M.; Furset, G. *J. Biomed. Biotechnol.* **2006**, 1.
(11) Sioud, M. *Trends Mol. Med.* **2006**, *12*, 167.
(12) White, P. J. *Clin. Exp. Pharmacol. Physiol.* **2008**, *35*, 1371.
(13) Tseng, Y. C.; Mozumdar, S.; Huang, L. *Adv. Drug Delivery Rev.* **2009**, *61*, 721.
(14) Howard, K. A. *Adv. Drug Delivery Rev.* **2009**, *61*, 710.
(15) Hall, T. M. T. *Structure* **2005**, *13*, 1403.
(16) Wang, Y. L.; Sheng, G.; Juranek, S.; Tuschl, T.; Patel, D. J. *Nature* **2008**, *456*, 209.
(17) Wang, Y. L.; Juranek, S.; Li, H. T.; Sheng, G.; Tuschl, T.; Patel, D. J. *Nature* **2008**, *456*, 921.
(18) Kornberg, R. *Angew. Chem., Int. Ed.* **2007**, *46*, 6957.
(19) Simonovic, M.; Steitz, T. A. *Biochim. Biophys. Acta* **2009**, *1789*, 612.
(20) Rivas, F. V.; Tolia, N. H.; Song, J. J.; Aragon, J. P.; Liu, J. D.; Hannon, G. J.; Joshua-Tor, L. *Nat. Struct. Mol. Biol.* **2005**, *12*, 340.
(21) Haley, B.; Zamore, P. D. *Nat. Struct. Mol. Biol.* **2004**, *11*, 599.
(22) Kariko, K.; Buckstein, M.; Ni, H. P.; Weissman, D. *Immunity* **2005**, *23*, 165.
(23) Kariko, K.; Weissman, D. *Curr. Opin. Drug Discovery Dev.* **2007**, *10*, 523.
(24) Manoharan, M. *Curr. Opin. Chem. Biol.* **2004**, *8*, 570.
(25) Bramsen, J. B.; et al. *Nucleic Acids Res.* **2009**, *37*, 2867.
(26) Zhang, H. Y.; Du, Q.; Wahlestedt, C.; Liang, Z. C. *Curr. Top. Med. Chem.* **2006**, *6*, 893.
(27) Loakes, D. *Nucleic Acids Res.* **2001**, *29*, 2437.
(28) Kool, E. T. *Annu. Rev. Biophys. Biomol. Struct.* **2001**, *30*, 1.
(29) Sherer, E. C.; York, D. M.; Cramer, C. J. *J. Comput. Chem.* **2003**, *24*, 57.
(30) Chizhikov, V.; Wagner, M.; Ivshina, A.; Hoshino, Y.; Kapikian, A. Z.; Chumakov, K. *J. Clin. Microbiol* **2002**, *40*, 2398.
(31) Janke, E. M. B.; Riechert-Krause, F.; Weisz, K. *J. Phys. Chem. B* **2011**, *115*, 8569.
(32) Burkard, M. E.; Turner, D. H. *Biochemistry* **2000**, *39*, 11748.
(33) Zlatev, I.; Vasseur, J. J.; Morvan, F. *Tetrahedron* **2007**, *63*, 11174.
(34) Jackson, A. L.; Burchard, J.; Leake, D.; Reynolds, A.; Schelter, J.; Guo, J.; Johnson, J. M.; Lim, L.; Karpilow, J.; Nichols, K.; Marshall, W.; Khvorova, A.; Linsley, P. S. *RNA* **2006**, *12*, 1197.
(35) Robbins, M.; Judge, A.; Liang, L.; McClintock, K.; Yaworski, E.; MacLachlan, I. *Mol. Ther.* **2007**, *15*, 1663.
(36) Compositions and Methods for Detecting and Modulating RNA Activity and Gene Expression. WO 91/10671, July 25, 1991.