Modification of Small Molecule Drug Methods to Confirm Drug Safety of Large Double Stranded siRNA Biotherapeutics

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BACKGROUND

The U.S. FDA has issued guidance for Metabolites in Safety Testing (MIST) which includes characterization and quantitation of drug metabolites used to determine safety. The guidance targets small molecule drug metabolites but the same guidance can be used to estimate the safety of larger biologic drugs, such as siRNA therapeutics. We used three commercial double stranded siRNA drugs in a series of inter-species, *in vitro*, qualitative and quantitative metabolite characterization experiments using modified small molecule procedures to confirm internal process to establish similar confidence for MIST.

METHODS

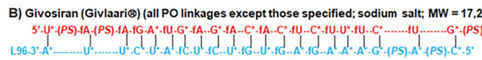
Example siRNA drugs Patisiran®, Givosiran®, and Lumasiran®, were incubated in rat, monkey, and human liver HepatoPac®. Each of the samples were solid-phase extracted, using Clarity OTX 96-well plates (mixed mode AX). All extracted samples were analyzed by UPLC/Q-TOF-HRMS (Shimadzu LC-30AD/Sciex 6600+) in the negative ESI mode. Chromatographic separation using an Acquity BEH C18, 130Å, 1.7 µm, 2.1 x 50 mm (Waters) column with the ion-pairing mobile phases A:aq:DIPA:HFIP and B: aq:MeOH:DIPA:HFIP.

Observed accurate mass molecular ions of parents and metabolites were used to confirm, identify, and profile the metabolites by comparing to its theoretically calculated M_w. The concentration of the parent strands and metabolites of the test siRNA's were calculated relative to an analogue IS. The peak areas of the metabolites were then compared to the corresponding parent strand peak areas. Only metabolites found to be above 1.0% of the administered dose were reported, which is 10x lower than outlined in MIST FDA guidance.

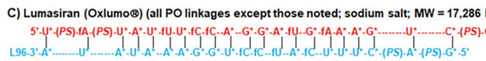
Structures of Three siRNA's Patisiran®, Givosiran®, Lumasiran®, and L-96 GalNAc Terminal Group

A) Patisiran (Onpattro®) (all PO linkages; sodium salt; MW = 14,304 Da; LNP formulation)

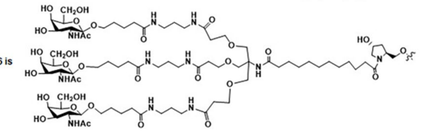
B) Givosiran (Givlaari®) (all PO linkages except those specified; sodium salt; MW = 17,245.56 Da)



C) Lumasiran (Oxlumo®) (all PO linkages except those noted; sodium salt; MW = 17,286 Da)



Where L96 is



(A) Patisiran (B) Givosiran and (C) Lumasiran . (B) and (C) are both siRNAs in which the sense (or passenger) strand is covalently linked to three GalNAc moieties facilitating the delivery via the guide strand, also containing two 3'-overhang ribonucleotides connected through phosphorothioate (PS) linkages. d = Thymine. A=Adenine, C=Cytosine, G=Guanine, U=Uracil; d = deoxy; f = 2'-fluoro; * = 2'-methoxy.

RESULTS and CONCLUSIONS

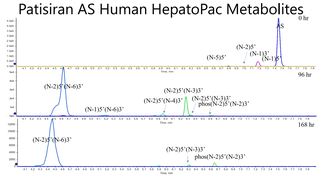
There were no disproportional nor unique human metabolites in any of the siRNA's so here we will only report HepatoPac results from human. Both AS and SS strands, in Patisiran showed extensive metabolism over 168 hrs. Givosiran and Lumasiran AS and SS strands were more resistant to metabolism than Patisiran, likely due to the phosphorothioate groups located near the 3' and 5' terminal ends. For all three antisense strands tested, two types of metabolites were observed: loss of oligo groups, primarily exonuclease activity at the (N-x)3' and (N-x)5' ends. Some of these metabolites with larger sections of the oligonucleotide cleaved could also be result of endonuclease activity.

The sense strand for all three test compounds also exhibited 3' and 5' exonuclease metabolism. The GalNAc terminal group for Givosiran and Lumasiran both also exhibited esterase metabolism resulting in the sequential cleavage of the *N*-acetyl-galactosamine (L-96) groups.

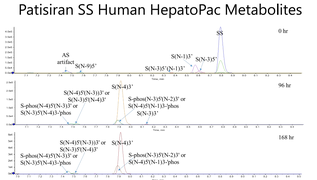
Quantitative calculations all assume similar extraction recovery of the metabolites, equal mass spectral response for parents and metabolites, and a linear HRMS response over the concentration range. These results should be considered semi-quantitative, however, still useful to identify preclinical coverage for metabolites which might require future testing. These results suggest that Patisiran would make an excellent positive control and Givosiran and Lumasiran excellent negative controls for future studies.

Patisiran®, Givisiran®, and Lumasiran® Metabolites Human HepatoPac Over 168 hr

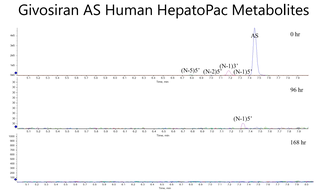
Patisiran AS Human HepatoPac Metabolites



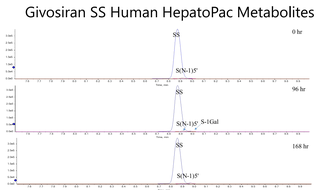
Patisiran SS Human HepatoPac Metabolites



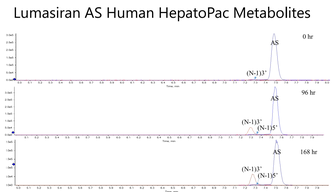
Givosiran AS Human HepatoPac Metabolites



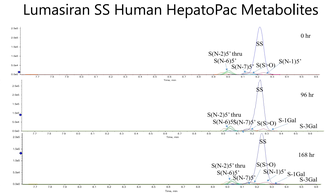
Givosiran SS Human HepatoPac Metabolites



Lumasiran AS Human HepatoPac Metabolites



Lumasiran SS Human HepatoPac Metabolites



Patisiran AS Human HepatoPac Metabolites

Component	Sequence	Ret.Time	MW (Da)	Percent of Total		
				0 hr	96 hr	168 hr
AS	rArUrGrGrArAmUrArCrUrCrUrUrGrGrUmUrArCdTdT	7.46	6656.92	91.0%	ND	ND
AS(N-1)5'	rUrGrGrArAmUrArCrUrCrUrUrGrGrUmUrArCdTdT	7.19	6327.87	0.1%	ND	ND
AS(N-1)3'	rArUrGrGrArAmUrArCrUrCrUrUrGrGrUmUrArCdT	7.18	6352.87	8.1%	ND	ND
AS(N-2)5'	rGrGrArAmUrArCrUrCrUrUrGrGrUmUrArCdTdT	7.05	6021.84	0.2%	ND	ND
AS(N-3)3'	rArUrGrGrArAmUrArCrUrCrUrUrGrGrUmUrA	6.84	5743.76	0.2%	ND	ND
AS(N-5)5'	rAmUrArCrUrCrUrUrGrGrUmUrArCdTdT	6.83	5002.70	0.7%	ND	ND
phos-AS(N-2)5'(N-2)3'	phos-rGrGrArAmUrArCrUrCrUrUrGrGrUmUrArC	6.58	5493.72	ND	0.3%	1.0%
AS(N-2)5'(N-2)3'	rGrGrArAmUrArCrUrCrUrUrGrGrUmUrArC	6.33	5413.75	ND	0.4%	ND
AS(N-2)5'(N-3)3'	rGrGrArAmUrArCrUrCrUrUrGrGrUmUrA	6.25	5108.71	ND	7.7%	0.4%
AS(N-2)5'(N-4)3'	rGrGrArAmUrArCrUrCrUrUrGrGrUmU	5.93	4779.66	ND	1.0%	ND
phos-AS(N-1)5'(N-6)3'	phos-rUrGrGrArAmUrArCrUrCrUrUrGrG	5.81	4539.58	ND	0.3%	ND
AS(N-1)5'(N-6)3'	rUrGrGrArAmUrArCrUrCrUrUrGrG	5.19	4459.62	ND	1.0%	ND
AS(N-2)5'(N-6)3'	rGrGrArAmUrArCrUrCrUrUrGrG	4.64	4153.59	ND	89.3%	98.5%

ND = not detected

Patisiran SS Human HepatoPac Metabolites

Component	Sequence	Ret.Time	MW (Da)	Percent of Total		
				0 hr	96 hr	168 hr
S	rGmUrArAmCmCrArArGrArGmUrAmUmUmCmCrAmUdTdT	8.92	6761.08	82.7%	ND	ND
S(N-1)3'	rGmUrArAmCmCrArArGrArGmUrAmUmUmCmCrAmUdT	8.69	6457.05	12.0%	ND	ND
S(N-3)5'	rAmCmCrArArGrArGmUrAmUmUmCmCrAmUdTdT	8.72	5766.94	3.3%	ND	ND
S(N-3)5'(N-1)3'	rGmUrArAmCmCrArArGrArGmUrAmUmUmCmCrA-phos	8.37	5462.89	0.2%	ND	ND
S(N-3)3'	rGmUrArAmCmCrArArGrArGmUrAmUmUmCmCrA	8.12	5832.94	ND	0.4%	ND
S(N-4)3'	rGmUrArAmCmCrArArGrArGmUrAmUmUmCmC	7.91	5503.89	ND	85.5%	72.0%
S-phos(N-3)5'(N-2)3'	phos-mAmCmCrArArGrArGmUrAmUmUmCmCrAmU	7.88	5238.81	ND	7.5%	14.0%
S(N-9)5'	rAmGmUrAmUmUmCmCrAmUdTdT	7.57	3796.62	1.3%	ND	ND
S(N-4)5'(N-3)3' or S(N-3)5'(N-4)3'	mCmCrArArGrArGmUrAmUmUmCmCrA rAmCmCrArArGrArGmUrAmUmUmCmC	7.53	4509..75	ND	2.9%	2.9%
S-phos(N-4)5'(N-3)3'	phos-mCmCrArArGrArGmUrAmUmUmCmCrA	7.48	4589.72	ND	3.8%	11.1%
S(N-9)5'(N-1)3'	rArGmUrAmUmUmCmCrAmUdT	7.14	3492.57	0.6%	ND	ND

ND = not detected

Givosiran AS Human HepatoPac Metabolites

Component	Sequence	Ret.Time	MW (Da)	Percent of Total		
				0 hr	96 hr	168 hr
AS	mUsfAsfAfGmAfUmGfAmGfAmCfAmCfUmCfUmUfUmCfUmGsmGsmU	7.27	7559.01	98.9%	73.7%	61.2%
AS(N-1)5' or AS(N-1)3'	fAsfAsfGmAfUmGfAmGfAmCfAmCfUmCfUmUfUmCfUmGsmGsmU or mUsfAsfAfGmAfUmGfAmGfAmCfAmCfUmCfUmUfUmCfUmGsmG	7.08	7223.99	1.1%	26.0%	38.2%
s(N-1)5'(N-1)3'	sfAsfAfGmAfUmGfAmGfAmCfAmCfUmCfUmUfUmCfUmGsmG	6.89	6864.04	ND	0.3%	0.6%

ND = not detected

Givosiran SS Human HepatoPac Metabolites

Component	Sequence	Ret.Time	MW (Da)	Percent of Total		
				0 hr	96 hr	168 hr
S	mCsmAsmGmAmAmAfGmAfGmUfGmUfCmUfCmAmUmCmUmUmA-L96	8.88	8732.02	98.0%	97.4%	95.9%
S-1Gal	mCsmAsmGmAmAmAfGmAfGmUfGmUfCmUfCmAmUmCmUmUmA-L96-1Gal	9.01	8528.94	0.6%	1.3%	2.6%
S(N-1)5'	mAsmGmAmAmAfGmAfGmUfGmUfCmUfCmAmUmCmUmUmA-L96	8.93	8396.99	1.5%	1.3%	1.5%

Lumarsiran AS Human HepatoPac Metabolites

Component	Sequence	Ret.Time	MW (Da)	Percent of Total		
				0 hr	96 hr	168 hr
AS	mUsfAsmUmAmUfUmUfCfCmAmGmGmAfUmGfAmAmAmGmUmCsmCsmA	7.48	7627.14	99.3%	90.0%	86.5%
AS(N-1)5'	fAsmUmAmUfUmUfCfCmAmGmGmAfUmGfAmAmAmGmUmCsmCsmA	7.40	7291.12	0.3%	0.3%	0.3%
AS(N-1)3'	mUsfAsmUmAmUfUmUfCfCmAmGmGmAfUmGfAmAmAmGmUmCsmC	7.25	7268.1	0.4%	9.7%	13.2%

Lumasiran SS Human HepatoPac Metabolites

Component	Sequence	Ret.Time	MW (Da)	Percent of Total		
				0 hr	96 hr	168 hr
S	mGsmAsmCmUmUmUfCmAfUfCfCmUmGmGmAmAmAmUmAmUmA-L96	9.24	8704.01	78.1%	77.2%	78.3%
S-3Gal	mGsmAsmCmUmUmUfCmAfUfCfCmUmGmGmAmAmAmUmAmUmA-L96-3Gal	9.56	8095.78	ND	0.1%	0.2%
S-1Gal	mGsmAsmCmUmUmUfCmAfUfCfCmUmGmGmAmAmAmUmAmUmA-L96-1Gal	9.34	8501.94	0.2%	0.5%	0.9%
S(N-1)5'	mAsmCmUmUmUfCmAfUfCfCmUmGmGmAmAmAmUmAmUmA-L96	9.26	8329.98	3.3%	3.2%	3.0%
S(S=O)	mGmAsmCmUmUmUfCmAfUfCfCmUmGmGmAmAmAmUmAmUmA-L96	9.16	8688.03	1.4%	1.5%	1.4%
S(N-2)5'	mCmUmUmUfCmAfUfCfCmUmGmGmAmAmAmUmAmUmA-L96	9.00	7970.93	2.4%	2.6%	2.3%
S(N-3)5'	mUmUmUfCmAfUfCfCmUmGmGmAmAmAmUmAmUmA-L96	9.04	7651.83	4.3%	4.5%	4.3%
S(N-4)5'	mUmUfCmAfUfCfCmUmGmGmAmAmAmUmAmUmA-L96	9.03	7331.83	2.2%	2.2%	2.1%
S(N-5)5'	mUfCmAfUfCfCmUmGmGmAmAmAmUmAmUmA-L96	9.02	7011.79	2.4%	2.5%	2.4%
S(N-6)5'	fCmAfUfCfCmUmGmGmAmAmAmUmAmUmA-L96	9.03	6691.73	3.7%	3.7%	3.5%
S(N-7)5'	mAfUfCfCmUmGmGmAmAmAmUmAmUmA-L96	8.89	6384.72	2.2%	1.8%	1.5%

ND = not detected