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Effect of pH on Metabolite Profiling and Identification of GalNAc Conjugated siRNA in *In Vitro* Metabolic System

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PURPOSE

The metabolites of GalNAc conjugated siRNA were compared in *in vitro* incubation systems with different pH values to evaluate their influence on the metabolism of GalNAc conjugated siRNA, and then help to select appropriate experiment conditions for siRNA *in vitro* metabolism evaluation.

METHOD(S)

Test article: siRNA-01, a commercial product, is a synthetic siRNA targeting to liver and conjugated to L96, with triantennary N-acetylgalactosamine carbohydrates (GalNAc) in its sense strand.

In vitro liver S9 incubation: siRNA-01 was incubated in mouse, rat, dog, monkey and human liver S9 fractions for 24 hours at 37°C with the buffer of pH 5, 6 and 8, respectively.

In vitro liver homogenate: siRNA-01 was incubated in mouse, rat, and monkey liver homogenate for 24 hours at 37°C with the buffer of pH 5, 6 and 8, respectively.

An LC-UV-MS/MS method was successfully developed with Waters® ACQUITY UPLC® PDA coupled with HRMS (Thermo Scientific™ Q-Exactive™ Plus) in full MS/ddMS^2 mode for analysis of siRNA-01.

The sample treatment process was involved in special liquid-liquid extraction or solid phase extraction.

RESULT(S)

Liver S9 Fractions

In the system with pH = 5, the major metabolite of antisense strand (AS) was AS 3' n-1, with relative abundance of 1.80%~6.18% in five species of liver S9 fractions. The relative abundance of intact AS was more than 93.5% in all five species. For sense strand (SS), the loss of one to three GalNAc groups was the major metabolites, total accounting for 43.92%~95.20%.

In the system with pH = 6, the major metabolites of AS were AS 3' n-1, and AS 5' n-5. The relative abundance of AS 3' n-1 in was 8.99~34.77% in five species. The AS 5' n-5 accounted 22.93% in rat, and <4.5% in other four species. The relative abundance of intact AS was 54.31%~78.79% in five species. For SS, the loss of one to three GalNAc groups was also the major metabolites, with relative abundance 18.36~57.17% in five species. Their proportion was obviously lower than that of pH = 5.

In the system with pH = 8, the major metabolites of AS were AS 3' n-1, AS 5' n-5 with and without 3' n-1. The relative abundance of AS 3' n-1 was 6.36%~28.42% in five species. The AS 5' n-5 with and without 3' n-1 accounted 15.17% and 24.10% in monkey, but <2.5% and <1% in other four species, respectively. For SS, the intact SS was the dominated component, with relative abundance of 98.63% to 100% in five species. Only one SS related metabolite (SS-1GalNAc) was detected with low relative abundance of 0.34~1.37% in mouse, dog, monkey and human. No SS related metabolites were detected in rat.

Liver Homogenate

In the system with pH = 5, only a small amount of AS metabolized. Total three to five AS related metabolites were detected in mouse, rat and monkey. The intact AS accounted between 96.42% to 97.13% in mouse, rat and monkey. For SS, top three metabolites (loss of one, two and three GalNAc groups metabolites) were detected with total relative abundance of 66.45%, 80.1% and 49.69% in mouse, rat and monkey, respectively.

In the system with pH = 6, total 12 to 14 AS related metabolites were detected in mouse, rat and monkey. The top metabolite AS 3' n-1 was accounted >30%. For SS, the top three metabolites (loss of one to three GalNAc groups metabolites) were detected with total relative abundance >48.58%. In addition to the top three metabolites, small amount of SS 3' n-2, n-3, n-4, n-5 and n-6 metabolites were also detected in rat.

In the system with pH = 8, total 6 to 7 AS related metabolites were detected in mouse, rat and monkey. The intact AS accounted between 94.01% to 95.61% in mouse, rat and monkey. For SS, only one SS related metabolite (SS-1GalNAc) was detected with minor amount (<1%). The intact SS accounted more than 99%.

Table 1. *Summary of The Relative Abundance of Metabolites of siRNA-01 in Liver S9 Fractions and Liver Homogenate with Different pH Incubation Conditions*

Metabolic Change	Liver S9 Fractions															Liver Homogenate								
	Mouse			Rat			Dog			Monkey			Human			Mouse			Rat			Monkey		
	pH=5	pH=6	pH=8	pH=5	pH=6	pH=8	pH=5	pH=6	pH=8	pH=5	pH=6	pH=8	pH=5	pH=6	pH=8	pH=5	pH=6	pH=8	pH=5	pH=6	pH=8	pH=5	pH=6	pH=8
AS 5' n-14	ND	ND	ND	ND	0.03	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.02	ND	ND	ND	ND
AS 5' n-6_3' n-1	ND	0.03	0.01	ND	0.04	ND	ND	0.02	ND	ND	0.02	ND	ND	0.02	0.02	ND	0.26	ND	ND	2.35	ND	ND	0.12	ND
AS 5' n-5_3' n-1_P	ND	0.24	0.71	ND	4.78	ND	ND	0.19	0.79	ND	1.11	15.17	ND	0.25	0.15	ND	0.25	ND	ND	9.51	ND	ND	2.75	0.02
AS 5' n-4_3' n-1_P	ND	0.16	0.44	ND	0.86	ND	ND	0.09	0.34	ND	0.31	1.77	ND	0.15	0.08	ND	0.17	ND	ND	1.15	ND	ND	0.69	0.01
AS 5' n-5_P	ND	1.22	2.48	ND	22.93	ND	ND	1.03	1.72	ND	4.14	24.10	ND	0.41	0.27	ND	0.26	0.50	0.04	8.84	0.34	ND	3.91	0.42
AS 5' n-6	ND	0.17	0.07	ND	0.43	ND	ND	0.10	ND	ND	0.15	ND	ND	0.06	ND	0.14	0.31	0.10	0.13	2.10	0.10	0.12	0.19	0.10
AS 5' n-4_P	ND	0.76	1.61	ND	4.18	ND	ND	0.55	0.69	ND	1.22	3.14	ND	0.22	ND	ND	0.13	0.35	0.04	1.32	0.19	ND	1.01	0.18
AS 3' n-11	ND	ND	ND	ND	0.01	ND	ND	ND	ND	ND	0.01	ND	ND	0.06	ND	ND	0.01	ND	ND	0.04	ND	ND	ND	ND
AS 3' n-10	ND	ND	ND	ND	0.05	ND	ND	ND	ND	ND	0.01	0.04	ND	ND	ND	ND	ND	ND	ND	0.07	ND	ND	0.01	ND
AS 3' n-9	ND	ND	ND	ND	0.24	ND	ND	ND	ND	ND	0.03	0.36	ND	0.37	0.11	ND	ND	ND	ND	0.28	ND	ND	0.01	ND
AS 3' n-7	ND	ND	0.20	ND	0.13	ND	ND	ND	0.05	ND	0.01	1.32	ND	0.62	0.21	ND	0.02	ND	ND	3.98	ND	ND	0.77	ND
AS 3' n-6	ND	0.20	0.46	ND	2.36	ND	ND	0.06	0.29	ND	0.95	4.16	ND	0.02	0.04	ND	0.02	ND	ND	0.01	0.01	ND	0.06	ND
AS 3' n-5	ND	0.06	0.06	ND	0.67	ND	ND	0.01	ND	ND	0.13	0.03	ND	0.24	0.08	ND	0.05	0.02	ND	0.72	0.02	ND	0.17	ND
AS 3' n-2	ND	0.15	ND	ND	ND	ND	0.04	0.14	ND	ND	ND	ND	ND	ND	ND	0.57	1.05	0.64	0.82	1.66	0.55	0.69	0.58	0.68
AS 3' n-1	2.64	18.42	17.06	1.80	8.99	6.36	2.30	15.67	24.63	4.30	17.37	16.92	6.18	34.77	28.42	2.16	46.65	2.77	2.55	30.96	4.49	2.18	30.46	4.58
AS-NH+O	ND	ND	ND	ND	ND	ND	ND	3.35	ND	ND	1.74	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.16	ND
AS	97.36	78.60	76.91	98.20	54.31	93.64	97.67	78.79	71.48	95.70	72.80	32.99	93.82	62.81	70.61	97.13	50.82	95.61	96.42	37.02	94.31	97.01	56.12	94.01
SS 3' n-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.45	ND	ND	ND	ND
SS 3' n-5	ND	ND	ND	ND	0.01	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.13	ND	ND	ND	ND
SS 3' n-4	ND	ND	ND	ND	0.01	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.18	ND	ND	ND	ND
SS 3' n-3	ND	ND	ND	ND	0.02	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.25	ND	ND	ND	ND
SS 3' n-2	ND	ND	ND	ND	0.05	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.03	ND	ND	ND	ND
SS	9.09	71.27	99.29	3.48	42.74	100.00	36.82	60.82	98.90	25.44	43.49	98.63	56.08	81.64	99.66	33.55	51.42	99.38	19.71	11.31	99.22	50.31	42.38	99.41
SS-1GalNAc	11.97	22.89	0.71	3.82	35.72	ND	31.30	31.70	1.10	27.44	37.81	1.37	27.41	10.76	0.34	14.24	31.66	0.62	7.45	23.41	0.78	23.47	39.97	0.59
SS-2GalNAc	17.84	3.95	ND	6.39	15.68	ND	17.14	5.73	ND	21.35	14.80	ND	10.19	7.23	ND	15.92	11.99	ND	8.87	28.44	ND	13.31	14.67	ND
SS-3GalNAc	61.10	1.89	ND	84.99	5.78	ND	14.74	1.75	ND	25.77	3.90	ND	6.32	0.37	ND	36.29	4.93	ND	63.69	32.80	ND	12.91	2.98	ND
SS-3GalNAc-100	ND	ND	ND	1.10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.28	ND	ND	ND	ND	ND
SS-3GalNAc-200	ND	ND	ND	0.16	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SS-3GalNAc-300	ND	ND	ND	0.06	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

P: Phosphorylation

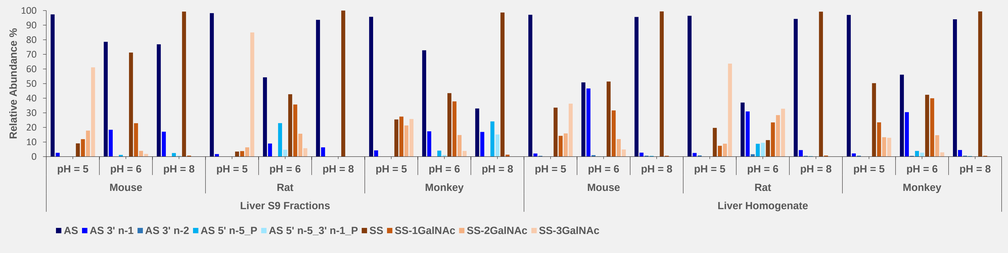

Figure 1. *Major Metabolites in Different pH Incubation Conditions.*

CONCLUSION(S)

1. In this study, higher GalNAc loss of SS was observed under lower pH conditions after incubation of siRNA-01 for 24 h, and the metabolism rate of SS in liver S9 fraction and liver homogenate was pH 5.0 > pH 6.0 > pH 8.0.
2. In the incubation system with pH = 6.0, both AS and SS of siRNA-01 had comprehensive metabolism in liver S9 and liver homogenate, which is close to their metabolism *in vivo* (Figure 2*).* It demonstrated the *in vitro* metabolic condition under pH = 6.0 is more suitable to predict the *in vivo* metabolism for siRNA-01.
3. The same conclusion was obtained with more GalNAc conjugated siRNAs evaluation under PH=6.0 condition. Several internal cases showed that this PH condition is also applicable to the metabolism of some non-GalNAc conjugated Oligos.

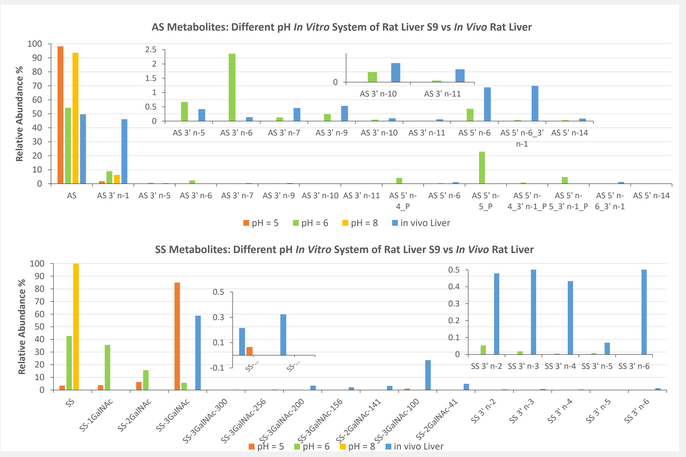

Figure 2. *The Metabolites of siRNA-01 in Different pH In Vitro System of Rat Liver S9 Comparing with In Vivo Rat Liver*

REFERENCES

[1] Babak Basiri, et al. Introducing an *In Vitro* Liver Stability Assay Capable of Predicting the *In Vivo* Pharmacodynamic Efficacy of siRNAs for IVIVC. Molecular Therapy: *Nucleic Acids Vol. 21 September 2020, 725-734.*

[2] Jaeah Kim, et al. *In vitro* metabolism of 2'-ribose unmodified and modified phosphorothioate oligonucleotide therapeutics using liquid chromatography mass spectrometry. Biomedical Chromatography. 2020, 34, e4839.