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A CASE STUDY FOR VISUALIZING OLIGONUCLEOTIDE THERAPEUTICS DISTRIBUTION IN RAT ORGANS USING MALDI MASS SPECTROMETRY IMAGING

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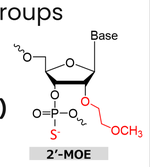
Objective

Therapeutic oligonucleotides (OGNs), which are part of next-generation pharmaceuticals, have been introduced to the market continuously since around 2016, and have been actively developed in recent years. Various quantitative methods (e.g. ligand binding assays utilizing hybridization, fluorescent-LC of the hybridized sample with the fluorescence probe, and LC-MS/MS using ion pair reagents or hydrophilic interaction liquid chromatography columns) have been reported for measuring OGNs in the body after administration. However, these methods cannot provide information on the localization of administered OGNs within tissues. On the other hand, mass spectrometry imaging (MSI) has attracted attention in recent years in the field of drug discovery research. This technique has allowed visualization of the spatial distribution of target compounds in biological samples without labeling. Therefore, the aim of this study is to visualize the distribution of OGNs in tissues using MSI.

Materials and Methods

OGNs used in this study: Nusinersen

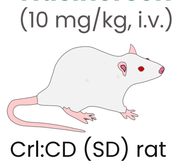
- Marketed as **SPINRAZA®**
- Treating **spinal muscular atrophy** (SMA)
- Antisense oligonucleotide which modulates splicing
- Replaced with **2′-*O* -2-methoxyethyl (2′-MOE)** groups
- Replaced with **phosphorothioate (PS)** linkages
- **Methylated with 5′ Cytosine**



5′-T^C^A^A^C^T^T^T^C^A^A^T^A^A^T^G^C^T^G^G-3′ (18 mer)

Underline; RNA with 2′-MOE modifications
^; phosphorothioate linkage

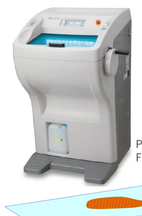
Nusinersen
(10 mg/kg, i.v.)



Crl:CD (SD) rat

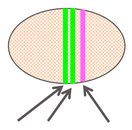
Tissue collection at 1, 8, 24, and 48 h after dose
(Kidney, Liver, Spleen, Testis, Brain, Thyroid, and Mandibular lymph node)

Cryosectioning from frozen tissues



Provided by Sakura Finetek Japan Co., Ltd.

Tissue slice
(10 μm)



Tissue block

for MSI **for LC-MS/MS**

Preparation step

- **Washing of tissue slices**

Wash (all 3 min):
1. EtOH*1
2. Carnoy's*2
3. 70% EtOH
4. EtOH

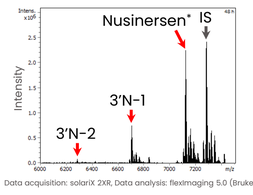
*1 EtOH (99.5%)
*2 EtOH: chloroform:acetic acid (6:3:1, v/v/v)
*3 3-Hydroxypicolinic Acid

- **Matrix coating**

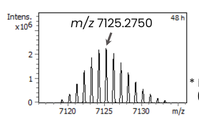
1. Matrix (15 mg/mL 3-HPA*3)
2. IS (10 μg/mL 23-mer phosphorotioate polyT)

- **MSI analysis**

Nusinersen:
5′-TCACTTTCATAATGCTGG-3′
Nusinersen 3′N-1:
5′-TCACTTTCATAATGCTG-3′
Nusinersen 3′N-1:
5′-TCACTTTCATAATGCT-3′



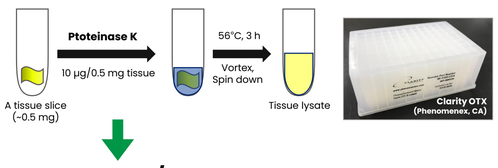
Data acquisition: solariX 2XR, Data analysis: flexiImaging 5.0 (Bruker)



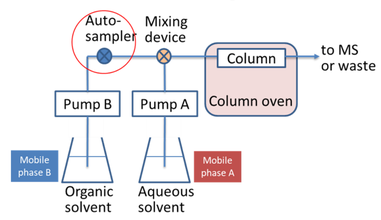
* Nusinersen (magnified)

Preparation of tissue slices

Proteinase K treatment (3 h, 56˚C)
Tissue lysate was collected
Solid-phase extraction (Clarity OTX)



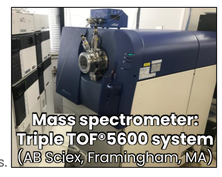
PAC-LC-MS/MS analysis



PAC-LC:
Peptide adsorption-controlled-Liquid Chromatograph
(Patent / P5144273)

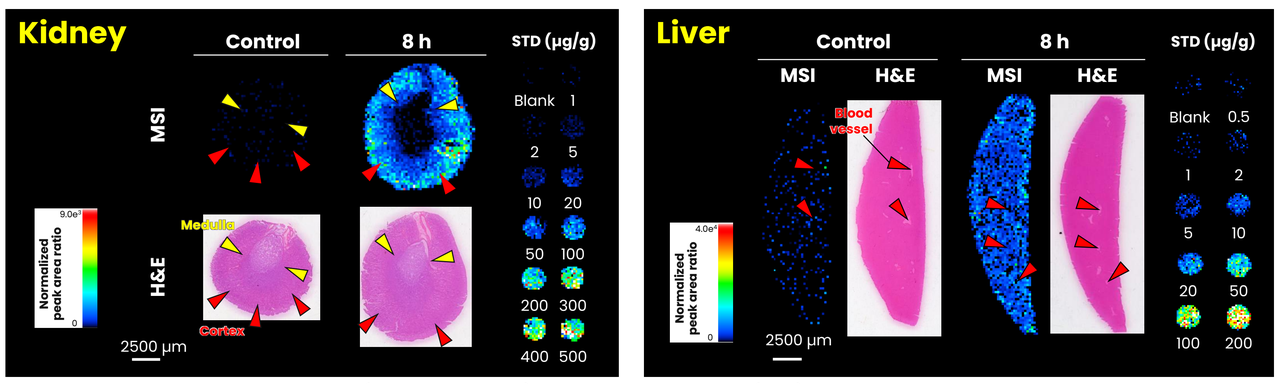
The feature of PAC-LC is that **a large volume of analytical sample with organic solvent can be injected to the analytical column.**

Goda R, Sudo K. Biomed Chromatogr. 2007;21(10):1005-15. with slight modifications.



Results

Spatial distribution of Nusinersen in kidney and liver



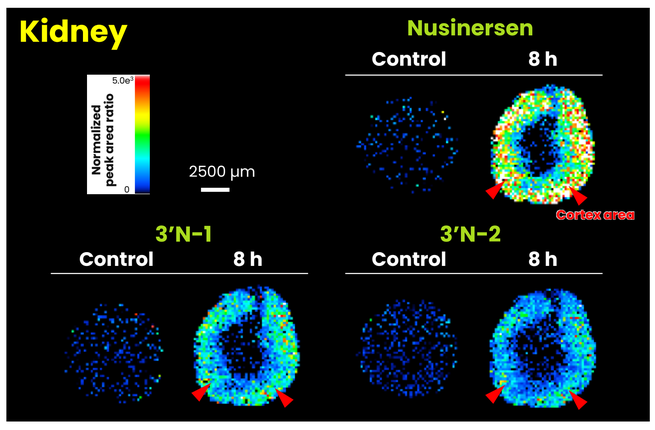
Samples: control and 8 h after dose (kidney and liver). Nusinersen standard (STD) for kidney: Nusinersen was spiked in 70% kidney homogenate, for liver: Nusinersen was spiked in 70% liver homogenate. The special resolution of MSI: 200 μm. Analyzed mass ranges: *m/z* 7125.2750±0.4 as Nusinersen. The images were normalized by IS. The characteristic tissue structures are indicated by colored arrows.

Quantitative values of qMSI and LC-MS/MS in slices of kidney and liver

Organ	Tissue samples (time after dose)	Nusinersen conc. (μg/g) qMSI	Nusinersen conc. (μg/g) PAC-LC-MS/MS
Kidney	Control	<5.00	<2.00
	1 h	67.9	60.4
	8 h	96.5	136
	24 h	108	133
	48 h	111	157
Liver	Control	<5.00	<0.500
	1 h	6.37	9.05
	8 h	14.3	17.7
	24 h	10.6	15.4
	48 h	9.83	12.9

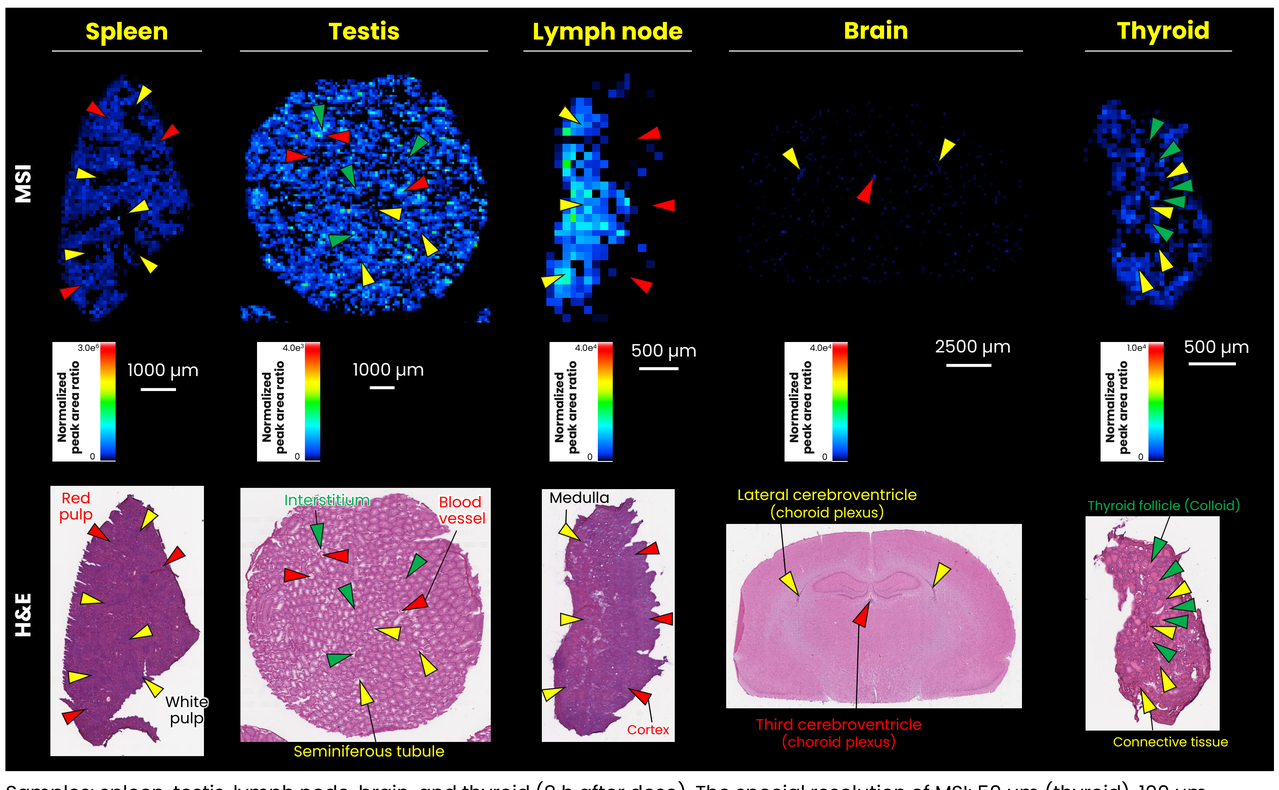
The quantification ranges of qMSI: kidney (5-300 μg/g tissue), liver (5-100 μg/g tissue). The quantification ranges of LC-MS/MS: kidney (2-1000 μg/g tissue), liver (0.5-250 μg/g tissue). Quantitative calculations were performed by selecting the entire tissue section as the region of interest. Measurements by qMSI were performed twice using serial sections for each tissue, and the average values were used as the quantitative values.

Distribution of Nusinersen and its metabolites in kidney



Samples: kidney of control and 8 h after dose. The special resolution of MSI: 200 μm. Analyzed mass ranges: *m/z* 7125.2750±0.4 as Nusinersen, *m/z* 6706.2080±0.4 as 3′N-1, 6287.1420±0.4 as 3′N-2. The images were normalized by IS.

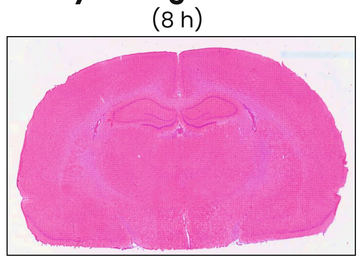
Spatial distribution of Nusinersen in other organs



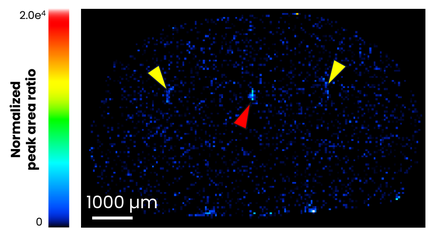
Samples: spleen, testis, lymph node, brain, and thyroid (8 h after dose). The special resolution of MSI: 50 μm (thyroid), 100 μm (spleen, testis, lymph node and brain). Analyzed mass ranges: *m/z* 7125.2750±0.4 as Nusinersen. The images were normalized by IS. The characteristic tissue structures are indicated by colored arrows.

High-sensitive analysis of testis and brain detecting PS linkages of OGNs

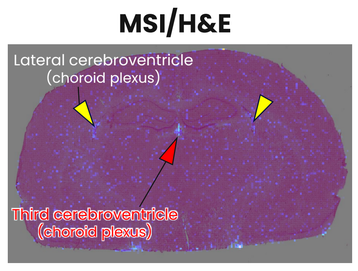
Analyzed region on brain
(8 h)



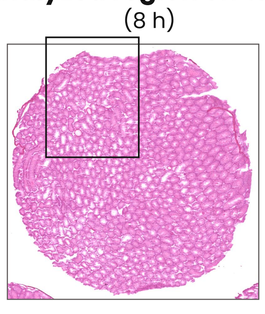
MSI on brain



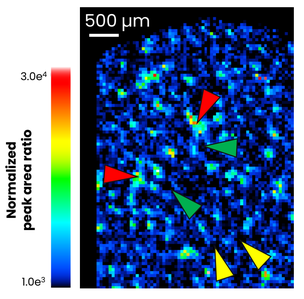
MSI/H&E



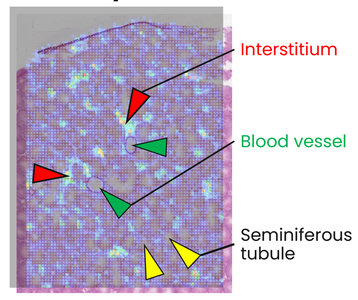
Analyzed region on testis
(8 h)



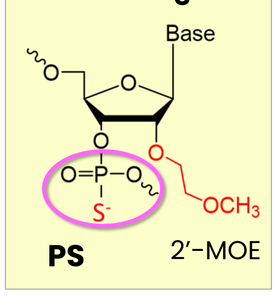
MSI on testis



MSI/H&E



PS linkage



Samples: testis and brain (8 h after dose). The special resolution of MSI: 50 μm (testis), 100 μm (brain). As a diagnostic fragment derived from the phosphorothioate backbone of OGNs is used for high-sensitive detection of Nusinersen and its metabolites. Analyzed mass ranges: *m/z* 94.9362±0.0002. The characteristic tissue structures are indicated by colored arrows.

Results Summary

- Nusinersen exhibited a tendency to predominantly localize in the renal cortex, red pulp of the spleen, interstitial tissue of the testes, medulla of the lymph nodes, and the choroid plexus of the brain (specifically within the lateral and third ventricles). Furthermore, high-sensitivity analysis using a method for detecting PS linkages clearly confirmed the presence of OGNs related to Nusinersen in the interstitial tissue of the testis and the choroid plexus of brain. In contrast, in the liver and thyroid gland, Nusinersen was observed to be more diffusely distributed throughout the entire tissue.
- Quantitative analysis was conducted on the MSI results for the kidney and liver at four time points up to 48 h after administration. The results showed that the concentration of Nusinersen in the kidney ranged from 67.9 to 111 μg/g tissue, and in the liver, it ranged from 6.37 to 14.3 μg/g tissue. Additionally, LC-MS/MS measurements were performed on serial sections of the same organs from the same individuals. These results indicated that the concentration of Nusinersen in the kidney was between 60.4 and 157 μg/g tissue, and in the liver, it was between 9.05 and 17.7 μg/g tissue.
- Two metabolites of Nusinersen (3'N-1 and 3'N-2) showed a tendency to localize in the renal cortex, similar to the distribution observed for Nusinersen. These two metabolites were not detected in other organs due to insufficient sensitivity (data not shown).

Conclusions

- **The distribution of Nusinersen across seven rat organs was successfully visualized.**
- **In the kidney, spleen, testes, lymph nodes, and brain, Nusinersen was observed to localize heterogeneously according to tissue structure. The distribution patterns across multiple organs suggest that the localization of Nusinersen is associated with areas of macrophage distribution.**
- **MSI results for the kidney indicated that the two metabolites of Nusinersen showed a distribution pattern similar to that of Nusinersen.**
- **Quantitative values from qMSI for the kidney and liver were 68.8-112% of the values obtained by LC-MS/MS, indicating a close similarity in trends.**

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COI disclosure:
We have the following financial relationships to disclose for our presentation contents.
Employees; Mediford Corporation (HA, HH, HW, KT, SN, NS, YN, TK, TH, TO, KM), Mitsubishi Tanabe Pharma Corporation (HK), Daiichi Sankyo Company, Ltd.(RG).

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