Research Article

A Novel Ultrasensitive Hybridization-Based ELISA Method for 2-Methoxyphosphorothiolate MicroRNAs and Its *In vitro* and *In vivo* Application

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Abstract. MicroRNAs (miRNAs) are endogenous, small non-coding RNAs that bind to target mRNAs and regulate their expression. Recent evidence has indicated the involvement of miRNAs in human malignancies. It has been suggested that aberrantly down-regulated or up-regulated miRNAs may be replaced with synthetic miRNAs or antagomiRNAs, respectively, and restore normal cell functions. As therapeutic development requires analytical support, we developed and validated an ultrasensitive and selective assay for quantification of synthetic 2'-methoxyphosphorothiolate-miRNA in mouse plasma and cell lysate for the first time. The method is based on a hybridization-ligation fluorescence enzyme-linked immunosorbent assay and has provided a linear dynamic range of 10-1,000,000 pM for three synthetic miRNAs both singly and in a mixture. The intra- and inter-day coefficients of variation were <20% and the accuracy values nearly 100%. Using this assay, we performed pharmacokinetic studies of three synthetic miRNAs in mice treated with a single i.v. bolus dose of 7.5 mg kg⁻¹. The 2-methoxyphosphorothiolate-miRNAs reached peak concentrations in the μ M and nM ranges in plasma and bone marrow, respectively, and remained measurable at 24 h. These concentrations are in a range that shows biological activities. We conclude that this method provides a general and valuable tool for the pharmacologic study and clinical development of synthetic miRNAs.

KEY WORDS: ELISA; 2'-MeOPSmRNAs; quantification; pharmacokinetics.

INTRODUCTION

Naturally occurring microRNAs (miRNAs) are 19-25 nucleotide (nt) transcripts cleaved from 70 to 100 nt hairpin primary precursors, which are encoded in the genomes of invertebrates, vertebrates, and plants (1). Although, the biological functions of miRNAs remain to be fully understood, for the most part, these non-coding RNAs seem to regulate protein

expression by either causing degradation or translation inhibition of the corresponding coding mRNA (2). Following earlier reports that link miRNA de-regulation in solid tumors and hematological malignancies, recent work indicates that miRNAs are involved in human tumorigenesis (3-12). A subset of miRNAs may act as oncogenes or tumor suppressors (4-7); for instance, the miRNA-17-92 clusters are up-regulated in diffused B-cell lymphomas and lung and stomach cancer (4,5). The ectopic expression of this family of miRNA induces proliferation, decreases apoptosis, and cooperates with c-Myc to develop lymphoma in mice (4). Over-expression of miRNA-155 has also been reported in high-grade B-cell malignancies, such as Burkitt's leukemia (13), B-cell lymphomas (14) and high risk chronic lymphocytic leukemia (CLL) (15,16), and causes acute leukemia in transgenic murine models (17). On the other hand, down-regulation of distinct miRNAs may also contribute to the malignant transformation by allowing re-expression of oncogenes that contribute to aggressive phenotypes. Cimmino *et al.* reported that miRNA-15a and miRNA-16-1, which are deleted or down-regulated in approximately 60% of B cell CLL (6), target the antiapoptotic gene BCL-2 (18). Similarly, the miRNA-29 family of miRNAs, including miRNA29a, b and c, is down-regulated in aggressive non-small cell lung cancer (7) and CLL (8). Further studies both by us and others have shown that these miRNAs target the oncogenes Tcl-1, Mcl-1, SP1, and DNMT3A and 3B (9,10,19,20). Indeed restoring miRNA-29

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expression induces apoptosis and hampers tumorigenesis in a xenograft model of lung cancer (10). Collectively, these data indicates that miRNAs might be potential therapeutic targets for synthetic RNA oligonucleotides that function as antagomiRNAs to down-regulate over-expressed endogenous miRNAs, or synthetic miRNAs to replace the under-expressed or depleted endogenous counterpart.

In order to move forward with these synthetic compounds to the clinic, a sensitive and specific analytical tool is needed for measurement of the drug levels in circulation and tissues, thereby allowing plasma pharmacokinetic characterization and tissue and intracellular distribution. The ultimate goal is to provide a detailed pharmacokinetic and pharmacodynamic relationship in animal models for dose-effect correlation and determination of therapeutic doses in the clinic.

With the anticipated low levels of these compounds, normal analytical methodologies, such as HPLC, and even capillary electrophoresis methods, are not likely to have adequate sensitivity. Several groups have described microarray methods for monitoring miRNA expression (21–26), and these efforts have provided a qualitative overview of miRNA expression patterns in cell lines, and in normal and diseased human tissues (5,9). Although gene expression microarrays and quantitative transcript-specific PCR assays have proven to be powerful tools for validating initial observations of their biological activities and their underlining mechanisms of endogenous miRNAs (16,27), none of these methods has been applied to characterize

the levels of synthetic miRNAs. Previously, we developed a hybridization-based fluorescence enzyme-linked immunosorbent assay (ELISA) methods for antisense oligonucleotides (28–30) with sensitivity limits at picomolar levels. Here, we have adapted the similar approach for the development of assay methods for synthetic miRNAs. As 2'-methoxy phosphorothioate (2'-MeOPS) oligonucleotides derivatives represent the most common structural modification for antisense oligonucleotides currently under clinical evaluation because of their stability and higher RNase activity, we selected the synthetic 2'-OMePS miRNAs and antagomiRNAs to demonstrate the applicability of such quantification assay and provide initial *in vivo* data. Because of our interest in these miRNAs as a potential therapeutic for the treatment of leukemia, these initial studies were conducted to characterize their preclinical pharmacokinetics and to assess the achievable concentrations in plasma, blood and bone marrow.

MATERIALS AND METHODS

Oligonucleotides, Reagents and Preparation of Buffers

The oligonucleotides and their sequences used for this study listed in the table below were purchased from Dharmacon Inc. (Lafayette, CO, USA), while the capture and detection probe were custom synthesized and acquired through Integrated DNA Technologies (Coralville, IA, USA).

Oligonucleotides	Sequence
2'-MeOPSmiRNA29b	5'-mU*mA*mG* mC*mA*mC* mC*mA*mU* mU*mU*mG* mA*mA*mA* mU*mC*mA* mG*mU*-3' (31)
Scrambled 2'-MeOPSmiRNA29b	5'-mA*mC*mG* mC*mA*mC* mT*mC*mA* mG *mC*mT* mA*mG*mT* mG*mA*mC* mC*mA*-3'
3'-N-1 2'-MeOPSmiRNA29b	5'-mU*mA*mG* mC* mA*mC* mC*mA*mU* mU*mU*mG* mA*mA*mA* mU*mC*mA* mG*-3'
3'-N-2 2'-MeOPSmiRNA29b	5'-mU*mA*mG* mC*mA*mC* mC*mA*mU* mU*mU*mG* mA*mA*mA* mU*mC*mA*-3'
3'-N-3 2'-MeOPSmiRNA29b	5'-mU*mA*mG* mC*mA*mC* mC*mA*mU* mU*mU*mG* mA*mA*mA* mU*mC*-3'
5'-N-1 2'-MeOPSmiRNA29b	5'-mA*mG* mC*mA*mC* mC*mA*mU* mU*mU*mG* mA*mA*mA* mU*mC*mA* mG*mU*-3'
2'-MeOPSmiRNA16-1	5'-mC*mG*mU*mU*mA*mG*mC*mC*mG*mU*mA*mC*mC*mG*mU* mU* mG*mA*mA*mU*mC*mC*-3'
3'-N-1 2'-MeOPSmiRNA16-1	5'-mC*mG*mU*mU*mA*mG*mC*mC*mG*mU*mA*mC*mC*mG*mU* mU* mG*mA*mA*mU*mC*-3'
3'-N-2 2'-MeOPSmiRNA16-1	5'-mC*mG*mU*mU*mA*mG*mC*mC*mG*mU*mA*mC*mC*mG*mU* mU* mG*mA*mA*mU*-3'
5'-N-1 2'-MeOPSmiRNA16-1	5'-mG*mU*mU*mA*mG*mC*mC*mG*mU*mA*mC*mC*mG*mU* mU* mG*mA*mA*mU*mC*mC*-3'
3'-MeOPSantago miRNA155	5'-mC*mC*mC*mC*mU*mA*mU*mC*mA*mC*mG*mA*mU*mU*mA*mG* mC*mA*mU*mU*mA*-3'
3'-N-1 2'-MeOPSantago miRNA155	5'-mC*mC*mC*mC*mU*mA*mU*mC*mA*mC*mG*mA*mU*mU*mA*mG* mC*mA*mU*mU*mA*-3'
3'-N-2 2'-MeOPSantago miRNA155	5'-mC*mC*mC*mC*mU*mA*mU*mC*mA*mC*mG*mA*mU*mU*mA*mG* mC*mA*mU*mU*-3'
5'-N-1 2'-MeOPSantago miRNA155	5'-mC*mC*mC*mU*mA*mU*mC*mA*mC*mG*mA*mU*mU*mA*mG* mC*mA*mU*mU*mA*-3'
Capture Probe for 2'-MeOPS miRNA29b	5'-TAAGTAGTG ACTGATTTCAAATGGTGATA-Biotin-3'
Capture Probe for 2'-MeOPS miRNA16-1	5'-TAAGTAGTG GGATTCAACGGTACGGCTAACG-Biotin-3'
Capture Probe for 2'-MeOPSantago miRNA155	5'-TAAGTAGTG GGAATGCTAATCGTGATAGGGG-Biotin-3'
Detection Probe	5'-CACTA GTTA-digoxigenin-3'

m = methoxy and * denotes phosphorothioate

The capture probe for 2'-MeOPSMiRNA-29b used in the two-step hybridization ELISA was designed as a 29mer DNA oligonucleotide with the first 20mer sequence from the 3'-end complementary to 2'-MeOPSMiRNA29b and the 3'-end was attached to a NeutrAvidin-coated 96-well plate via biotin. The 9mer overhang (5'-TAA CTA GTG-3') serves as a template for the detection probe. A 9-mer DNA phosphorothioate with digoxigenin at the 3'-end and a sequence complement to the 5'-end 9mer overhang of the capture probes for 2'-MeOPSMiRNA29b is used as an appropriate detection probe following the hybridization ligation reaction.

Similarly for 2'-MeOPSMiRNA16-1 and 2'-MeOPSantagomiRNA155, we have designed the respective capture probes as shown in the table above. The purity and identity of each oligomer was verified by HPLC-UV/Mass spectrometry as a single peak and matched molecular weight (Ion trap mass spectrometer Model: LCQ, Finnigan, San Jose, CA, USA).

2'-MeOPSMiRNA and antagomiRNA standards were diluted in Tris-HCl and EDTA (TE) buffer containing 10 mM Tris-HCl and 1 mM EDTA (pH=8.0). The hybridization buffer used in preparation of capture probe solution contained 60 mM sodium phosphate, pH 7.4, 1.0 M NaCl, 5 mM EDTA, and 0.2% Tween 20. The ligation buffer was prepared as a mixture of 66 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 5 U mL⁻¹ T4 DNA ligase (Amersham Biosciences, Piscataway, NJ, USA) and 100-nM detection probe oligonucleotide. The washing buffer used throughout the assay contained 25 mM Tris-HCl, pH=7.2, 0.15 M NaCl, and 0.2% Tween 20.

Cell Culture, Transfection, and Cell Lysate Preparation

MV4-11 and K562 cells were grown in RPMI1640 medium supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO₂ incubator. Harvested cells (10⁶ per aliquot) were lysed with 1 mL lysis buffer (10 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1.0% Triton-X 100) for ELISA. Synthetic 2'-OMePSmiRNA29b or miRNA16-1 at the indicated final concentrations, were introduced into cells by nucleoporation (Lonza, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Hybridization ELISA Procedure

This method is based on a two-step hybridization, first by base pairing 2'-MeOPSMiRNA(s) with the capture probe(s) with an overhang, followed by hybridization with a detection probe p-5'-TAA CTA GTG-digoxigenin-3', which is ligated to the analyte. The general procedure of the method is described as follows. Basically, 100 µL of the capture template solution (200 nM) was added to 100 µL 10% mouse plasma or 10% cell lysate diluted with TE buffer containing the 2'-MeOPSMiRNA, and the solution was mixed in a 96-well raised PCR plate (VWR International, Bridgeport, NJ, USA). Next, the mixture was incubated at 37°C for 2.5 h for hybridization. Then 150 µL of the solution was transferred to a NeutrAvidin-coated 96-well plate (Pierce, Rockford, IL, USA), which was incubated at 37°C for 30 min to allow the attachment of biotin labeled capture template to NeutrA-

vidin-coated wells. The plate was washed six times with washing buffer and 150 µL ligation solution containing 5 U mL⁻¹ T4 DNA ligase and 100 nM detection probe was added to each well. The plate was incubated overnight at 25°C. The plate was washed three times with washing buffer and three times with deionized (DI) water to remove the unligated detection probe. Following the addition of 60 U S1 nuclease (per well) solution in 100 mM NaCl, the plate was incubated at 37°C for 2 h to cleave the truncated duplex. After washing with DI water six times, the plate was blocked with 1:1 superblock buffer and antibody dilution buffer followed by addition of 150 µL anti-digoxigenin-AP (Roche, Indianapolis, IN, USA) diluted with 1:2,500 super BSA block buffer (Roche, Indianapolis, IN, USA) into each well. The plate was then incubated at room temperature for 0.5 h with gentle shaking. After washing six times with DI water, 150 µL substrate solution [36 mg Attophos in 60 mL diethanolamine buffer (Promega, Madison, WI, USA)] was added to each well, and the plate was incubated at 37°C for 0.5 h. Finally, fluorescence intensity was measured at Ex 430/Em 560 (filter=530 nm) using a Gemini XS fluorescence microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

Method Validation

The hybridization-ligation ELISA method for each of the 2'-MeOPSMiRNAs or 2'-MeOPSantagomiRNAs in 10% mouse plasma and in 10% K562 cell lysate was validated. Linearity at the concentration range between 10 and 5,000 pM was evaluated. Within-day and between-day precision and accuracy were determined at 10 pM (low quality control, QC), 20 pM (medium low, QC), 100 pM (median QC), and 2,000 pM (high QC) with six replicates in each matrix and reported as % coefficient of variation (CV) and percent from the spiked nominal concentrations, respectively. Since the drug concentration ranges in animal and cell samples are likely to exceed the upper limit of the calibration curve, extension of the dynamic range was evaluated by dilution. Mouse plasma (Innovation Research Inc., Southfield, MI, USA) spiked with 10, 50, and 200 nM 2'-MeOPSMiRNAs was diluted with 10% mouse plasma to 10, 100, 200, and 1,000-fold, respectively (*n*=6). These standards were assayed and the dilution recovery was calculated by the dilution factors. Additionally, as an alternate procedure for validation of the synthetic miRNAs in cell matrix, to simplify the procedure and to conserve the cell matrix, aliquots of these samples were diluted by appropriate volumes of 10% mouse plasma and treated as plasma samples, and a set of each was compared with those of the original matrices.

Stability of 2'-MeOPSMiRNAs

Only the stability of 2'-MeOPSMiRNA29b was investigated in detail. 2'-MeOPSMiRNA29b (1.5 µM) was incubated in heparin-pretreated mouse plasma separately at -20, 4, 25, 37°C, and 100 µL aliquots each of these samples were collected at 0, 30 min, 1, 2, 4, 8, and 24 h. These aliquots were stored in a -80°C freezer until analysis. All measurements were performed in triplicates.

Specificity and Selectivity of the Hybridization-Ligation ELISA

The specificity and selectivity of the ELISA was evaluated with the putative 3'-chain-shortened and 5'-N-1 metabolites and scrambled controls (scrambled oligonucleotides). The cross-reactivity of 3'-N-1, N-2, N-3 and 5'-N-1 putative metabolites and scrambled oligonucleotide of 2'-MeOPSmRNA29b at the concentration range between 5 pM and 10 nM in 10% mouse plasma in TE buffer was evaluated. The observed fluorescence-concentration profiles of these oligonucleotides were compared with those of the parent 2'-MeOPSmRNA29b. The cross-reactivity of each metabolite toward 2'-MeOPSmRNA29b was determined as the percentage of their EC₅₀ values to that of 2'-MeOPSmRNA29b. Similarly, the cross-reactivity of 3'-N-1, N-2, N-3 and 5'-N-1 putative metabolites and scrambled oligonucleotide of 2'-MeOPSmRNA16-1 and 2'-MeOPSantagomiRNA155 at the same concentration range as those of 2'-MeOPSmRNA29b in the same medium were also evaluated. All EC₅₀ values were calculated by the nonlinear regression model in SigmaPlot (SPSS, Chicago, IL, USA). All experiments were performed in duplicates.

Assay of 2'-MeOPSmRNAs in a Mixture Containing 2'-MeOPSmRNA29b, 2'-MeOPSmRNA16-1, and 2'-MeOPSantagomiRNA155

The procedure was basically the same as those for the single synthetic miRNAs. One hundred microliter of the individual capture template solution (200 nM, miRNA29b, miRNA16-1 or miRNA155 template solution) was added into 100 μ L 10% mouse plasma (cell lysate or tissue extracts) containing the mixture of 2'-MeOPSmRNA29b, 2'-MeOPSmRNA16-1, and 2'-MeOPSantagomiRNA155 and the solution was mixed in a 96-well plate (VWR International, Bridgeport, NJ, USA). Next, the mixture was incubated at 37°C for 2.5 h for hybridization. Then 150 μ L of the solution was transferred to a NeutrAvidin-coated 96-well plate as processed as described above for the single synthetic miRNAs. The calibration curves for the individual 2'-MeOPSmRNA29b, 2'-MeOPSmRNA16-1, and 2'-MeOPSantagomiRNA155 were made using 10% mouse plasma (also for cell lysate) spiked with a mixture of these miRNAs, each ranged between 10 and 3,000 pM. The individual synthetic miRNA concentrations in plasma or tissues were calculated by their respective calibration curves.

Method Validation for 2'-MeOPSmRNA29b, 2'-MeOPSmRNA16-1 and 2'-MeOPSantagomiRNA155 in Plasma as a Mixture

The hybridization-ligation ELISA method for each of 2'-MeOPSmRNA29b, 2'-MeOPSmRNA16-1 and 2'-MeOPSantagomiRNA155 as a mixture in 10% mouse plasma was also validated. Linearity at the concentration range between 10 and 3,000 pM was evaluated. Within-day and between-day precision and accuracy were determined at 50, 250, and 1,000 pM in six replicates each and reported as percentage

CV and percentage from the spiked nominal concentrations, respectively.

Pharmacokinetics of 2'-MeOPSmRNA29b, 2'-MeOPSmRNA16-1 and 2'-MeOPSantagomiRNA155 in Mice

C57BL/6 mice (~20 g; Harlan, Indianapolis, IN, USA) were used in this study. All animal procedures were performed according to a protocol in compliance with The Ohio State University Laboratory Animal Resources policies, which also adheres to the guidelines as defined in Principles of Laboratory Animal Care by the National Institutes of Health. For intravenous bolus administration, approximately 100 μ L (adjusted by body weight) 2'-MeOPSmRNA29b was dissolved in sterile normal saline as a 2 mg mL⁻¹ solution was injected through the tail vein resulting in an intravenous bolus dose of 7.5 mg kg⁻¹. The blood was removed by cardiac puncture under CO₂ anesthesia at the time schedule of 0 (pre-dose), 0.08, 0.15, 0.25, 0.5, 1, 2, 4, 7, and 24 h after dosing and was mixed with 3% (v/v) sodium heparin. The blood samples were centrifuged at 1,000 g for 5 min, and the supernatant and peripheral blood cell (PBC) of each were collected and kept at -80°C until analysis. The bone marrow (BM) samples of these mice were also collected at 4, 7, and 24 h. These samples were processed to remove blood contamination and membrane-bound oligonucleotides according to our previous studies with Bcl-2 antisense oligonucleotides G3139 (28). Since the yield for the purified BM from the mouse was rather low, in order to cover a more extensive time period, for the subsequent studies with 2'-MeOPSmRNA16-1 and 2'-MeOPSantagomiRNA155, the levels of the oligonucleotide in the collected BM samples (without further processing) were measured and the BM samples were collected at 15, 30 min, 1, 2, 8, 22, and 24 h for 2'-MeOPSmRNA16-1, and 15 min, 2, 8, 22, and 24 h for 2'-MeOPSantagomiRNA155. For the processed BM, PBC, and plasma samples, they were diluted properly based on our previous pharmacokinetics study of anti-sense oligonucleotides and these diluted samples were analyzed according to the procedures for plasma and cell lysate (28). 2'-MeOPSmRNAs and 2'-MeOPSantagomiRNAs levels in plasma, in PBC (only for 2'-MeOPSmRNA29b) and in BM were measured using the aforementioned ELISA. Protein levels in PBC lysate and BM samples were determined with BCA assay (Pierce), and were used to normalize the miRNA concentrations in BM (pg μ g⁻¹ protein) and to convert miRNA levels in PBC to nM. The latter utilized a conversion factor of 2 $\times$ 10⁶ cells to 1 μ L cell volume or 1 μ g protein to 0.035 μ L cell volume (28). Plasma and PBC concentration-time data were analyzed by WinNonlin computer software (Pharsight 5.0, Mountain View, CA, USA) using appropriate pharmacokinetic models.

Quantitative RT-PCR

Transfected cells were harvested 24 h after transfection and the total RNA was isolated by Trizol (Invitrogen). DNMT3a, DNMT3b, and Bcl2 mRNA level was detected by using TaqMan gene expression assays with GAPDH as the internal control. Reactions were carried out in triplicate.

Relative expression was calculated by the comparative Ct method.

Western Blot

Transfected cells were harvested and lysed 48 h after transfection. Proteins were separated by SDS-PAGE gel, transferred to a nitrocellulose membrane and analyzed by immunoblotting. Antibodies to DNMT3a and 3b were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), to DNMT1 from New England BioLabs (Ipswich, MA, USA), to Bcl2 from Cell Signaling Technology (Danvers, MA, USA) and to loading control β -actin from Sigma (St. Louis, MO, USA).

RESULTS

Method Development and Validation

A previously established two-step hybridization-ligation ELISA method for DNA antisense oligonucleotide compounds (28,29) was adapted to quantify 2'-MeOPSmRNAs and 2'-MeOPSantagomiRNA in mouse plasma and leukemia cell lysate. The principle of the method has been documented previously (28,29,32). The linearity of this method for quantification of 2'-MeOPSmRNA29b was initially evaluated in TE buffer (data not shown), and then in 10% mouse plasma and 10% K562 cell lysate. The assay was indeed found to be linear in these matrices in a range between 10 and 5,000 pM with a regression coefficient >0.990 . The upper limit for the calibration curve was set at 5,000 pM, as above this value the fluorescence response reached a plateau in both plasma and cell lysate. At concentrations $\geq 5,000$ pM, serial dilution with 10% plasma in TE buffer could extend the linear dynamic range to 1,000,000 pM, with a mean dilution recovery value of $96.2 \pm 11\%$ ($n=15$). Therefore, the linear dynamic range of the assay could be extended from 10 to 1,000,000 pM with serial dilution without loss of precision and accuracy. The lowest limit of detection was found to be 5 pM and the lowest limit of quantification (LLOQ) was found to be 10 pM following blank subtraction, respectively. No interference from the endogenous miRNAs with the assay was found. Similar characteristics were found for 2'-MeOP-

SmRNA16-1 and 2'-MeOPSantomiRNA155 (data not shown).

The precision and accuracy of the method were then determined. The within-day precision for 2'-MeOPSmRNA29b were found to be 15.4%, 6.6%, 14.9%, and 11.0% CV and accuracy values were found to be 110%, 97.6%, 104%, and 96.0% ($n=6$) at 10, 20, 100, and 2,000 pM, respectively, in 10% mouse plasma (Table I). The between-day precision values were found to be 22.8%, 18.1%, 5.3%, and 4.0% CV and accuracy values were 105.9%, 93.85%, 87.2%, and 93.5% at 10, 20, 100, and 2,000 pM in 10% mouse plasma, respectively.

For 2'-MeOPSmRNA16-1, the within-day precision values were determined to be 18.0%, 9.5%, 3.3%, and 7.2% CV and accuracy values to be 90.5%, 96.5%, 102.1%, and 96.6% ($n=6$) at 10, 20, 100, and 2,000 pM, respectively, in 10% mouse plasma (Table I). The between-day precision values were 22.7%, 20.2%, 7.9%, and 7.7% CV and accuracy values were 99.7%, 107.0%, 112.5%, and 96.4% at 10, 20, 100, and 2,000 pM in 10% mouse plasma, respectively.

For 2'-MeOPSantagomiRNA155, the within-day precision values were 11.0%, 9.2%, 8.5%, and 15.0% CV and accuracy values were 98.4%, 114.8%, 114.8, and 97.4% ($n=6$) at 10, 20, 100, and 2,000 pM, respectively, in 10% mouse plasma (Table I). The between-day precision values were 21.6%, 13.0%, 7.5%, and 6.1% CV and accuracy values were 101.8%, 88.1%, 105.7%, and 103.5% at 10, 20, 100, and 2,000 pM in 10% mouse plasma, respectively.

In 10% K562 cell lysate, the within-day precision values for 2'-MeOPSmRNA29b were found to be 6.32%, 8.57%, 6.30%, and 3.43% and accuracy values were 81%, 93%, 107%, and 99% at 10, 50, 500, and 5,000 pM, respectively (Table II). The between-day precision values were 19.9%, 12.9%, 10.6%, and 6.2% CV and accuracy values were 107.8%, 105.7%, 115%, and 103% at 10, 50, 500, and 5,000 pM in 10% cell lysate, respectively.

As an alternative procedure to using pure cell lysate for validation of these oligonucleotides in the cell lysate matrices, we found that the cell lysate samples could be treated as plasma samples with an appropriate dilution of the initial cell lysate samples with 10% mouse plasma with no additional interference or compromised assay validation characteristics. Therefore, for 2'-MeOPSmRNA16-1 and 2'-MeOPSantago-

Table I. Within-run and between-run Validation Parameters of 2'-MeOPSmRNAs Diluted in 10% Mouse Plasma (all $n=6$)

Concentrations, pM	Analytes	Within-day		Between-day	
		CV%	Accuracy%	CV%	Accuracy%
10	2'-MeOPSmRNA29b	15.4	109.8	22.8	105.9
	2'-MeOPSmRNA16-1	18.0	90.5	22.7	99.7
	2'-MeOPSantagomiRNA155	11.0	98.4	21.6	93.0
20	2'-MeOPSmRNA29b	6.6	97.6	18.1	93.9
	2'-MeOPSmRNA16-1	9.5	96.5	20.2	107.0
	2'-MeOPSantagomiRNA155	9.20	114.8	13.04	88.1
100	2'-MeOPSmRNA29b	14.9	104.3	5.3	87.2
	2'-MeOPSmRNA16-1	3.31	102.1	7.88	112.5
	2'-MeOPSantagomiRNA155	8.48	114.8	7.46	105.7
2000	2'-MeOPSmRNA29b	11.0	96.0	4.0	93.5
	2'-MeOPSmRNA16-1	7.2	96.6	7.7	96.4
	2'-MeOPSantagomiRNA155	14.95	97.4	6.06	103.5

Table II. Within-day and between-day Validation Parameters of 2'-MeOPSmRNA29b in 10% K562 Cell Lysates (all $n=6$)

Conc. (pM)	Within-day CV%	Within-day Accuracy%	Between-day CV%	Between-day Accuracy%
10	6.3	81.3	19.9	107.8
50	8.6	93.0	12.9	105.7
500	6.3	107.4	10.6	115.0
5000	3.4	98.9	6.2	103.1

miRNA155 in K562 cell lysates, they were validated as plasma samples and the results were similar to those in plasma (data not shown).

Method Validation for 2'-MeOPSmRNA29b, 2'-MeOPSmRNA16-1 and 2'-MeOPSantagomiRNA155 in Plasma as a Mixture

No interference among the three synthetic miRNAs was found when they were spiked in plasma as a mixture and the background was very low, similar to the single synthetic miRNAs assay. The assay for the individual synthetic miRNAs was linear from 10 to 2,000 pM monitored (data not shown). The within-day validation data for the individual synthetic miRNAs are shown in Table III. As shown, the within-day coefficients of variation and accuracy values for the individual assays of 2'-MeOPSmRNA29b, 2'-MeOPSmRNA16-1 and 2'-MeOPSantagomiRNA155 as a mixture in plasma were similar to those as single components.

Selectivity

miRNAs are endogenous small RNAs existing in precursor and mature forms intracellularly, and as circulating nucleic acid in human serum (33) and plasma (34). The endogenous miRNAs, therefore, may represent a potential source of interference in an analytical system. Therefore, we examined our methods for interferences from endogenous miRNAs and from scrambled oligonucleotides. The fluorescence responses from blank cell matrices and plasma evaluated were found to be negligible and showed no difference from that of the PBS control, indicating a lack of interference from endogenous substances. Furthermore, the scrambled 2'-MeOPSmRNA oligonucleotides for all two 2'-MeOPSmRNA and the 2'-MeOPSantagomiRNA did not seem to

compete with the analytes in plasma as shown by their negligible fluorescence signal (Fig. 1), thereby supporting the selectivity of our assay.

Next, we examined the cross-reactivity of the assay with chain-shortened metabolites. The hybridization-ligation ELISA demonstrated its selectivity toward the parent analyte. Compared to the concentration-response curve of 2'-MeOPSmRNA29b, its putative 3'-N-1 metabolites gave significantly lower fluorescence intensity. The cross-reactivity value for 3'-N-1 2'-MeOPSmRNA29b was determined to be 2.2% (Fig. 1a). No significant cross-reactivity with 3'-N-2 and 3'-N-3 putative metabolites or with the scrambled 2'-MeOPSmRNA29b was observed (Fig. 1a). Thus, the assay is considered single nucleotide resolution at the 3'-end. However, the cross-reactivity of the assay with the less likely putative 5'-N-1 metabolite was about 90% (Fig. 1a); therefore, this method is considered highly selective, but not specific.

Similarly, the selectivity studies of 2'-MeOPSmRNA16-1 and 2'-MeOPSantagomiRNA155 with their respective putative chain-shortened metabolites indicated a low cross-reactivity with the 3'-N-1 (0.08% and 6.0%, respectively) and an insignificant overlap for 3'-N-2 and 3'-N-3 (all <0.1%) oligonucleotides was found (Fig. 1b and c). However, as was the case with 2'-MeOPSmRNA29b, the cross-reactivity of 2'-MeOPSmRNA16-1 and 2'-MeOPSantagomiRNA155, with the less likely 5'-N-1 metabolites, was 95% and 88%, respectively (Fig. 1b and c).

Assay of Specific 2'-MeOPSmRNAs in a Mixture. We have shown that synthetic miRNAs may be assayed as a single entity or in a mixture without cross-reactivity with each other. Figure 2 demonstrated that using the specific individual capture templates, 2'-MeOPSmRNA29b (Fig. 2a), 2'-MeOPSmRNA16-1 (Fig. 2b), and 2'-MeOPSantagomiRNA155

Table III. Within-run and between-run Validation Parameters of 2'-MeOPSmRNAs in a Mixture in 10% Mouse Plasma (all $n=6$)

Concentrations, pM	Analytes	Within-day		Between-day	
		CV%	Accuracy%	CV%	Accuracy%
50	2'-MeOPSmRNA29b	15.9	103.3	20.9	101
	2'-MeOPSmRNA16-1	25.7	103.7	23.8	121.4
	2'-MeOPSantagomiRNA155	10.8	96.8	27.1	114.4
250	2'-MeOPSmRNA29b	3.4	104.0	2.5	99.9
	2'-MeOPSmRNA16-1	8.0	90.9	6.1	104.4
	2'-MeOPSantagomiRNA155	4.8	99.0	6.5	98.6
1000	2'-MeOPSmRNA29b	3.3	101	6.4	99.7
	2'-MeOPSmRNA16-1	3.3	92.6	6.7	100.6
	2'-MeOPSantagomiRNA155	12.4	109.0	5.9	100.4

Fig. 1. **a** Cross-reactivity of putative metabolites (5'-N-1, 3'-N-1, 3'-N-2, and 3'-N-3), and scrambled 2'-MeOPSMiRNA29b with 2'-MeOPSMiRNA29b. The small insert showed the cross-reactivity at low concentrations; **b** cross-reactivity of putative metabolites (5'-N-1, 3'-N-1, 3'-N-2, and 3'-N-3), and scrambled 2'-MeOPSMiRNA16-1 with 2'-MeOPSMiRNA16-1; **c** cross-reactivity of putative metabolites (5'-N-1, 3'-N-1, 3'-N-2, and 3'-N-3), and scrambled 2'-MeOPSantagomiRNA155 with 2'-MeOPSantagomiRNA155. The symbols represent mean data of duplicate determinations

(Fig. 2c) could be quantified individually in a mixture and showed no appreciable cross-reactivity with each other. Additionally, the good accuracy data in the mixture (Table III) comparable to those of single synthetic miRNAs (Table I) also supports the selectivity of the assay. These data,

coupled to the lack of cross-reactivity with the 3'-chained shortened metabolite, indicates that the assay is highly selective and able to distinguish and quantify different synthetic miRNAs with similar sequences.

Stability of 2'-MeOPSMiRNA29b in Mouse Plasma

The stability of 2'-MeOPSMiRNA29b was evaluated in PBS and in heparin-treated mouse plasma. Although 2'-MeOPSMiRNA29b was found to be quite stable in PBS for 24 h (data not shown), it decomposed biexponentially in heparinized-treated mouse plasma in a temperature dependent manner. At -20°C , the 24 h level of 2'-MeOPSMiRNA29b showed no statistical difference with the initial concentration;

Fig. 2. Assay of Specific miRNAs in a mixture. **a** Cross-reactivity of 2'-MeOPSMiRNA29b in a mixture of three miRNAs; **b** cross-reactivity of 2'-MeOPSMiRNA16-1 in a mixture of three miRNAs; **c** cross-reactivity of 2'-MeOPSantagomiRNA155 in a mixture of three miRNAs. The symbols represent data of a single run

however, at all other temperatures, the 24 h levels all showed significant decline compared with the initial level ($p < 0.001$). Therefore 2'-MeOPSmRNA29b is considered stable at -20°C . However, at other temperatures evaluated, there was an initial steep decline of 2'-MeOPSmRNA29b to 60 min followed by a slower decline with half-lives of 326, 84, and 86 h at 4°C , 25°C , and 37°C , respectively. The nature of degradation is not clear so is the degradation kinetics at this time (Fig. 3).

Pharmacokinetics of 2'-MeOPSmRNA29b in Mice

Using the ultra-sensitive ELISA method, the pharmacokinetics of 2'-MeOPSmRNA29b was investigated in C57BL/6 mice following an i.v bolus dose at 7.5 mg kg^{-1} formulated in saline as a 2 mg mL^{-1} solution. As shown in Fig. 4a, plasma concentrations of 2'-MeOPSmRNA29b reached $\sim 1.9 \mu\text{M}$ at 5 min and declined biexponentially with time to $\sim 0.0013 \mu\text{M}$ at 24 h post-dose. Analysis of the data with a two-compartment model showed the initial half-life of 44.29 min, the terminal half-life of 8.5 h, AUC $115.1 \text{ min } \mu\text{M}$, the total body clearance (CL) $0.087 \text{ mL min}^{-1} \text{ kg}^{-1}$ and the volume of distribution at steady-state 8.9 mL kg^{-1} (Table IV). Interestingly, the 2'-MeOPSmRNA29b levels in PBC were measurable and reached the C_{max} of $1.67 \mu\text{M}$ at 5 min then declined over time to $\sim 0.0025 \mu\text{M}$ at 24 h (Fig. 4b). Comparison of 2'-MeOPSmRNA29b plasma and PBC levels indicated that plasma levels were always higher during the first 7 h after dosing (Fig. 4b inset), suggesting the existence of a lower uptake gradient for 2'-MeOPSmRNA29b in PBC. More importantly and germane to our future studies in leukemia patients, 2'-MeOPSmRNA29b was also taken up in bone marrow, where it achieved a concentration of $0.31 \text{ pg } \mu\text{g}^{-1}$ protein ($0.0020 \mu\text{M}$) at 4 h, peaked at $1.73 \text{ pg } \mu\text{g}^{-1}$ protein ($0.0072 \mu\text{M}$) at 7 h, and then decreased to $0.76 \text{ pg } \mu\text{g}^{-1}$ protein ($0.0036 \mu\text{M}$) at 24 h (Fig. 4c). Of note, the synthetic miRNAs in bone marrow at 24 h was still higher than the concentration observed in PBC and plasma at the same time point.

Pharmacokinetics of 2'-MeOPSmRNA16-1 in Mice

The pharmacokinetics of 2'-MeOPSmRNA16-1 was also investigated in C57BL/6 mice following an i.v bolus dose at 7.5 mg kg^{-1} formulated in saline as a 2 mg mL^{-1} solution. As shown in Fig. 5, plasma concentrations of 2'-MeOPSmRNA16-1

Fig. 3. The stability profiles of 2'-MeOPSmRNA29b in heparin-treated mouse plasma at different temperatures. The symbols represent mean data with standard deviations indicated in the upper or lower bars

Fig. 4. **a** Plasma concentration-time profile of 2'-MeOPSmRNA29b in mice following an intravenous bolus dose at 7.5 mg kg^{-1} . Squares represent mean of measured concentrations, upper and lower bars represent SD with $n=6$, and the line represents the fitted curve to a two-compartment model with instantaneous input. **b** Intracellular concentration-time profiles of 2'-MeOPSmRNA29b in peripheral blood cells and **c** bone marrow in mice following an intravenous bolus dose at 7.5 mg kg^{-1} . The small insert compares the level of 2'-MeOPSmRNA29b in plasma and PBC at earlier time points

reached $\sim 6 \mu\text{M}$ at 5 min and declined biexponentially with time to $\sim 0.007 \mu\text{M}$ at 24 h post-dose. Analysis of the data by a two-compartment model showed an initial half-life of 19 min and the terminal half-life of 16 h, AUC $144.7 \text{ min } \mu\text{M}$, the CL of $0.01 \text{ mL min}^{-1} \text{ kg}^{-1}$ and the volume of distribution at steady-state 1.97 mL kg^{-1} (Table IV). Also, 2'-MeOPSmRNA16-1 was found to be taken up in bone marrow, where it achieved a concentration of $1.9 \text{ pg } \mu\text{g}^{-1}$ protein at 0.25 h, increased to $2.9 \text{ pg } \mu\text{g}^{-1}$ protein at 2 h, and then continued to climb and sustained at $3.7 \text{ pg } \mu\text{g}^{-1}$ protein ($0.0154 \mu\text{M}$) at 24 h (Fig. 5b). The latter was higher than the concentration observed in plasma at the same time point ($0.067 \mu\text{M}$).

Table IV. Relevant Pharmacokinetic Parameters of 2'-OMePSmiRNA29b, 2'-OMePSmiRNA16-1 and 2'-OMePSantagomiRNA155 in Mice Following Their Separate i.v. Dosing at 7.5 mg/kg Each

PK Parameter	Units	Analytes	Estimate	CV (%)
C_0	μM	2'-MeOPSmiRNA16-1	4.2	26.7
		2'-MeOPSmiRNA29b	1.7	11.7
		2'-MeOPSantagomiRNA155	5.2	28.9
Alpha	Min^{-1}	2'-MeOPSmiRNA16-1	0.04	15.9
		2'-MeOPSmiRNA29b	0.0157	8.9
		2'-MeOPSantagomiRNA155	0.076	14.8
Beta	Min^{-1}	2'-MeOPSmiRNA16-1	0.000104	61.1
		2'-MeOPSmiRNA29b	0.00137	36.1
		2'-MeOPSantagomiRNA155	0.0012	25.9
Alpha-HL	Min	2'-MeOPSmiRNA16-1	19.3	15.9
		2'-MeOPSmiRNA29b	44.3	NA
		2'-MeOPSantagomiRNA155	9.17	NA
Beta-HL	Min	2'-MeOPSmiRNA16-1	975.	61.1
		2'-MeOPSmiRNA29b	507.	NA
		2'-MeOPSantagomiRNA155	561.	NA
AUC	$\text{Min} \times \mu\text{M}$	2'-MeOPSmiRNA16-1	145.	16.9
		2'-MeOPSmiRNA29b	115.	8.3
		2'-MeOPSantagomiRNA155	87.2	16.2
CL	$\text{l min}^{-1} \text{ kg}^{-1}$	2'-MeOPSmiRNA16-1	0.01	16.9
		2'-MeOPSmiRNA29b	0.087	8.5
		2'-MeOPSantagomiRNA155	0.012	16.3
V_{ss}	l kg^{-1}	2'-MeOPSmiRNA16-1	1.97	73.9
		2'-MeOPSmiRNA29b	8.93	15.5
		2'-MeOPSantagomiRNA155	2.13	41.1

C_0 initial concentration, α first exponential decay rate constant, β second exponential decay rate constant, HL half-life, CL total body clearance, AUC area under the curve, V_{ss} volume of distribution at steady-state

Fig. 5. Plasma concentration-time profile (a) and bone marrow levels (b) of 2'-MeOPSmiRNA16-1 in mice following an i.v. bolus dose at 7.5 mg kg⁻¹. Square symbols with error bars show mean $\pm$ SD, $n=6$ and the line represents fitted curve to a two-compartment model

Pharmacokinetics of 2'-MeOPSantagomiRNA155 in Mice

The pharmacokinetics of 2'-MeOPSantagomiRNA155 in C57BL/6 mice following an i.v. bolus dose at 7.5 mg kg⁻¹ displayed a similar two compartment kinetics (Fig. 6). Plasma concentrations of 2'-MeOPSantagomiRNA155 reached $\sim 5 \mu\text{M}$ at 5 min and declined biexponentially with time to $\sim 0.005 \mu\text{M}$ at 24 h post-dose. Analysis of the data with a two-compartment model showed an initial half-life of 9.7 min and the terminal half-life of 9.4 h, AUC 87.2 min $\times\mu\text{M}$, the CL of 0.012 mL min⁻¹ kg⁻¹ and the volume of distribution at steady-state of 2.13 mL kg⁻¹ (Table IV). 2'-MeOPSantagomiRNA155 was also found to be taken up in bone marrow, where it achieved a concentration of 1.3

Picogram per microgram protein (0.0054 μM) at 0.25 h, peaked at 2.3 pg μg^{-1} protein at 2 h, and then decreased to 1.6 pg μg^{-1} protein (0.0067 μM) at 24 h (Fig. 6b). The latter was higher than the observed concentration in plasma at the same time point (0.0041 μM).

Thus, pharmacokinetics of these three synthetic miRNAs and antagomiRNA generally show similar characteristics but with some differences in plasma half-lives. With this assay, these oligonucleotides were detectable in circulation and in bone marrow up to the monitored period of 24 h.

Synthetic 2'-MeOPSmiRNA29b and 2'-MeOPSmiRNA16-1 Down-regulate Their Bio-targets in Leukemia Cells

To confirm that synthetic 2'-MeOPSmiRNAs are biologically functional, we transfected 2'-MeOPSmiRNA29b and 2'-MeOPSmiR16-1 into MV4-11 cell line and assessed mRNA

Fig. 6. Plasma concentration-time profile (a) and bone marrow levels (b) of 2'-MeOPSantagomiRNA155 in mice following an i.v. bolus dose at 7.5 mg kg⁻¹. Square symbols with error bars show mean ± SD, *n*=6, and the line represents fitted curve to a two-compartment model

and protein levels of several known targets of miRNA29b and miRNA16-1. Transfection of 2'-MeOPSMiRNA29b significantly decreases levels of DNMT3a and DNMT3b (Fig. 7a and b). Similarly, transfection of 2'-MeOPSMiRNA16-1, reduces endogenous Bcl2 mRNA and protein levels in MV4-11 cells (Fig. 7c and d).

DISCUSSION

To date, microarray (4,7–9,18,19,21–23,25,35,36), northern blot analysis (6,18,24,37), and RT-PCR (7,11,16,27,38,39) have been reported for screening endogenous miRNAs. However, none of these methods were used to characterize exogenous, synthetic miRNAs. Furthermore, hybridization-based ELISA methods have been successfully developed for the determination of polynucleotides in biological matrices (28–30,32,40–42). However, these ELISA methods have not been applied to quantify endogenous miRNAs and their applicability for measuring these endogenous substances remains uncertain because of the extremely low endogenous miRNA levels. The current results demonstrated that the ELISA was unable to detect the endogenous miRNAs.

Previously, using the hybridization-based ELISA method, we have successfully developed highly sensitive methods for quantification of two antisense DNA oligonucleotides (i.e., G3139 and GTI-2040) tested in the clinic (28–30). The current method differs from the earlier methods, which were designed for DNA oligonucleotides, in several

ways. First, we successfully adapted this approach to the detection and quantification of structurally modified RNA oligonucleotides such as 2'-MeOPSMiRNA29b, 2'-MeOPSMiRNA16-1, and 2'-MeOPSantagomiRNA155. Second, we designed and selected a detection probe, which is different from the earlier reports (29,32), and found it to be superior in robustness and sensitivity for the ELISA method and first tested for GTI-2040 antisense (28). Subsequently, we applied this probe for the current studies and several other antisense and synthetic miRNAs and found similar excellent characteristics (data not shown). From this detection probe, we designed appropriate capture probes with its sequence complementary to each of the synthetic miRNAs with a common 3' 9-mer overhang with its sequence complementary to that of 9-mer detection probe and 5'-end digoxigenin (Dig-label). We have found that both these two 2'-MeOPSMiRNAs and a 2'-MeOPSantagomiRNA were able to effectively hybridize to the designed capture probe and ligate to the detection probe in the presence of T4 ligase and ATP. These formed well bound duplexes (e.g., 29-mer duplex for 2'-MeOPSMiRNA29b), which were resistant to the S1 digestion and were subsequently detected by the dig fluorescence system. The third major modifications were the use of optimal concentration of the S1 nuclease and other conditions, which has been discussed in our recent publication (28).

Although no metabolism study for synthetic miRNAs has been published, it is anticipated that, *in vitro* and *in vivo*, the 3'-end chain of 2'-MeOPSMiRNAs may be subjected to 3'-exonucleases similar to antisense compounds to generate chain-shortened metabolites. These potential metabolites may interfere with the quantification of 2'-MeOPSMiRNAs. Therefore, the cross-activity of these 2'-MeOPSMiRNAs and 2'-MeOPSantagomiRNA with their putative metabolites 3'-N-1, 3'-N-2, and 3'-N-3 was tested. We found that only the parent 2'-MeOPSMiRNAs and 2'-MeOPSantagomiRNA have a rather low cross-activity with the 3'-N-1, 3'-N-2, and 3'-N-3 putative metabolites. On the other hand, the assays show a high (about 90%) cross-reactivity with the 5'-N-1 putative metabolite. However, this may not pose a significant problem in practice, since 5'-end metabolites for antisense compounds are commonly known to form only at very low levels (28,29,43–45).

Therapeutically, it is anticipated that synthetic miRNAs may be used in combination with each other or with small molecules. While the assay platform will not be interfered by small molecules, the ability to distinguish with other synthetic miRNAs must be demonstrated. To this end, using a mixture of these three synthetic miRNAs, we have demonstrated that individual synthetic miRNAs were quantifiable with the similar assay characteristics as the single synthetic miRNAs.

Assay Validation in Cell Lysates

In the cell lysate matrix, we did not find any interference from endogenous substance with the assay of antisense compounds or with 2'-MeOPSMiRNA29b and we have satisfactorily validated 2'-MeOPSMiRNA29b in K562 cell lysate. We have also found that cell lysate can be substituted with plasma by dilution of the cell lysate with plasma. The resulting sample was conveniently treated as the plasma sample and the calibration results were essentially similar to

Fig. 7. Synthetic 2'-MeOPSmiRNA29b and 2'-MeOPSmiRNA16-1 decrease their corresponding targets at both mRNA and protein levels in MV4-11 cells. **a** DNMT3a and DNMT3b mRNA expression 24 h after nucleoporation of serial concentrations (30, 100, 500, and 1,000 nM) of synthetic miR29b in MV4-11 cells. Histograms show the relative ratio of DNMTs mRNA over the internal control of GAPDH. **b** The protein levels of DNMT3a and DNMT3b in 2'-MeOPSmiRNA29b-transfected MV4-11 cells. Cells exposed to 2.5 μ M decitabine (dec) for 24 h was used as the positive control. **c** Bcl2 mRNA levels after nucleoporation of synthetic 2'-MeOPSmiRNA16-1 (3, 10, 30 nM) into MV4-11 cells. Histograms show the relative ratio of Bcl2 mRNA over GAPDH. **d** Bcl2 protein expression in 2'-MeOPSmiRNA16-1-transfected MV4-11 cells ($*p < 0.05$, $**p < 0.01$) as compared with vehicle-transfected samples. Densitometry was performed to quantify each western blot lane and the ratio of each target protein over loading control β -actin is presented under the blots

those of cell matrix alone. Thus, we have adapted this modified procedure for the validation of 2'-MeOPSmiRNA16-1 and 2'-MeOPSantagomiRNA155 in K562 cell lysate using a plasma dilution method and the results were similar to those in cell lysate alone. This is especially important in clinical situations, where the source of untreated (control) cells is extremely limited for use as calibration purposes and now the cell samples may be determined by plasma dilution method.

Following an evaluation of selectivity, the method was validated and the results were found to meet the commonly accepted validation criteria (45). Therefore, the validated hybridization-ligation ELISA method was applied to *in vivo* pharmacokinetic studies for the first time. For the pharmacokinetic characterization of 2'-MeOPSmiRNA29b in C57BL/6 mice, plasma concentrations of 2'-MeOPSmiRNA29b were measured up to 24 h after an intravenous bolus of a 7.5 mg kg⁻¹ single dose and the mean terminal half-life of this oligonucleotide was determined to be about 8 h. The observed highest mean plasma concentration was nearly 2 μ M, which declined to 1.3 nM at 24 h. Before 6 h, the concentrations were well above 20 nM, which is the concentration range previously used for transfection experiments. Mott *et al.* found that cholangiocarcinoma

KMCH cells transfected with 20-50 nM miRNA29b precursors showed a decreased Mcl-1 reactivity (19,46,47). Mersey *et al.* reported that when HEK293 cells were transfected with as little as 0.2 nmol of miRNA29b, they were able to achieve BCKD activity in releasing CO₂ for over 24 h (48-51). Fabbri *et al.* transfected A549 and H1299 cells with 50 nM miRNA29b to target DNMTs and found an increased expression of hypermethylated FHIT and WWOX genes (48-51). Therefore, the achievable *in vivo* microRNA concentrations in our experiments appear to be at or above the active concentration range of several microRNAs previously reported to be active *in vitro*. We performed additional *in vitro* experiments with 2'-MeOPSmiRNA29b and 2'-MeOPSmiRNA16-1 as proof of principle. Our results showed that the two synthetic miRNAs could down-regulate their corresponding biomarkers, such as DNMT3a, 3b and Bcl2, at nM to μ M range in human leukemia cell lines.

Finally, our first *in vivo* studies with these 2'-MeOPSmiRNAs and 2'-MeOPSantagomiRNA have demonstrated that, at a dose as low as 7.5 mg kg⁻¹, micromolar concentrations of the synthetic microRNA in circulation can be achieved with no apparent toxicity. This dose is tenfold lower than the doses used for other cholesterol-modified synthetic RNAs or antagomiRs (>80 mg kg⁻¹) previously

reported (48,51,52). Further, this concentration range has been shown to have biological activity *in vitro* by others as well as by us. If higher concentrations are needed, higher doses could be used. This data may be important to serve as a guide for future planning of large scale preclinical pharmacology, *in vivo* efficacy, and toxicology studies of synthetic microRNAs.

CONCLUSION

A fluorescence hybridization-ligation ELISA assay for quantification of 2'-MeOPSmRNA29b, 2'-MeOPSmRNA16-1 and 2'-MeOPSantagomiRNA155 singly and in a mixture in mouse plasma and human leukemia cell lysate has been developed and validated. Our method represents the first quantification method for exogenous microRNAs and is ultra-sensitive with an LLOQ of 10 pM with acceptable precision and accuracy. This assay has also been successfully applied to measure plasma and bone marrow levels of 2'-MeOPSmRNA29b, 2'-MeOPSmRNA16-1 and 2'-MeOPSantagomiRNA155 in mice for the first time, following their separate intravenous administrations at a bolus dose of 7.5 mg kg⁻¹. The pharmacokinetics of 2'-MeOPSmRNAs and 2'-MeOPSantagomiRNAs showed measurable plasma and bone marrow concentrations and a terminal half-life comparable to those for currently clinically used antisense drugs and these concentration ranges are biologically active *in vitro*. With the design of appropriate capture and detection probes, this assay may be universally applicable in the quantification of exogenous synthetic miRNAs and antagomiRNAs in a variety of biological matrices, as our method has been successfully applied to two 2-MeOPSmRNAs and 2-MeOPSantagomiR. Additionally, it is expected that this ELISA method will be a valuable tool for pharmacokinetics and pharmacodynamics studies which will guide the future development of this class of therapeutic agents.

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Conflict of Interest Statement A provisional US patent application for the analytical method has been filed by some of the authors (KKC, ZL, ZX, SL, RG, CMC, JCB, NM, and GM) through the Ohio State University.

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