Pharmacokinetics, pharmacodynamics and therapeutic activity of a single-stranded miR-29b mimic in murine with human acute myeloid leukemia

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Running title: Single-stranded miR-29b mimic is active in AML
Abstract

**Background:** In acute myeloid leukemia (AML), aberrant miR-29b downregulation contributes to leukemogenesis, thus restoring normal miR-29b levels by synthetic microRNA (miR) mimics may result in antileukemic activity and represent a novel molecular targeting approach. To date, the majority of studies on miR replacement therapies has used double-stranded mimics leaving unanswered question as of whether the single-stranded counterparts can be efficiently delivered, uploaded into the RISC (RNA-induced Silencing Complex) and biologically active. We report here a comprehensive and integrated study of pharmacokinetics (PK), pharmacodynamics (PD) and antileukemic activity of a single-stranded 2′-O-methyl phosphorothioate miR-29b (2′-MeOPSmiR-29b) in AML.

**Methods:** Level of synthetic single-stranded 2′-MeOPSmiR-29b in plasma, tissues and cells were quantified by ELISA and mRNA and protein levels of putative targets were determined by qRT-PCR and immunoblotting, respectively. Global DNA methylation was analyzed by liquid chromatography-tandem mass spectrometry. Antileukemia activity was evaluated *in vivo* in MV4-11 cells-engrafted mice.

**Results:** 2′-MeOPSmiR-29b displayed a three-compartmental plasma PK profile, wide tissue distribution, substantially high organ concentrations (except brain) and slow elimination. Intravenous and intraperitoneal administration resulted in similar exposure. Multiple daily dosing led to tissue accumulation, robust plasma and intracellular level of 2′-MeOPSmiR-29b, efficient *miR-29b* target downregulation and significantly improved survival in an aggressive AML-like disease murine model. Incorporation of 2′-MeOPSmiR-29b into transferrin-conjugated nanoparticle (NP) formulation results in a similar biologic activity, but at a 10-fold lower dose.
Conclusions: Administration of 2′-MeOPSmiR-29b results in robust concentration in peripheral organs including bone marrow with a significant antileukemic activity. Changes to a liposomal drug formulation may reduce the total amount of mimic miR necessary to achieve a robust pharmacologic activity. Our results may represent a key step for translation of miR-29b-based therapy to the clinic for AML patients.
Introduction

Despite recent progress in understanding the mechanisms of myeloid leukemogenesis and implementation of molecular risk assessment and patients’ stratification to risk-adapted treatments, only 40% of younger (<60 years) and <10% of older (>60 years) patients with acute myeloid leukemia (AML) achieve long-term complete remission. Therefore, novel molecularly targeting treatments to improve otherwise poor clinical results are highly needed (1, 2).

miRs are short non-coding RNAs that are known to be deregulated in leukemia and other types of cancer. In AML, downregulation of miR-29b contributes to myeloid leukemogenesis and increases disease aggressiveness by enabling aberrant expression of DNA methyltransferases (DNMTs) and subsequent DNA hypermethylation and silencing of tumor suppressor genes (3, 4). We also reported that miR-29b targets the transcription factor Sp1 which participates in a Sp1/NFκB transcription complex that transactivates the expression of the wild-type or mutated oncogenes KIT and FLT3 (5, 6). Additionally, miR-29b downregulation leads to upregulation of the antiapoptotic protein Mcl-1 in myeloid blasts (7). Altogether, these data support a crucial role of miR-29b downregulation in AML and suggest that restoring normal levels of miR-29b in leukemia blasts may result in a clinical benefit.

Recently, we demonstrated that small molecule compounds could induce miR-29b upregulation in AML blasts (6, 8); however, these compounds often have off-target activities and undesirable side effects. Thus, we turned to the use of miR-29b mimics for a direct correction of its aberrantly low miR levels in AML. We previously reported an antileukemia activity of a double-stranded miR-29b mimic (11); this moiety however cannot be accurately monitored in vivo.
thereby making relatively difficult to assess pharmacokinetic parameters necessary for a prompt development of an optimal regimen to be used in human. In contrast, single-stranded mimic is much simpler to be measured in plasma and other different biologic matrices and is less costly to synthesize. Thus, we tested the pharmacokinetics (PK), pharmacodynamics (PD) and in vivo antileukemia activity of a single-stranded miR-29b mimic, 2’-O-methyl phosphorothioate (2’-MeOPS) miR-29b.
Methods

Synthetic miR mimics

Synthetic 2′-MeOPSiR-29b and 2′-MeOPS scrambled miR (2′-MeOPSiR-sc) oligonucleotides were purchased from Girindus (Boston, MA). The sequences of these oligos were previously reported (9). Differently from oligos previously used (9, 10), 2′-MeOPSiR-29b analyzed here met the Good Manufacturing Practice (cGMP) standards and could be produced in large-scale for in vivo study. This compound was studied “naked’ and encapsulated into transferrin-conjugated nanoparticles NP (Tf-NP-2′-MeOPSiR-29b) (11) to facilitate a targeted delivery to leukemia cells. Briefly, 2′-MeOPSiR-29b or 2′-MeOPSiR-sc was mixed with polyethylenimine (Sigma-Aldrich) to form a core and entrapped in empty NPs, which were composed of 1,2-dioleoyl-sn-bly-cero-2-phosphoethanolamine (DOPE, Avanti Polar Lipids), linoleic acid (Sigma-Aldrich) and 1,2-dimyristoyl-sn-glycerol-methoxypolyethylene glycol (DMG-PEG, Avanti Polar Lipids) in a ratio of 50/48/2. Tf was then incorporated onto the miR-loaded NP by a post-insertion method. The size of NP was analyzed by a NICOMP Particle Sizer Model 370 (Particle Sizing Systems).

Toxicity, plasma PK and tissue distribution of 2′-MeOPSiR-29b

Male CD2F1 mice (6-7 week-old, Harlan, Indianapolis, IN) were used for toxicity and PK studies. All animal experiments were carried out according to protocols approved by the Institutional Animal Care and Use Committee at The Ohio State University. Three 2′-MeOPSiR-29b doses (16, 24, and 32 mg/kg) were tested to determine dose-limiting toxicity (DLT). For these studies, a single bolus of 2′-MeOPSiR-29b was administered at each dose level through the tail vein. 2′-MeOPSiR-29b was prepared in sterile saline at concentrations of
1.6, 2.4 and 3.2 mg/ml, respectively, and injected at 10 ml/kg mouse weight (i.e., 0.2 ml per 20 g mouse). Mice behaviors and body weight were closely monitored throughout 48 hours post-injection. Animal toxicity was defined by hunched posture, inactivity, lethargy, rough hair coat, lack of righting reflex, anorexia, weight loss greater than 20% and mortality. DLT was defined as the dose at which toxicity or death occurs in at least one of the tested animals.

PK studies were done at the same dose levels; 2’-MeOPSMiR-29b was administered by a single i.v. (16, 24 or 32 mg/kg) or i.p. (24 mg/kg) bolus. Plasma and major organ tissues, including bone marrow (BM), spleen, liver, kidney, heart, lung and brain, were collected at sequential time points (5, 10, 15, 30, 60, 120, 240, 480, 1320, 1440 and 2880 minutes) following administration using 6 (for i.v.) or 3 (for i.p.) mice at each time point (9). A hybridization-based ELISA assay was used to quantify 2’-MeOPSMiR-29b concentration in plasma and tissues, as previously reported by our group (9, 10). Plasma concentration-time data were analyzed using Phoenix 64 v. 6.3 (Pharsight, Mountain View, CA) to calculate PK parameters using appropriate models. Compartmental model fitting of data was characterized as determined by Akaike information criterion (AIC), weighted sum of square, and visual goodness-of-fit of naïve pooled data as described previously (10).

To test for drug accumulation or chronic toxicity, CD2F1 mice (n=3 per group) were given an i.v. bolus of 24 mg/kg/day 2’-MeOPSMiR-29b for four consecutive days or twice/week for 2 weeks (in either case, they received a total of four doses). BM and spleen were collected for analysis 24 hours after the last dose.
For the study with the formulated Tf-NP-2′-MeOPSmiR-29b, CD2F1 mice received one single or three consecutive i.v. daily doses of 2.4 mg/kg. Plasma and BM were collected at 24 hours following the last injection to measure 2′-MeOPSmiR-29b levels.

**Cell culture, transfection and in vitro treatment**

MV4-11 cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Invitrogen, Carlsbad, CA). For *in vitro* studies, MV4-11 cells were transfected with 1 or 10 µM 2′-MeOPSmiR-29b or 2′-MeOPSmiR-sc by nucleoporation (Lonza, Gaithersburg, MD) according to manufacturer’s instructions. In contrast, no electroporation was necessary for *in vitro* delivery of Tf-NP-2′-MeOPSmiR-29b.

**Western blotting**

The cell lysates were prepared, resolved by SDS-PAGE (50 µg protein per lane) and immunoblotted for DNMT1, DNMT3a, DNMT3b and Sp1 as previously reported (9,10,12). Protein levels were quantified by densitometry using Image J software (Scion Image, Scion, Frederick, MD) and normalized to the loading control β-actin.

**Quantitative RT-PCR**

Total RNA was extracted from cells by Trizol reagent (Invitrogen) and reverse transcribed by transcriptase (Applied Biosystems, Foster City, CA). Levels of *FoxD3* mRNA and primary transcripts of *miR-29b* (*pri-miR-29b-1* and *pri-miR-29b-2*) were determined by Taqman gene expression assays (Applied Biosystems). Expression was calculated with the comparative *Ct* method and raw data was normalized by internal controls GAPDH (for human) or actin (for mouse).
Global DNA methylation (GDM) analysis

Genomic DNA was extracted from cells using the DNeasy Tissue Kit (Qiagen, Minneapolis, MN), hydrolyzed and analyzed with liquid chromatography-tandem mass spectrometry as previously reported (13). The ratio of 5-methyl-2'-deoxycytidine (5mdC) to the internal standard 2-deoxyguanosine (2dG) in mass signal was used to quantify GDM level.

Mice treatment regimens for PD and survival studies

For in vivo studies, 4–6 week-old NSG mice (NOD.Cg-Prkdscid Il2rgtm1Wjl/SzJ, The Jackson Laboratory, Bar Harbor, ME) were injected with 0.3x10^6 in vivo-adapted MV4-11 cells through the tail vein (11,14). The complete blood count, cytospin and FACS analysis of CD44 and FLT3 expression were examined weekly to monitor the progression of leukemia.

The mice were treated with a single i.v. bolus of saline, 2'-MeOPSMiR-sc (24 mg/kg) or 2'-MeOPSMiR-29b (16 or 24 mg/kg) two weeks after the engraftment upon first evidence of circulating leukemia cells. MNCs were isolated from BM of the treated mice 12, 24 or 48 hours following treatment and DNMTs and Sp1 levels were measured as described above.

For the survival studies, treatments started one week after mice were engrafted with adapted MV4-11 cells. The mice were followed longitudinally for disease progression and mortality. Three trials were conducted. In Trial 1 (i.v.-i.p.), mice were given four consecutive daily i.v. injection of saline (n=7), 2'-MeOPSMiR-sc (n=8) or 2'-MeOPSMiR-29b (n=8) at the dose of 24 mg/kg in week 1 followed by four consecutive daily i.p. doses in week 2. This two-week treatment cycle was repeated for two additional cycles (total 6 weeks of treatment). In Trial 2
(i.p.), mice received four consecutive daily i.p. doses of saline (n=7), 2′-MeOPSiR-sc (n=6) or 2′-MeOPSiR-29b (n=6) at the dose of 24 mg/kg every week for 6 weeks. In Trial 3, we investigated the in vivo antileukemic activity of Tf-NP-2′-MeOPSiR-29b. The formulation was freshly prepared every day before mice injection, as previously reported (11). AML engrafted-mice were treated with four consecutive daily i.p. doses of either Tf-NP alone (n=12), Tf-NP-2′-MeOPSiR-sc (n=8), Tf-NP-2′-MeOPSiR-29b (n=8) or naked (unformulated) 2′-MeOPSiR-29b (n=4) at the dose of 2.4 mg/kg each week for 8 weeks.

**Statistical analysis**

Data are presented as mean ± SE of at least six replicates performed in 2 independent experiments. Statistical significance was evaluated by Student’s two-tailed t test and p value < 0.05 was considered significant. Mice survival was analyzed using the Kaplan-Meier method and pair-wise multiple comparisons were performed by the log-rank test using GraphPad Prism Program (San Diego, CA).
Results

Acute toxicity, plasma PK and tissue disposition

No acute toxicity was observed with 2′-MeOPSmiR-29b given i.v. at 16, 24, and 32 mg/kg through the tail vein, and DLT was not achieved. Following administration, the 2′-MeOPSmiR-29b plasma concentration reached a peak at 5 minutes, declined tri-exponentially over time and remained measurable up to 48 hours. The plasma concentration-time profiles for these three were best fitted to a three-compartmental model. Estimated plasma peak concentration (\(C_{\text{max}}\)) and area-under-the-curve (\(AUC_{0-\infty}\)) suggested the 2′-MeOPSmiR-29b exposure was increased disproportionately with dose (Table 1). \(C_{\text{max}}\) of 11, 65 and 472 µM and \(AUC_{0-\infty}\) of 140, 484 and 1340 min*µM were estimated respectively for the 16, 24 and 32 mg/kg doses. While there was clearly a rapid distribution process, plasma levels remained high for several hours after administration followed by a relatively long elimination phase (termination half-life estimates ranged from 5 to 50 hours across all three dose levels). High plasma concentrations were also achieved in 5 minutes for i.p.-administered 2′-MeOPSmiR-29b, supporting rapid systemic drug availability with an estimated terminal half-life relatively longer compared to that of the i.v.-administration route (Table 1). However, the \(AUC_{0-\infty}\) (484 min*µM) for the i.p. administered 2′-MeOPSmiR-29b was nearly identical to that for the i.v.-administered drug (Table 1), making the two administration routes relatively comparable.

With regard to tissue disposition, i.v. 2′-MeOPSmiR-29b had a relatively slow elimination rate and robust concentrations in all tested organs throughout 48 hours except brain (Fig.1a-g), implying that an intact blood-brain-barrier has a limited permeability to synthetic miR. At the dose of 24 mg/kg, organs with the greatest 2′-MeOPSmiR-29b levels were kidney, liver and
spleen, Post-injection 2′-MeOPSmiR-29b levels had a single peak at 5 minutes followed by a decay phase in, spleen, lung, and heart (Fig. 1a-e), while a secondary peak was observed at 6 hours only in liver and kidney displayed (Fig. 1f-g). Relatively stable levels at pharmacologically active concentrations (1-5 µg/ml) were detected in BM over time (Fig. 1a).

Since maintaining drug tissue concentration within an appropriate therapeutic range may be crucial for clinical activity, next we examined PK for multiple dose treatments. We tested the dose of 24 mg/kg given i.v. either on four consecutive days or twice per week for two weeks. 2′-MeOPSmiR-29b concentrations in BM and spleen were determined 24 hours following the last dose. BM and spleen 2′-MeOPSmiR-29b concentrations after four consecutive doses were approximately twice those of single dose [3.3 ± 0.5 µg/ml for multiple doses vs. 1.5 ± 0.1 µg/ml for single dose in BM; 76.7 ± 8.6 µg/ml for multiple doses vs. 36.3 ± 15.5 µg/ml for single dose in spleen (p<0.05, Fig. 1h-i)]. In contrast, the twice-per-week doses did not demonstrate a significant increase in tissue concentrations relative to a single dose (Fig. 1h-i).

Together, these data demonstrated that 2′-MeOPSmiR-29b is safe and widely distributed in all tissues, including the hematopoietic compartments, after either i.v. or i.p. administration. Plasma and tissue concentrations could generally be increased with higher doses throughout the dose range tested, and multiple dosing on consecutive days results in drug accumulation and achievement of robust drug concentrations in hematopoietic tissues.

*In vivo* biological activity of 2′-MeOPSmiR-29b in systemically treated AML mice
We next investigated whether the single-stranded 2’-MeOPSiR-29b was functional after cellular uptake. We have previously demonstrated that Sp1 and DNMTs are miR-29b targets (4, 5). After electroporation, 2’-MeOPSiR-29b intracellular levels were 4.6 ± 0.2 and 18.7 ± 1.5 nM in 1 or 10 μM treated MV4-11 cells, respectively (Fig. 2a). These intracellular concentrations resulted in time- and dose-dependent Sp1 and DNMTs downregulation (Fig. 2b). Consistent with these results, we also observed ~65% reduction in global DNA methylation (GDM) and ~50% increase in the mRNA expression level of the hypermethylated and silenced tumor suppressor gene FoxD3 (15) in 2’-MeOPSiR-29b treated cells compared to the 2’-MeOPSiR-sc-treated controls (Fig. 2c-d).

For the in vivo studies, MV4-11 cells were engrafted into immunodeficient NSG mice (14). The engrafted mice developed an AML-like disease (Fig. 3a) with elevated circulating blasts, an extensive BM and spleen blast infiltration and 4 week survival from the time of engraftment (14,11). The animals received i.v. bolus of 16 or 24 mg/kg 2’-MeOPSiR-29b x 1 dose two weeks after cells transplantation, upon evidence of circulating blasts. Mice injected with saline or 2’-MeOPSiR-sc were used as controls. We selected these two doses because their peak plasma levels after a single injection achieved concentrations in the range of those that were shown to downregulate targets in vitro (Table 1; Fig. 3b). Furthermore, the disproportionally high concentrations achieved in plasma but not tissue with the 32 mg/kg dose suggested a saturated kinetics at this dose level. 2’-MeOPSiR-29b treated mice showed dose and time-dependent decreased levels of Sp1, DNMT3a and DNMT3b protein in BM mononuclear cells (MNCs) compared with controls (Fig. 3b). Approximately 80% decreases in DNMT3a and DNMT3b protein levels occurred at 24-hour for mice treated with the 24 mg/kg dosing and at 48-hour for
those treated with the 16 mg/kg dosing. Sp1 protein was downregulated as early as 12-hours and nearly depleted at 48-hour at both dose levels. Since Sp1 is a component of a miR-29b transcription repression complex in AML blasts (5), we also measured endogenous pri-miR-29b levels, which as expected was found to be upregulated by the mimic miR-29b treatment (Fig. 3c), further demonstrating the fine pharmacologic activity of 2′-MeOPSmiR-29b.

2′-MeOPSmiR-29b extends AML mice survival

The in vivo antileukemic activity of 2′-MeOPsmiR-29b was evaluated in MV4-11-engrafted NSG mice. One week after transplant, mice were treated with 2′-MeOPSmiR-29b, 2′-MeOPSmiR-sc or sterile saline and followed longitudinally for disease progression and survival. We conducted two initial trials. In Trial 1, mice were given 24 mg/kg/day i.v. x 4 days/week alternated with 24 mg/kg/day i.p. x 4 days/week x 3 cycles (Fig. 3d upper panel). The alternating i.v.-i.p. dosing was selected in order to reduce the technical difficulty of frequent tail i.v. injections. The median survival of saline and 2′-MeOPSmiR-sc-treated AML mice was 28 and 30 days respectively, as compared to 40 days for 2′-MeOPSmiR-29b treated mice (p<0.01 for each of the pair-wise comparisons, Fig. 3d middle panel). In Trial 2, mice received the drug only by i.p.. The median survival of 2′-MeOPSmiR-29b treated AML mice increased to 37 days as compared to saline or 2′-MeOPSmiR-sc treated controls both of 29 days (p<0.001 for each of the pair-wise comparisons, Fig. 3d lower panel). Although a trend for a better antileukemia activity was observed with the i.v.-i.p regimen compared with the i.p. regimen, this difference was not statistically significant. Of note, although 2′-MeOPSmiR-sc induced a non-specific target downregulation at the dose of 24 mg/kg when compared with saline-treated mouse (Fig. 3b), it did not elicit any beneficial effects for mice survival by two administration routes (Fig. 3d).
Tf-conjugated nanoparticle (NP) formulation improves 2′-MeOPSmiR-29b potency

In order to minimize potential toxicity, increase selectivity toward leukemia cells and reduce the dose of miR agent, we also sought to optimize the 2′-MeOPSmiR-29b formulation. We previously observed a high Tf receptor (TfR) surface expression on MV4-11 cells and AML patient blasts, and reported that a double-stranded miR-29b mimic-loaded Tf-conjugated NP yielded antileukemic activity (11). Therefore, we utilized the same formulation to encapsulate the single-stranded 2′-MeOPSmiR-29b to target leukemia cells expressing TfR (Fig. 4a). In *in vitro*-treated MV4-11 cells, 1 µM Tf-NP-2′-MeOPSmiR-29b reduced Sp1, DNMT1 and DNMT3a protein expression by 50-80%, completely depleted DNMT3b and increased the endogenous *miR*-29b transcripts by 2-fold following 48 hours incubation (Fig. 4b-c). In *in vivo* study, multiple daily *i.p.* doses of 2.4 mg/kg Tf-NP-2′-MeOPSmiR-29b led to a better BM drug accumulation than a single dose (11.7 ± 3.1 µg/ml for the multiple daily-dosed group vs. 4.0 ± 1.7 µg/ml for the single-dosed group; Fig. 4d). Despite being delivered at a concentration one-log lower than the “naked” drug, Tf-NP-2′-MeOPSmiR-29b achieved BM concentrations approximately 2-fold higher (Fig. 4d and Fig. 1h). Daily administration of 2.4 mg/kg Tf-NP-2′-MeOPSmiR-29b by *i.p.* injection on four consecutive days per week x 8 weeks (similar to Trial 2 regimen, Fig. 3d) resulted in a significantly prolonged survival of the MV4-11-engrafted mice as compared to the Tf-NP-2′-MeOPSmiR-sc-treated mice (median survival 43 vs 30 days, *p*< 0.001, Fig. 4e) or the naked 2′-MeOPSmiR-29b given at the same 2.4 mg/kg dose (median survival 43 vs 33 days, *p*<0.001, Fig. 4e). The therapeutic efficacy of the 2.4 mg/kg Tf-NP-2′-MeOPSmiR-29b was similar to that of the naked 2′-MeOPSmiR-29b given at a 10-fold higher
dose (24 mg/kg), indicating that the NP formulation significantly improved the potency of 2′-MeOPSmiR-29b (Fig. 3d and 4e).
Discussion

miRs are aberrantly expressed in leukemia and therefore may represent novel therapeutic targets. We and others reported that miR-29b target multiple genes that contribute to leukemogenesis including those encoding for epigenetic modifiers (i.e., DNMTs), kinases (i.e., KIT, FLT3) and antiapoptotic proteins (i.e., MCL-1). This miR is downregulated in AML cells, thereby providing an optimal model to study therapeutic miR-replacement strategies (4, 5, 6, 7). Furthermore, given that aberrant miR-29b downregulation has been found in other types of human cancers (3, 16-23) and non-malignant diseases (24-26), preclinical and clinical studies of therapeutic replacement of aberrantly low miR-29b with synthetic mimics appear to be relevant for much broader applications than leukemia “only”. However, information on translation of miR mimics from the bench to the clinic is limited.

We previously reported preliminary results of the activity of a single-stranded miR-29b mimic 2′-MeOPSmiR-29b (9,10). However, this study was not optimal to assess fully the toxicity, pharmacological and clinical activities and the feasibility of a scaled up production of this mimic compound because the dose was too low and did not meet cGMP standards. We reported here a comprehensive study of toxicity and PK, PD and antileukemic activities of a novel single-stranded miR-29b mimic distinctly formulated according to cGMP standards and administered at different doses and schedules. Currently, the majority of miR mimic therapies reported in the literature have used double-stranded RNA since it has been unclear whether the single-stranded form can be uploaded into the RISC (RNA-induced Silencing Complex). We structurally modified miR-29b mimic with 2′-MeOPS moiety; this modification was previously shown to successfully increase drug stability and nuclease resistance without affecting in vivo
pharmacologic activity in other synthetic oligonucleotides (27-31). In addition, we applied a sensitive pharmacoanalytical tools for quantitative analyses of single-stranded 2′-MeOPS-miRs in different biologic matrices (9). Here, we demonstrated that single-stranded miR mimic is a biologically and therapeutic feasible approach that can be easily studied from a pharmacoanalytical standpoint. Furthermore, single-stranded miR mimics is likely economically more advantageous as they can be produced at a lower cost than the corresponding double-stranded form. We also showed that plasma drug levels of 2′-MeOPSmiR-29b following i.v. administration exhibited a multi-exponential decay that was best fitted to a three-compartment model. While plasma concentrations and exposures increased with dose, the changes were not dose-proportional suggesting that active cellular uptake of 2′-MeOPSmiR-29b may be saturated at dose higher than a given threshold. Therefore, we selected the 16 and 24 mg/kg rather than higher oses for further in vivo studies.

Tissue PK demonstrated 2′-MeOPSmiR-29b was broadly distributed in all tissues except brain. Secondary drug concentration peaks were observed in liver and kidney, which we interpreted either as a gradual extracellular bound or intracellular accumulation of the compound in these tissues or as the generation and accumulation of 2′-MeOPSmiR-29b metabolites, not readily distinguishable from the parent compound using pharmacoanalytical assays (9). The relative equivalence for plasma drug exposure with i.v. and i.p. administrations suggested the i.p. route is a suitable and practical dosing route for repeated injections, especially in preclinical mouse studies where the i.v. route is technically challenging.
Together these data informed the dose, administration route and schedules for the subsequent in vivo studies of the single-stranded 2′-MeOPSmiR-29b in AML MV4-11 cells-engrafted mice (11,14). Effective target downregulation (i.e., DNMTs and Sp1) and significant antileukemic activity were shown in 2′-MeOPSmiR-29b treated leukemic mice compared to the miR-sc treated controls. As 2′-MeOPSmiR-29b also markedly decreased Sp1 expression and relieved miR-29b transcription repression, we observed an increase in the endogenous miR-29b transcript levels thereby indicating two possibly concurrent mechanisms of activity for the synthetic 2′-MeOPSmiR-29b. Targets downregulation after drug administration may be achieved through the pharmacological binding of the synthetic mimic to target mRNAs and/or by release of transcription of the endogenous miR-29b followed by a subsequent physiologic target downregulation.

In order to improve the efficacy and/or potency of 2′-MeOPSmiR-29b while lowering potential toxicity and decreasing costs, we also investigated a novel formulation to selectively deliver lower doses of the miR mimics to leukemic cells. We previously developed a Tf-conjugated lipopolyplex nanocarrier system for double-stranded miR mimic delivery (11, 32). We adapted this formulation to encapsulate the single-stranded 2′-MeOPSmiR-29b. This targeted delivery system markedly increased the potency of 2′-MeOPSmiR-29b in vitro. BM levels, PD and antileukemic activity of Tf-NP-2′-MeOPSmiR-29b were also dramatically enhanced in vivo as compared to the equivalent dose of the “naked” miR compound. These effects were not increased with higher dose of Tf-NP-2′-MeOPSmiR-29b (i.e., 2.4 vs 7.2 mg/kg), suggesting saturation of the TfR-mediated uptake at higher doses.
In summary, to our knowledge, this is the first study providing the concurrent evaluation of PK, PD and antileukemic activities of single and multiple doses, variuos schedules and different formulations of 2′-MeOPSmiR-29b. We believe that our data fill a knowledge gap for the development of synthetic single-stranded miR mimics in leukemia (36, 37). Further toxicological and PK-PD modeling studies of 2′-MeOPSmiR-29b and in other animal species will provide insights for a rapid transition of miR mimic therapies into the clinic.

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