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A Comprehensive Bioanalytical Strategy of *In Vivo* Studies for RNAi Therapeutics in Early Discovery

Nan Zhao, Jiaming Jiang, Qian Cui, Hefeng Zhang, Siyu Liu, Junjun Tian, Zhiren Yu, Hongmei Wang, Zhiyu Li, Lili Xing, Yi Tao, Liang ShenDMPK Service Department, WuXi AppTec, 31 Yiwei Road, Waigaoqiao Free Trade Zone, Shanghai, P.R. China, 200131

PURPOSE

The goal of this study was to establish a comprehensive bioanalytical strategy to speed up the early discovery of RNAi therapeutics. This strategy provides appropriate bioanalytical solutions to support *in vivo* studies for lead compound selection, such as biotransformation, pharmacodynamics (PD) and pharmacokinetics (PK) of small interference RNA (siRNA), and pharmacokinetics of RNA-induced silencing complex (RISC) loading siRNA.

OBJECTIVE(S)

Here, we present how to utilize multiple analytical techniques including the liquid chromatography-tandem mass spectrometry (LC-MS/MS), liquid chromatography-high resolution mass spectrometry (LC-HRMS), reverse transcription quantitative polymerase chain reaction (RT-qPCR), and enzyme-linked immunoassay (ELISA) to determine the PK and PD properties of a GalNAc-conjugated siRNA in mice.

METHOD(S)

siRNA-X is a GalNAc-conjugated siRNA with an asymmetric RNA/dTdT overhang (Figure 1), which is under development for the treatment of complement-mediated diseases by suppressing liver production of complement 5 (C5) protein. An animal study was conducted to evaluate PK and PD properties of siRNA-X in mice following the administration of a single subcutaneous (SC) dose. Three groups of 24 mice were treated at either 0.1, 1 or 5mg/kg of siRNA-X and sacrificed at 1, 6 hours and 1, 2, 3, 7, 14 and 21 days for sample collection. We have developed a LC-MS/MS method to measure concentrations of both sense and antisense strands of siRNA-X in mouse liver samples to determine liver PK parameters. PD were evaluated by measuring target mRNA levels in liver samples and protein levels in serum using RT-qPCR and ELISA, respectively. The comparative RT-qPCR method was applied to measure the fold-change in expression of target gene to evaluate degradation of the targeted mRNA. The expression of the C5 protein was measured by ELISA. To investigate PK/PD relationship, the RISC loading siRNA was measured by stem-loop RT-qPCR with immunoprecipitation, which used a similar strategy to the one described by Nair et al. [1].

RESULT(S)

Quantitation of siRNA-X by LC-MS for in vivo liver samples

Following a single SC administration of siRNA-X, the concentrations of sense and antisense strands of siRNA were determined in liver samples by LC-MS/MS. With the LC-MS/MS method, both the sense strand and antisense strand (AS) were monitored. However, the AS of the siRNA-X with dTdT overhang was not detected, while the sense strand concentration showed a good dose relationship in liver samples. The liver samples were then detected by LC-HRMS in the full scan mode, and the results showed that overhang of siRNA-X was cleaved very quickly *in vivo* to generate an active shortmer (AS (n-2)3'shortmer-siRNA-X). The LC-MS/MS method of AS (n-2)3'shortmer-siRNA-X quantitation in mouse liver was developed and used to reanalyze all liver samples. The concentration of AS (n-2)3'shortmer-siRNA-X increased dose proportionally as dose levels increased from 0.1 mg/kg to 1 mg/kg and from 1 mg/kg to 5 mg/kg after siRNA-X's treatment (Table 1).

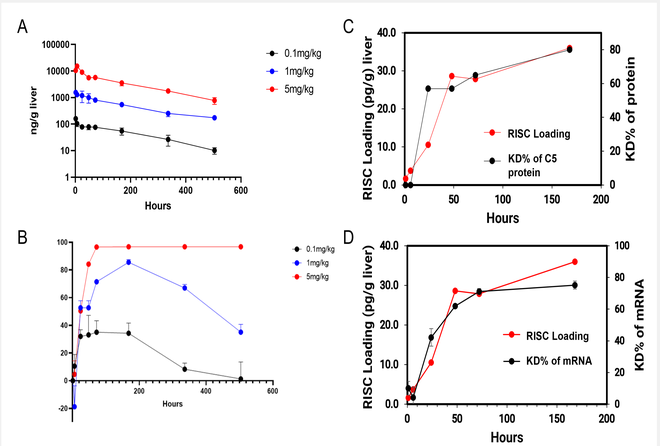

Figure 1. *(A) Concentration-time profiles of AS (n-2)3'shortmer-siRNA-X in mice liver by LC-MS/MS method. Units are in ng antisense siRNA per gram of liver (ng/g). (B) C5 protein level in mice serum by ELISA. (C) Knockdown efficiency of C5 protein in mice liver (black line) by a single subcutaneous dose (1 mg/kg). RISC-loaded antisense siRNA in mice liver (red line) was quantified by stem-loop RT-qPCR coupled with immunoprecipitation. Units are in pg antisense siRNA per gram of liver (pg/g). (D) Knockdown efficiency of target mRNA expression in mice serum (black line) by a single subcutaneous dose (1 mg/kg). RISC-loaded antisense siRNA in mice liver (red line) was quantified by stem-loop RT-qPCR coupled with immunoprecipitation. Units are in pg antisense siRNA per gram of liver (pg/g).*

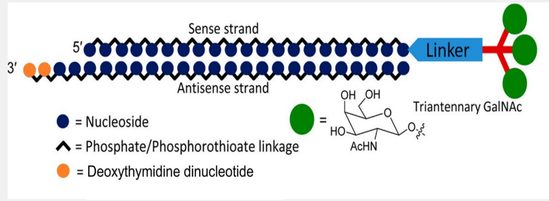

Figure 2. *Structure of siRNA-X.*

Table 1. *Concentrations of AS and AS (n-2)3'shortmer in mouse liver samples by LC-MS/MS method.*

Sample time	Group 1, 0.1 mg/kg		Group 2, 1 mg/kg		Group 3, 5 mg/kg	
	Mean Concentration (ng/g)		Mean Concentration (ng/g)		Mean Concentration (ng/g)	
	AS	AS (n-2)3' shortmer	AS	AS (n-2)3' shortmer	AS	AS (n-2)3' shortmer
1h	ND	193	ND	2867	26	13900
6h	ND	191	ND	2452	67	19267
24h	ND	94	ND	1667	ND	9417
48h	ND	109	ND	1745	27	7483
168h	ND	72	ND	810	ND	4808
336h	ND	36	ND	286	ND	2008
504h	ND	ND	ND	136	ND	1123

Measurement of PK/PD properties of GalNAc-conjugated siRNA

The previous studies had confirmed that liver PK and siRNA levels in RISC in hepatocytes were better predictors of PD than plasma PK [2]. The liver concentration-time profiles of AS (n-2)3'shortmer-siRNA-X in mice were shown in Figure 2A, which demonstrated a continuous increase in effect with increasing dose. The reduction of protein expression also increased dose proportionally as dose levels increased from 0.1 mg/kg to 1 mg/kg and from 1 mg/kg to 5 mg/kg after siRNA-X's treatment (Figure 2B). Gene knockdown can be detected as early as 6 hours and could last up to more than 7 days. The concentration of RISC loading siRNA was too low to be detected by LC-MS/MS method, and was quantified by stem-loop RT-qPCR method coupled with immunoprecipitation. The siRNA-X level in RISC showed a direct correlation with target gene knockdown and C5 protein knockdown (Figure 2C and 2D).

CONCLUSION(S)

We develop a comprehensive bioanalytical platform including LC-MS/MS, LC-HRMS, ELISA and RT-qPCR, which is critical for the early discovery of RNAi therapeutics. Compared to LC-MS/MS or LC-HRMS method, RT-qPCR method could provide ultrahigh sensitivity in the pg/mL range for siRNA quantitation and support to build PK/PD modeling by using in early discovery of RNAi therapeutics. However, LC-MS-MS or LC-HRMS method can accurately quantify and discriminate shortmers from the full-length siRNA. The level of target gene and downstream protein could be evaluated for efficacy assay by using RT-qPCR and ELISA, prospectively.

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