T1330-06-35

Comparative Metabolite Profiling and Identification of a GalNAc-Conjugated siRNA, siRNA01, in Plateable Rat Hepatocytes and *in vivo* Liver using LC-UV-HRMS

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PURPOSE

Hepatocytes are recommended as a good *in vitro* metabolism model for oligonucleotides because they closely mimic the uptake into specific compartments of hepatocytes *in vivo*. However, there are some challenges in using hepatocytes to study the metabolism of GalNAc-conjugated siRNAs compared to that of conventional small molecules. In this study, we selected siRNA01, a commercially available GalNAc-conjugated siRNA, as a model compound for a comprehensive investigation into both *in vitro* and *in vivo* metabolism. A detailed comparison of *in vivo* and *in vitro* metabolite profiles in rats was conducted.

OBJECTIVES

- To optimize incubation conditions for plateable hepatocytes that could simulate the *in vivo* metabolism of siRNA01.
- To provide valuable insights into the metabolic characteristics of GalNAc-conjugated siRNAs.

METHODS

- ✓ siRNA01 was incubated in plateable rat hepatocytes for 24 hours.
- ✓ The SD rats were dosed subcutaneously with siRNA01 with a dosage of 50 mg/kg one time.
- ✓ An LC-UV-MS/MS method was successfully developed with Waters ACQUITY UPLC-PDA coupled with HRMS (Q-Exactive Plus) in full MS/ddMS^2 mode for analysis of siRNA01.
- ✓ Liquid-liquid extraction and/or solid-phase extraction was involved in the sample treatment process.

RESULTS

In order to investigate and optimize the *in vitro* hepatocyte incubation system to simulate the *in vivo* metabolism of siRNA01, we first performed the *in vivo* metabolite identification in rat liver to understand the pattern of siRNA01 metabolism in vivo.

Full-length AS and its 3’ n-1 were observed as the major components, with the relative abundance of 47.63% and 48.00%, respectively. The metabolites with 5 to 7, 9 to 11 nucleotides from 3’-end truncated, and 4 to 6 nucleotides from 5’-end truncated metabolites with or without one nucleotide 3´-end, were also observed and identified as minor metabolites (**Table 1**).

For the sense strand (SS), observed metabolites were mostly the cleavage products in the triantennary GalNAc moiety, resulting from the loss of GalNAc groups, complete loss of the triantennary targeting moiety, or cleavage in the middle of the targeting moiety. So SS-3GalNAc and further hydrolysis metabolite SS-3GalNAc-100 were the major metabolites in rat liver *in vivo*. In addition, 7 metabolites from the loss of GalNAc and further loss of linker were detected and identified with a relative abundance of less than 4%.

The above *in vivo* liver metabolite identification results provided a basis for optimizing hepatocyte incubation conditions. Following this, a series of *in vitro* hepatocyte incubation conditions were optimized, including incubation medium, compound concentrations, etc.

Under the optimized conditions for plateable rat hepatocytes, AS 3' n-1 was the major metabolite of AS strand with a relative abundance of 50.00%, which is comparable to the relative abundance of 47.63% in the *in vivo* liver.

For the SS strand, only minor intact SS was detected in the plateable hepatocytes with a relative abundance of 4.37%. The main metabolites were the cleavage products in the triantennary GalNAc moiety, including SS-related metabolites resulting from the loss of GalNAc groups, complete loss of the triantennary targeting moiety, or cleavage in the middle of the targeting moiety. These results are very similar to the *in vivo* liver results.

Table 1. Comparative Metabolite Profiling of siRNA01 in Plateable Rat Hepatocytes and In Vivo Rat Liver Metabolism

Strand	Code	Metabolic Changes	Relative Abundance (MS Peak Area %Total)	
			In Vitro Plateable Hepatocytes [a]	*In Vivo* Liver
AS	M1	AS 5' n-6_3' n-1	ND	1.34
	M2	AS 5' n-5_3' n-1_A6+P	ND	0.01
	M3	AS 5' n-4_3' n-1_A5+P	ND	0.02
	M4	AS 5' n-5_A6+P	ND	0.01
	M5	AS 5' n-6	ND	1.16
	M6	AS 5' n-4_A5+P	ND	0.01
	M7	AS 3' n-11	ND	0.10
	M8	AS 3' n-10	ND	0.11
	M9	AS 3' n-9	ND	0.63
	M10	AS 3' n-7	ND	0.41
	M11	AS 3' n-6	ND	0.15
	M12	AS 3' n-5	ND	0.42
	M13	AS 3' n-1	50.00	48.00
	AS	AS	50.00	47.63
SS	M14	SS 3' n-6	ND	1.24
	M15	SS 3' n-5	ND	0.09
	M16	SS 3' n-4	ND	0.50
	M17	SS 3' n-3	ND	0.98
	M18	SS 3' n-2	ND	0.57
	M19	SS-3GalNAc-300	18.28	0.13
	SS	SS	4.37	ND
	M20	SS-3GalNAc-256	ND	0.33
	M21	SS-3GalNAc-200	28.67	2.48
	M22	SS-3GalNAc 5' n-5	ND	0.11
	M23	SS-3GalNAc-156	ND	2.70
	M24	SS-2GalNAc-141	ND	2.78
	M25	SS-3GalNAc-100	30.96	21.19
	M26	SS-2GalNAc-41	ND	3.70
	M27	SS-3GalNAc	17.72	63.20

[a]: Values represent the average data, n = 2; ND: Not detected.

$$\%\text{Total} = \frac{\text{Peak Areas of AS or SS Related Component}}{\text{Peak Areas of Total AS or SS Related Components}} \times 100$$

CONCLUSIONS

- ✓ We have successfully established an *in vitro* model using plateable rat liver hepatocytes.
- ✓ Under the optimized incubation conditions of plateable rat hepatocytes, the metabolism of both the AS and SS of siRNA01 was similar to that *in vivo*.
- ✓ This demonstrates that our *in vitro* metabolic system can effectively predict *in vivo* liver metabolism of siRNA01, providing a valuable tool for further research in this field.
- ✓ This research not only provides deeper insights into the metabolic characteristics of siRNA01, but also lays a crucial foundation for future metabolism studies of other types of siRNAs using plateable hepatocytes, thereby helping to promote the optimization and development of oligonucleotide therapeutics.

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