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Synthetic microRNAs Cassette Dosing: Pharmacokinetics, Tissue Distribution and Bioactivity

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Abstract

MicroRNAs (miRs) are deregulated in cancer and leukemia. Restoring aberrantly downregulated tumor suppressor miRs or antagonizing overexpressed oncogenic miRs in malignant cells by synthetic RNA oligonucleotides represents a potentially novel therapeutic approach in cancer and leukemia. However, given the complex networking and concurrent deregulation of miRs in malignant cells, an effective approach may require concurrent targeting of multiple miRs. Cassette dosing involves simultaneous administration of a mixture of oligonucleotides from same or different structural classes. However, information on cassette dosing pharmacokinetics, tissue distribution and bioactivity of synthetic miRs is lacking. In this study, three synthetic 2'-methoxyphosphorothioate-miRs (2'-MeOPSmir16-1, 2'-MeOPSmir29b and 2'-MeOPSmir155) were administered *i.v.* to C57BL/6 mice as a mixture, each at 7.5mg/kg. Analysis of concentrations of individual miR in plasma and major organ tissues (bone marrow, spleen, liver, brain, heart, kidney and lung) was performed. The mRNA and protein levels of miR's bio-targets were monitored sequentially after dosing up to 24 hours. Our results demonstrated that these synthetic miRs retain their different individual pharmacokinetic properties and all display three-compartmental pharmacokinetics. 2'-MeOPSmir16-1 has the longest plasma gamma half-life of 2508 minutes and lowest total body clearance of 0.0054 L/min*kg, whereas 2'-MeOPSmir29b has the shortest gamma half-life of 510.6 minutes and highest total body clearance of 0.042 L/min*kg. The tissue concentrations of all three 2'-MeOPS-modified miR(s)/antagomiR were measurable from 5 minutes to at least 24 hours after dosing, indicating that these concurrently delivered oligonucleotides can reach organ tissues. Importantly, there were biological activities of the concurrently administered miRs which persisted, as shown by the downregulation of specific targets in tested tissues, albeit with variations. Brain was one of the most sensitive tissues with respect to downregulation of mRNA and protein levels of four measured bio-targets (e.g. Bcl-2, Mcl-1, DNMT3a and DNMT3b) despite its relatively low miR/antagomiRs levels. We conclude that cassette dosing is applicable to 2'-MeOPS-modified synthetic miRs that are tissue-deliverable and biofunctional without any additional formulation requirement. This study supports future exploration of miR-involved combination therapies.

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Keywords

2'-MeOPSMiRs; cassette dosing; pharmacokinetics; tissue distribution; pharmacodynamic

INTRODUCTION

MicroRNAs (miRs) are endogenous, small non-coding RNAs (18–25 nucleotides) that bind to target mRNAs and negatively regulate their expression through translational repression or mRNA degradation^{1–2}. Recent work indicates that miRs may act as oncogenes or tumor suppressors during cancer and leukemia development and progression^{3–4}. Overexpression of *miR-155* has been reported in acute myeloid leukemia (AML)^{5–6}, high-risk chronic lymphocytic leukemia (CLL)⁷, lymphomas^{8–9} and other solid tumors^{10–11}, suggesting a pivotal role in cancer pathogenesis. On the other hand, downregulation of tumor suppressive miRs may also contribute to the malignant transformation by allowing re-expression of oncogenes. *miR-15a* and *miR-16-1* clusters were identified as the first tumor suppressive miRs. When deleted or downregulated in CLL¹², these miRs resulted in overexpression of the antiapoptotic gene *BCL-2*¹³. Another set of tumor suppressive miRs, *miR-29* family, that has been reported to be downregulated in AML⁵, high-risk CLL⁷ and aggressive non-small cell lung cancer¹⁴, directly or indirectly targets several oncogenes including *TCL-1*¹⁵, *MCL-1*^{16–17}, *SP1*¹⁸, *DNMT1*¹⁹, *DNMT3a* and *DNMT3b*^{19–20}.

Collectively, these studies suggest that miRs might be potential biomarkers for diagnosis and prognosis²¹, and serve as novel targets for therapeutic intervention in cancer and leukemia. Therefore, antagonizing overexpressed miR or restoring downregulated miR by synthetic RNA oligonucleotides represents a potentially novel therapeutic approach.

One major advantage of miR over siRNAs or shRNAs-based therapy is that a single miR can regulate multiple targets²². Previous studies have suggested that each cancer and leukemia type has a unique profile (signature) of deregulated miRs. Thus, it is likely that several miR(s)/antagomiR(s) in the same family of their synthetic mimics could be used in concert for more effective miR-based treatments. Cassette dosing involves simultaneous administration of a mixture of several compounds that are from same structural class to a single animal²³. This approach was developed more than two decades ago and is now widely used as a drug discovery tool²⁴. We have now adapted it to study concurrent administration of synthetic miRs, (i.e., synthetic miR16-1, miR29b and antagomiR155). We selected 2'-methoxyphosphorothioate (2'-MeOPS)-modified miR/antagomiR compounds because oligonucleotide compounds with these structural modifications are being under clinical evaluation^{25–26}.

By using a sensitive and specific ELISA recently developed in our laboratory²⁷ that can simultaneously measure single synthetic miR from a mixture, we performed pharmacokinetic (PK) studies of three synthetic miRs/antagomiR in mice under a cassette dosing, quantified the tissue level of individual miR/antagomiR and determined the expression levels of their corresponding targets. To our knowledge, this is the first study to provide experimental evidences of the feasibility of cassette dosing PK, tissue distribution and pharmacodynamics (PD) of synthetic miR(s)/antagomiRs in murine model.

MATERIALS AND METHODS

Oligonucleotides

2'-MeOPSMiRs, including miR16-1, miR29b and antagomiR155 were purchased from Dharmacon Inc. (Lafayette, CO). Their sequences were provided in our previous paper²⁷.

Mouse treatment, tissue collection and PK analysis

C57BL/6 male mice (6~8-week-old) were purchased from Harlan (Indianapolis, IN). Upon receipt, mice were acclimated for one week before the study. All animal experiments were carried out according to a protocol approved by the Institutional Animal Care and Use Committee at The Ohio State University. A mixture of three miRs/antagomiR, including 2'-MeOPSMiR16-1, 2'-MeOPSMiR29b and 2'-MeOPSantagomiR155, was prepared in sterile saline and administered via the tail vein at the dose of 7.5 mg/kg for each. Mice were sacrificed at the time points of 5, 10, 15, 30, 60, 120, 240, 480, 1320 and 1440 minutes after injection. Each time point had 5~6 mice. Plasma and tissues were collected immediately, tissues were briefly rinsed with PBS, blotted with gauze-pad, and snap-shot frozen until further analysis. Plasma concentration-time data were analyzed by WinNonlin computer software (Pharsight 5.0, Mountain View, CA) using appropriate PK models.

Tissue processing and hybridization-based ELISA for synthetic miRs

After a brief thaw at room temperature, the tissue block (30~50 mg) was homogenized and lysed in 1 ml ice-cold lysis buffer (10 mM Tris-HCl, pH 8.0, 0.5mM EDTA, 1.0% Triton-X 100). The lysate was cleared by centrifugation at 2,000 g for 5 minutes, and the supernatant was analyzed by ELISA developed in our laboratory²⁷. Briefly, the method is based on hybridization of 2'-MeOPSMiR to the biotin-labeled capture probe, followed by ligation with digoxigenin-labeled detection probe. The bound duplex is then detected by anti-digoxigenin-alkaline phosphatase using Attophos (Promega, Madison, WI) as substrate. The capture and detection probes were custom synthesized by Integrated DNA Technologies (IDT, Coralville, IA) and their sequences were listed previously²⁷. Of note, we did not detect any ELISA signal in the untreated (blank) mouse tissues, suggesting that there was no interference from the endogenous miRs for this assay.

Quantitative RT-PCR

Tissues were homogenized by a power homogenizer, total RNA was extracted from tissues using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed by transcriptase (Applied Biosystems, Foster City, CA). mRNA expression levels of Bcl-2, Mcl-1, DNMT3a, DNMT3b were determined by SYBR Green gene expression assays (Applied Biosystems). The following primers, synthesized by IDT were used for PCR amplification: mouse Bcl-2 (5'-CCAGCGTGTGTGTGCAAGTGTAAG-3' and 5'-ACACTCCGGCTTCAC TGAGAATGT-3'); mouse Mcl-1 (5'-AGTCAGCACAGCTTTCCTGTCAGA-3' and 5'-TGCCAATCCAAGAATGCCAATCCC-3'); mouse DNMT3a (5'-CAGCACCATTCC TGGTCATGCAAA-3' and 5'-TCCTGTGTGGTAGGCACCTGAAAT-3'); mouse DNMT3b (5'-ACGCAGGAAAGATTGGAACATGCG-3' and 5'-TACCCAACTCCTT GAGCACCAAGT-3'); mouse GAPDH (5'-CTCATGACCACAGTCCATGC-3' and 5'-TTCAGCTCTGGGATGACCTT-3' and). mRNA level of C/EBP β was determined by TaqMan gene expression assays (Applied Biosystem). Real-time PCR reactions were carried out in triplicate by using a StepOne Plus system (Applied Biosystems). The expression was calculated with comparative *Ct* method and the raw data was normalized with the internal control GAPDH.

Western blotting

Powdered tissues were homogenized and lysed in ice-cold lysis buffer (20 mM, pH 7.0 HEPES, 150 mM NaCl, 0.1% NP40 supplemented with 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 mM NaF, 1 mM benzamide and 1 mM phenylmethylsulfonyl fluoride with protease inhibitors) for 45 minutes on ice. The lysate was centrifuged at 14,000 g for 10 min at 4 °C. The supernatants were resolved on 4~15% SDS-PAGE, transferred to nitrocellulose

membranes and probed with corresponding antibodies. Antibody to Bcl-2 from Cell Signaling (Danvers, MA); to DNMT3a was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); to DNMT3b from Abcam (Boston, MA); to β -actin from Sigma (St. Louis, MO). The protein expression was quantified by densitometry and normalized to β -actin.

Statistics

Two-side *Student's t*-test was performed to evaluate differences between the groups (10 minutes, 1 hour and 24 hours) in the mRNA level of bio-targets.

RESULTS

Cassette Dosing Plasma PK

A mixture of three synthetic miRs/antagomiR were administered intravenously to C57BL/6 mice. The dose of each synthetic miRs/antagomiR was 7.5 mg/kg. Plasma was collected at different time points from 5 minutes up to 24 hours following drug administration. Under the cassette dosing, the plasma levels of all three synthetic miRs/antagomiR compounds were measurable up to at least 24 hours. The mean plasma concentration-time profile for each miR/antagomiR from five or six mice showed multiple exponential decays (Figure 1) and the data were found to be best fitted to a three compartmental intravenous bolus PK model as determined by Akai Information Criterion (AIC), weighted sum of square, and visual goodness-of-fit (Figure 1). The PK parameters for the individual synthetic miR/antagomiR were analyzed by using the compartment and non-compartment methods (Table). The initial half-lives (*Alpha-HL* and *Beta-HL*) were comparable for all synthetic miRs in the range of 5–6 minutes for *Alpha-HL* and 30–50 minutes for *Beta-HL*, respectively, but the terminal half-life (*Gamma-HL*) was the longest for 2'-MeOPSmIR16-1 (2508 minutes), and shorter for 2'-MeOPSmIR155 (782.8 minutes) and 2'-MeOPSmIR29b (510.6 minutes) as shown in Table. The total body clearances (*CL*) were in the reverse rank order, with 2'-MeOPSmIR29b being the fastest and 2'-MeOPSmIR16-1 the slowest. The area under the curve (*AUC*) for 2'-MeOPSmIR29b was ~ 4 and ~ 7 fold lower than that of 2'-MeOPSmIR155 and 2'-MeOPSmIR16-1, respectively. The volumes of distribution at steady-state (*V_{ss}*) ranged from 3.4~7.5 L/kg and were relatively similar for the three miRs/antagomiRs.

Tissue Distribution and PK of Synthetic miRs /antagomiRs

Preclinical development of synthetic miRs requires information on tissue distribution, as with other small molecular drugs. Using our validated ELISA, we performed analysis of the three synthetic miRs/antagomiRs individually in tissues, following a cassette *i.v.* bolus dose at 7.5mg/kg for each.

Tissue concentrations of all the three miRs/antagomiR in brain, heart and lung showed that they peaked at 5 minutes, then declined rapidly until 480 minutes post-injection and sustained through 24 hours monitored (Figure 2A–2C). Tissue concentrations of all the three miRs/antagomiR in liver and kidney displayed high and sustained levels up to 24 hours with minor initial fluctuation (Figure 2D and 2E). BM had a distinctly different time-course of tissue concentrations: the concentrations of miRs/antagomiR peaked at 120 min, followed by a quick drop until 240 minutes and then remained stable through 24 hours (Figure 2F). The three miRs/antagomiRs appeared to share similar kinetic patterns in each individual tissue except spleen (Figure 2G).

The organ tissue peak concentrations among miRs/antagomiRs differed slightly. For 2'-MeOPSmIR16-1 the highest peak concentration was reached in the kidneys and for 2'-

MeOPSmir29b and 2'-MeOPSantagomiR155 in BM. The lowest peak concentrations were in brain for all three miRs/antagomiRs, and also in heart for 2'-MeOPSmir16-1 and 2'-MeOPSantagomiR155 (Figure 2D–2G). Of note, in examining the concentrations of miRs/antagomiR between the plasma and organ tissues, the sustained drug organ tissue concentrations relative to those in blood indicates that it is unlikely that blood concentrations contributed significantly to those in organ tissues, although possibility still exists. In some organ tissues (e.g. liver and kidney) the synthetic miRs/antagomiRs concentrations sustained and were higher than those in plasma at later times, indicating that plasma PK may not have captured the true terminal phase.

Regulation of Targets Expression by miRs in Mouse Organ Tissues

Since the synthetic miRs levels in mouse organ tissue were measurable and remained relatively stable up to 24 hours, we next determined the biological effects of the miRs/antagomiRs in these tissues. Consistent with the established functions of these miRs, we measured mRNA and protein expression levels of several known major targets of our miRs. *BCL-2* and *MCL-1* are targets of *miR-16-1*¹³, whereas *MCL-1*, *DNMT3a* and *DNMT3b* are targets of *miR-29b*^{16–20}; and *C/EBPβ* is a target of *miR-155*^{28–29}. Since the mature mouse and human sequences of *miR-16-1*, *miR-29b* and *miR-155* are conserved (*mmu-miR-16* MIMAT0000527; *hsa-miR-16* MIMAT0000069; *mmu-miR-29b* MIMAT0000127; *hsa-miR-29b* MIMAT0000100; *mmu-miR-155* MIMAT0000165; *hsa-miR-155* MIMAT0000646), we postulate that our synthetic miRs/antagomiR designed to target human miRs would modulate these targets in mouse tissues. We selected brain and liver tissues to determine the miR's targets effects as proof of principle. In brain tissue, mRNA expressions of *Bcl-2*, *Mcl-1*, *DNMT3a* and *DNMT3b* were all significantly downregulated 24 hours after injection despite low levels of the miRs/antagomiRs. The 24-hour level of each target mRNA was about 40~60% of its 10-minute level (Figure 3A). However, there was no change in *C/EBPβ* mRNA expression after cassette dosing (Figure 3A). Protein level of *Bcl-2* and *DNMT3b* were reduced even only 1-hour post-injection (Figure 3C). In liver tissues, *Bcl-2* and *Mcl-1* mRNA levels showed tendency to decrease at 24 hours, although the decrease was not statistically significant (Figure 3B). *C/EBPβ* mRNA level showed tendency to increase in 1-hour samples but decrease in 24-hour samples (Figure 3B). Protein level of *Bcl-2* was also reduced at 24 hours (Figure 3D). In contrast, mRNA and protein expression of *DNMT3b* was not altered by synthetic miRs in liver (Figure 3C and 3D). Notably, the *DNMT3a* protein levels in both brain and liver tissues were variable among mice (Figure 3C and 3D).

DISCUSSION

Cassette dosing technique, widely used in pharmaceutical industry, has been regarded as a screening method to rapidly place test compounds in rank order based on their PK properties. Although proved to be useful in anticancer drug development^{30–31}, this technology still holds considerable concerns, such as compound-compound interactions, analytical challenges and formulation problems^{24,32}.

Although cassette dosing may not be appropriate for all chemical entities³¹, synthetic miR may be suitable for cassette dosing as multiple miRs are known to be deregulated in cancer and leukemia. Thus concurrently therapeutic correction of multiple miRs may require a cassette dose approach. To our knowledge, we report here the first example of this approach *in vivo* describing the PK and PD evaluation of three cassette-dosed synthetic miRs/antagomiR. Using a quantification method with excellent specificity and selectivity²⁷, we showed that individual miR or antagomiR levels can be accurately determined under the cassette dosing.

Interestingly, although the miRs were concurrently administered, they displayed their individual PK parameters. Comparing with their single doses²⁷, the peak concentration (5 minute concentration or C_5) decreased slightly under the cassette dosing (2'-MeOPSMiR16-1: 3.1 μM for the cassette vs. 6.0 μM for the single; 2'-MeOPSMiR29b: 0.91 μM for the cassette vs. 1.7 μM for the single; 2'-MeOPSantagomiR155: 3.9 μM for the cassette vs. 5.2 μM for the single). The cassette dosing however increased the terminal half-life for 2'-MeOPSMiR16-1 (2508 minutes for the cassette vs. 975 minutes for the single) and, to a much less extent, for 2'-MeOPSantagomiR155 (782.8 minutes for the cassette vs. 561 minutes for the single), but retained similar values for 2'-MeOPSMiR29b (510.6 minutes for the cassette vs. 507 minutes for the single). For 2'-MeOPSMiR16-1, there was a 50% reduction in CL between from the single dose and this was possibly in part due to an increase in terminal half-life (~2.5 fold), although the apparent V_{ss} also increased. Cassette dosing decreased the AUC of 2'-MeOPSMiR29b (~4-fold) but did not change those of the other two miRs (2'-MeOPSMiR16-1: 188.4 $\text{min} \cdot \mu\text{M}$ for the cassette vs. 144.7 $\text{min} \cdot \mu\text{M}$ for the single; 2'-MeOPSantagomiR155: 112.8 $\text{min} \cdot \mu\text{M}$ for the cassette vs. 87.2 $\text{min} \cdot \mu\text{M}$ for the single) significantly. The decrease in AUC value for 2'-MeOPSMiR29b did not cause an increase in CL , since its apparent V_{ss} was significantly decreased (~2 fold) with no change in the terminal half-life.

In drug development, the aim is to target the drug to the effect organ to exert its pharmacological effect. While the pharmacological activity is a key endpoint, achievement of adequate concentrations in target organ tissue is also important. Tissue distribution study helps to provide disposition information of the studied agent. Our data showed that all three synthetic 2'-MeOPS-modified miRs/antagomiR can be directly delivered to all major organ tissues without any formulation. The tissue(s) level is measurable at all time points monitored, from 5 minutes up to 24 hours after dosing. BM, kidney, liver and spleen have relatively higher levels (3~35ng/mg tissue), whereas brain, heart and lung have lower levels (<1 ng/mg tissue). The dose for each miR is 10-fold lower than previously reported doses used for other synthetic RNAs or antagomiRs (>80mg/kg)³³⁻³⁴. The high concentration of miRs/antagomiR in BM and spleen renders them ideal for development to leukemia therapeutics because BM and spleen are primary sites for leukemia cell filtration.

Ideally, the drug should remain relatively inactive or with low activity in the non-target organ tissue. The selectivity is especially important criteria with antitumor agents, they should preferentially elicit antitumor effects on the tumor, while sparing other host organ tissues. For synthetic miRs, their functions on various organs are less known. It is possible that the same miR may have vastly different effects on various organ tissues, or even opposing effects, e.g. *miR-99b* and *miR-125b* are upregulated in color cancer, but downregulated in breast cancer³⁵. While the intent is to use synthetic miRs to augment endogenous *miRs* and modulate their targets, the knowledge of their effects on physiological, pharmacological and/or toxicological functions is equally important. Therefore, in the present study, not only that we quantify the tissue distribution of these compounds but we also measured their effects on some normal organ tissues in terms of their known targets. Surprisingly, we found that brain is sensitive to synthetic miRs. The expression levels of all four miRs molecular targets are decreased 30~60% in brain tissue. Liver is much less responsive to synthetic miRs in modulating bio-targets, although high concentrations of miRs are present in this tissue. Since this work was primarily designed for PK assessment, we did not include scrambled miRs as controls for biological activity analysis. In addition, we did not have 0-minute time point tissue level of miRs' bio-targets for comparison. We used 10-minute time point levels as the basal levels, since we expected that there was no significant difference between 0-minute and 10-minute levels in those targets due to their relatively long half-lives (Bcl-2 $t_{1/2} = 10\sim 24\text{ h}$ ³⁶⁻³⁷; Mcl-1 $t_{1/2} = 1.5\sim 3\text{ h}$ ³⁷; DNMT3a $t_{1/2} = 8\text{ h}$ ³⁸; DNMT3b $t_{1/2} = 4\text{ h}$ ³⁹).

Collectively, this study will open a door for future exploitation of appropriate combination of synthetic miR and/or antagomiR from the same or different families, or even with antisense or RNAi^{33–34}. Future studies on the PK and PD profiles of miR/antagomiR in tumor-bearing mouse models will provide further information on the selectivity of synthetic miR/antagomiR between tumor and normal organ/tissues.

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Figure 1.

Plasma concentration-time profiles of individual synthetic 2'-MeOPSMiRs/antagomiR following their cassette dosing in C57BL/6 mice. *Dots* represent means of measured (observed) concentrations; *bars* represent SE with $n=5\sim6$. *Lines* represent the fitting curves by using a triexponential disposition bolus input model.

Figure 2. Tissue levels of individual synthetic 2'-MeOPSMiRs/antagomiR in C57BL/6 mice following an *i.v.* bolus injection of their mixture at 7.5mg/kg for each: (A) Brain; (B) Heart; (C) Lung; (D) Liver; (E) Kidney; (F) BM; (G) Spleen. Dots represent means of measured concentrations; bars represent SE with n=5~6.

Figure 3.

Target effects of synthetic 2'-MeOPSMiRs/antagomiR in C57BL/6 mice tissues following cassette dosing. (A–B) mRNA expression of Bcl-2, Mcl-1, DNMT3a, DNMT3b and C/EBP β at the indicated time points post-injection in mouse brain (A) and liver (B) tissues, respectively. Histograms show normalized ratio of target mRNA over the internal control GAPDH. The results are presented as the mean $\pm$ SE, $n=5\sim6$. ** $p<0.01$, *** $p<0.001$ when compared to the 10 minute-samples. (C–D) Protein levels of bio-targets in mouse brain (C) and liver (D), respectively, at the indicated time points after injection. Densitometry was

performed to quantify each lane and the ratio of target over the loading control β -actin is presented under each plot.

Table

Relevant PK parameters of 2'-MeOPSmir16-1, 2'-MeOPSmir29b and 2'-MeOPSantagomiR155 given as cassette dosing at 7.5mg/kg each

	C _s (μM)	Alpha (min ⁻¹)	Beta (min ⁻¹)	Gamma (min ⁻¹)	Alpha-HL (min)	Beta-HL (min)	Gamma-HL (min)	AUC _{0-∞} (min*μM)	CL (L/min*kg)	V _{ss} L/kg
miRNA16-1 (SE)	3.1	0.1486 (0.0882)	0.02215 (0.0026)	0.000276 (0.000203)	4.68 (2.76)	31.28 (3.707)	2508.0 (1840.0)	188.38 (40.0)	0.0054 (0.0011)	7.5 (6.23)
miRNA29b (SE)	0.91	0.1147 (0.0544)	0.01325 (0.00376)	0.001375 (0.00060)	6.04 (2.87)	52.32 (14.85)	510.6 (222.2)	27.3 (3.53)	0.042 (0.0057)	4.5 (1.38)
antagomiRNA-155 (SE)	3.9	0.1189 (0.0362)	0.02378 (0.00733)	0.000895 (0.00020)	5.82 (1.77)	29.14 (8.97)	782.8 (176.0)	112.8 (8.24)	0.009 (0.0006)	3.4 (0.8)

All analyses were performed with the pooled mice data for each time point: C_s measured concentrations at 5 minutes; Alpha first exponential decay rate constant; Beta second exponential decay rate constant; Gamma terminal decay rate constant; HL half-life; AUC_{0-∞} area under the curve to time infinity (estimated by trapezoidal rule plus extrapolation); CL total body clearance (compartmental); V_{ss} volume of distribution at steady state (compartmental); Standard error (SE) values were derived from model fitting.