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Short, terminally modified 2′-OMe RNAs as inhibitors of microRNA†

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We applied 14-mer 2′-OMe RNAs as inhibitors of selected micro RNAs. To improve their properties, we introduced a trimethoxystilbene residue at the 5′-terminus and three 2′-fluoro-2′-deoxynucleotides at the 3′-terminus to obtain potent inhibitors, whose mismatch discrimination is substantially better than that of typically applied >18-mers.

Recently discovered micro ribonucleic acids (miRNAs) are 20–24-mer duplexes,[1] which are known to participate in post-transcriptional control of gene expression and are responsible for regulation of numerous cellular events, such as apoptosis, proliferation or differentiation.[2] The biological activity of miRNAs can be inhibited by oligonucleotides (ODN) and their analogues, which typically bind to the seed region of the miRNA sequence. The affinity of the inhibitors toward target miRNAs is increased upon increasing their length, whereas their ability to discriminate between the fully matched and mismatched sequences (specificity) negatively correlates with this parameter. Traditionally used 18–22-mer inhibitors bind fully matched target miRNAs strongly, but also have substantial affinity to miRNAs with single nucleotide mismatches. For example, Gao, Zamore and co-workers have recently applied recombinant adeno-associated virus (rAAV) vectors with miRNA 'tough decoys' (TuDs), which generate *in vivo* long inhibitors flanked by double stranded stretches and having 22-mer recognition sequences.[3] These inhibitors down-regulate not only desired, fully matched miRNAs (let-7a, 12.1 fold), but also significantly affect targets with single mismatches (let-7c, 7d, 7f, miR-98, 5.0–11.0 fold). Moreover, synthetic 2′-OMe RNA-based TuD's have been found to be unable to discriminate a target having 2 mismatches.[4] Koch and co-workers have studied different length inhibitors for their ability to discriminate single mismatches in the down-regulation of apoB mRNA expression in mice.[5] In particular, they have compared the activity of fully matched 16-, 14- and 12-mer locked nucleic acid (LNA)–DNA hybrids with that of the ones containing single mismatches. As expected, they have found that the shortest, 12-mer inhibitors are substantially more sequence specific than their longer analogues. Few other <18-mer inhibitors have been described.[6] According to these reports carefully designed 12–16-mer LNA–DNA hybrids are suitable for specific targeting of intracellular RNAs, whereas 8-mers can be used for inhibition of entire miRNA families.

>18-mer 2′-OMe RNAs are excellent inhibitors of miRNA.[1b,7] The monomers used for their synthesis are commercially available and are substantially cheaper than those required for synthesis of LNAs or peptide nucleic acids (PNAs). Herein we report on the development of short (14-mer) 2′-OMe RNA inhibitors, which exhibit single mismatch discrimination and retain high affinity towards their target miRNAs (Fig. 1).

To enable the efficient binding of the inhibitor to miRNAs under physiological conditions we introduced a trimethoxystilbene (Cap) at the 5′-terminus and three 2′-fluoro-2′-deoxynucleotides ($^{F}A^{F}A^{F}C$, Fig. 1) at the 3′-terminus. These moieties were selected from the result of screening several known, duplex stabilizing terminal modifications: positively charged, at pH 7, an aminohexyl residue and *N*,*N*,*N*′*N*′-tetramethylrhodamine dye, hydrophobic cholesterol, a cholic acid residue, a pyrene and anthraquinone residue as well as modified nucleosides like 2′-fluoro-2′-deoxynucleotides and LNA monomers.[8] The modifiers were attached either directly to the termini or one to three 3′-terminal 2′-OMe RNA monomers were substituted for either 2′-fluoronucleotides or LNA monomers. All together, we generated 59 chemically modified 14-mer inhibitors. Their structures, synthesis details and properties are provided in the ESI.†

As the target miRNA we selected hsa-miR-92 (further miR-92), a member of the oncogenic miR-17-92 cluster, whose expression is increased in a number of cancer cells, but is usually low in healthy cells.[9] We selected HeLa cells for further experimentation, since miR-92 is expressed in these cancer cells in moderate to high levels (data not shown). For monitoring the inhibitor activity directly in cells a dual luciferase reporter system was used. Inhibitors were transfected along with the reporter constructs. As a positive control we used an unmodified 21-mer

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† Electronic supplementary information (ESI) available: Additional data on inhibitors (structures, labelling scheme, synthesis details, characterization, data on inhibitory activity and cellular stability). See DOI: 10.1039/c3cc43174f

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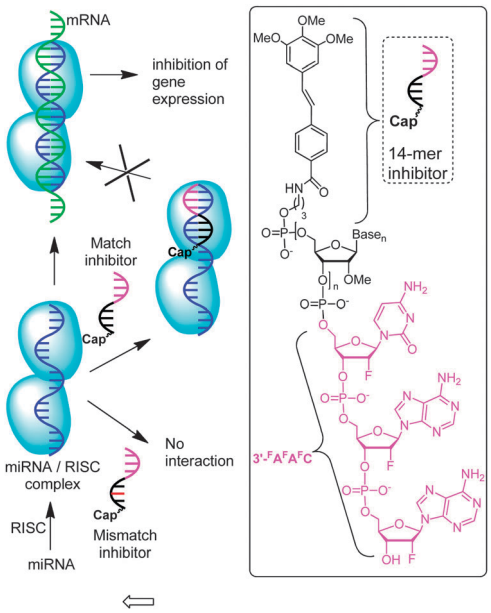

Fig. 1 A possible mode of interaction of the terminally modified 14-mer inhibitor with the microRNA–RISC complex; matching nucleobases are shown as black and violet sticks; a mismatch nucleobase is shown as a red stick; violet sticks and backbone represent 2′-fluoro-2′-deoxynucleosides $^{F}A^{F}A^{F}C$; structure of inhibitors is given in the box; base$_n$ – nucleobases A, G, C or U; inhibitor sequences are given in Scheme 1.

2′-OMe RNA (21-mer92), which is entirely complementary to the leading strand of miR-92 (miR-92l, Scheme 1).

This inhibitor demonstrated a concentration dependent increase in the luciferase signal intensity and reached saturation at concentrations ≥50 nM (Fig. 2). For example, in the presence of 50 nM 21-mer92 the luciferase signal is increased by 7.3 ± 2.6 fold, whereas the truncated 14-mer 2′-OMe RNA (14-mer92, 50 nM), which is complementary to 6 out of 7 nucleobases of the seed region of miR-92 (Scheme 1), facilitates the luciferase expression by only 1.7 ± 0.5 fold. As a negative control we used 2′-OMe RNA with an anti-Bantam sequence (scr, Scheme 1). Since bantam is a *Drosophila* micro RNA, it has no homologs in humans. As expected, it facilitates the luciferase expression in our assay by only 1.1 ± 0.3 fold, which is comparable to the effect of 14-mer92. 14-mer 2′-OMe RNA terminally modified with 5′-Cap and 3′-$^{F}A^{F}A^{F}C$ (14-mer*92, 50 nM, Fig. 1) was found to up-regulate luciferase expression slightly better than the positive control: 8.7 ± 3.2 fold *vs.* 7.3 ± 2.6 fold, respectively. Interestingly, at the higher inhibitor concentration (100 nM) 14-mer*92 is even more active, whereas the activity of the control 21-mer92 remains practically unchanged: 11.9 ± 3.4 *versus* 7.6 ± 0.5 fold (Fig. 2A).

miR-92p: 3'-UCGUAACGUUGGCUAGGGUUGGA
miR-92l:[ii] 5'- UAUUGCACUUGUCCCGGCCUGU
21-mer92: 3'-AUAACGUGAACAGGGCCGGAC
14-mer92: 3'- AACGUGAACAGGGC
14-mer*92: 3'-$^{F}A^{F}A^{F}$CGUGAACAGGGC-Cap
miR-30b: 5'- UGUAAACAUCCUACACUCAGCU
21-mer30b: 3'-CAUUUGUAGGAUGUGAGUGAA
14-mer30b: 3'-CAUUUGUAGGAUGU
14-mer*30b: 3'- $^{F}C^{F}A^{F}$UUUGUAGGAUGU-Cap
Scr: 5'-AUCAGCUUUCAAAAUGAUCUCAdT

Scheme 1 Sequences of inhibitors, scrambled sequence and miR-92 and miR-30b targets (for miR-92 both leading and passenger sequences: miR-92l, miR-92p); the seed sequence of the targets is labelled in blue color.

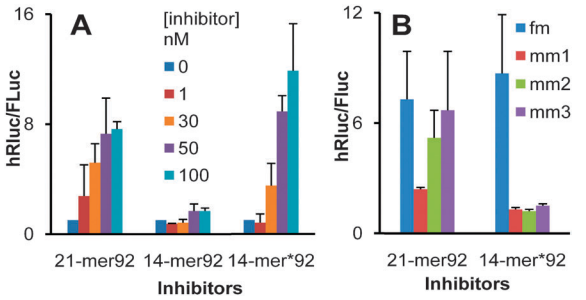

Fig. 2 (A) Inhibition of miR-92 in HeLa cells (expressed in fold increase of hRluc expression relative to Fluc expression: hRluc/Fluc) in the presence of 21-mer92, 14-mer92 and 14-mer*92 at 0–100 nM; (B) comparison of sequence specificity of 21-mer92 and 14-mer*92: fm – full match (21-mer92 or 14-mer*92), mm1 (G13 → U13 mismatch: 21-mer92_a or 14-mer*92_a), mm2 (G12 → U12 mismatch: 21-mer92_b or 14-mer*92_b), mm3 (G11 → U11 mismatch: 21-mer92_c or 14-mer*92_c); concentration of inhibitors – 50 nM; at least three independent experiments were conducted to obtain the data shown in this figure; these data were used to calculate standard deviations shown as error bars.

Potentially, this difference can be even higher at >100 nM since, in contrast to 21-mer92, the biological activity of 14-mer*92 is not saturated at 100 nM (Fig. 2A). The stronger effect of the modified inhibitor probably indicates that 14-mer*92/miR-92 interaction is more disrupting for the complex of miR-92 with RISC than that of 21-mer92/miR-92. It seems that both terminal modifications are important for the activity. In particular, removal of the cap (conjugate A9-RNA-B6, Scheme S1, Tables S1 and S2, ESI†) leads to reduction of the activity of the conjugate from 8.7 to 2.3, whereas removal of the fluorinated sugars (conjugate A10-RNA-B3) leads to reduction of the activity to 4.3. At lower inhibitor concentrations (<50 nM) the effect of control 21-mer92 (IC_{50} = 16 ± 12 nM) is stronger than that of 14-mer*92 (IC_{50} = 36 ± 6 nM, Fig. 2A), which can be explained by the higher affinity of 21-mer92 to the RNA target. The latter conclusion has been confirmed by UV-melting experiments (Fig. S5, ESI†). In particular, we observed that the duplex of 21-mer92 with the leading strand of miR-92 (miR-92l) melts at >85 °C, whereas the 14-mer*92/miR-92l duplex melts at 77.1 ± 0.5 °C. It is notable that though 14-mer*92 exhibits substantially better inhibitory activity than unmodified 14-mer92, duplexes of both compounds with miR-92l melt at similar temperatures as determined using UV-visible spectroscopy: T_m = 77.1 ± 0.5 °C for 14-mer*92/miR-92l and 76.5 ± 0.8 °C for 14-mer/miR-92l. These data indicate that the affinity of 2′-OMe RNAs to the leading strand of target micro RNAs does not at least in our case correlate with their inhibitory activity. Next, we studied the interaction of the inhibitors with the intact miR-92 duplex, in which the fluorescent dye (fluorescein, FAM) was attached to the 3′-terminus of the passenger strand (miR-92p-FAM) and the quencher (Q) was attached to the 5′-end of the leading strand (miR-92l-Q, Scheme 1). In the duplex miR-92p-FAM/miR-92l-Q, the FAM dye is quenched due to the proximity of the quencher Q (Fig. 3A). Its fluorescence is restored upon duplex dissociation in the presence of inhibitors that allows studying the interaction of inhibitors with intact miR-92 using fluorescence spectroscopy. By using this assay we observed that 21-mer92 induces dissociation of up to 82% of the miR-92 duplex, whereas its truncated analogue, 14-mer92, induces only 38% duplex opening (Fig. 3A).

In contrast to the unmodified 14-mer the terminally modified 14-mer*92 induces dissociation of up to 68% of the miR-92 duplex,

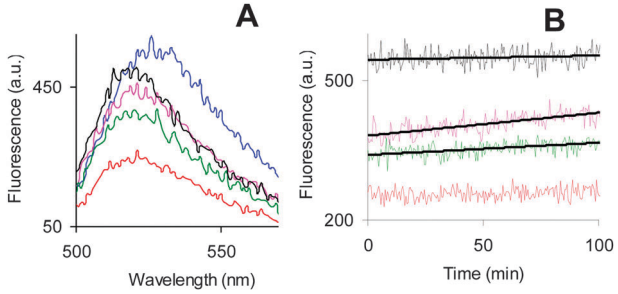

Fig. 3 (A) Fluorescence spectra (λ_{ex} = 490 nm) of single stranded miR-92p-FAM (blue trace), miR-92p-FAM/miR-92l-Q (red trace) and mixtures of miR-92p-FAM/miR-92l-Q with inhibitor 14-mer*92 (magenta trace), 21-mer92 (black trace) and 14-mer92 (green trace); concentration of each inhibitor was 1 μM; concentration of the labelled duplex was 20 nM; DPBS buffer (150 mM) was used for all mixtures; (B) rate of the fluorescence change observed upon addition of the inhibitors to miR-92p-FAM/miR-92l-Q; the measurement was started 2 min after addition of the inhibitors to the solution of the duplex; linear fits of the data are shown as black, straight lines; all experimental conditions were the same as in A.

which is close to the effect of the 21-mer control (Fig. 3A). These data are in agreement with the data on the biological activity of the inhibitors discussed above (Fig. 2A). We also used the same labelled RNA duplex to study the kinetics of interaction of the inhibitors with their target. In particular, we observed that the reaction of the 21-mer with the miR-92 duplex is completed within less than 2 min (black trace, Fig. 3B). In contrast, the duplex opening in the presence of either unmodified 14-mer92 or 14-mer*92 is dramatically slower. However, the 14-mer*92-induced reaction is still 1.9 fold faster than that induced by the unmodified 14-mer. Thus, the selected chemical modifications of the 14-mer enhance not only the ability of the inhibitor to induce dissociation of the dsRNA target, but also increase the rate of inhibitor–target interaction. Though the biological activity data (Fig. 2) correlate with our *in vitro* data (Fig. 3), it should be mentioned that in cells miRNAs are bound to proteins, *e.g.* RISC. The effects of these proteins can further modulate the interaction of the inhibitors with micro RNAs.

To study sequence specificity of the 14-mer*92, we introduced into its sequence three different mutations to obtain 14-mer*92_a (G13 → U13), 14-mer*92_b (G12 → U12) and 14-mer*92_c (G11 → U11). Mutated 21-mer analogues (21-mer92-a, 21-mer92-b, 21-mer92-c) were prepared for comparison. The activity of these compounds as inhibitors of miR-92 at 50 nM was studied by using the dual luciferase assay as described above (Fig. 2B). We were pleased to observe that all short 14-mers containing single mismatches in their sequences practically do not enhance luciferase expression: a 1.2–1.5 fold increase was observed. In contrast, analogous mismatched 21-mer 2′-OMe RNAs enhanced luciferase expression by 2.4–6.7 fold. These data indicate that the 14-mer inhibitor described here is substantially more sequence specific than traditionally used 21-mers.

To prove the general applicability of modified 14-mers as micro RNA inhibitors we designed a binder (14-mer*30b) targeting miRNA with an unrelated seed sequence: hsa-miR-30b (further miR-30b, Scheme 1). This inhibitor binds to the seed region of miR-30b analogous to the one targeting miR-92. We observed that the unmodified 14-mer (14-mer30b, 50 nM) inhibitor does not affect miR-30b-controlled expression of hRluc (hRluc/Fluc = 0.7 ± 0.1), whereas the modified 14-mer (14-mer*30b) inhibitor increased the luciferase signal by 4.1 ± 2.3 fold. It was still lower than that of the respective unmodified 21-mer (10.6 ± 0.5 fold hRluc enhancement), indicating dependence of the inhibitor effect on the particular sequence and/or structure.

In summary, we demonstrated that terminally modified 14-mer 2′-OMe RNAs are potent and highly sequence specific inhibitors of representative miRNAs, hsa-miR-92 and hsa-miR-30b. The 14-mers exhibit better mismatch discrimination than the usually used 21-mers and are stable in cells (Fig. S6, ESI†). Therefore, they can potentially find applications in biological research for specific targeting of individual miRNAs in the pool of homologous miRNAs that could allow dissecting roles of these biomolecules in cells.

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