PK/PD evaluation of antisense oligonucleotides in tissues and cells

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Purpose

Distribution and activity of antisense oligonucleotides (ASOs) are limited to some tissues when administered systemically, which is a barrier to use as a therapeutic drug for a wide range of diseases. Now that, various drug delivery systems have been developed to overcome these problems. It is known that the distribution of ASO is not evenly in tissues and cells, evaluation method for distribution of ASOs has not been sufficiently constructed. Detailed evaluation of the distribution of ASO in tissues and cells would be needed to select appropriate targets, evaluate the efficiency of drug delivery systems, and validate their concepts. In this study, we constructed methods for evaluating PK/PD in more detail *in vivo* and *in vitro*.

Summary

First, we developed a double staining method of IHC with anti-PS-ASO antibody and ISH of target mRNA. Using this method, the distribution of ASO and mRNA knockdown could be simultaneously assessed in the histological section. Distribution and knockdown activity in specific cells and regions in tissue could be assessed.

Second, we developed hybridization ELISA methods to measure the concentration of ASO in cultured cells and nuclei. Using this method, we can know the relationship between the number of nucleic acids and mRNA knockdown in cells and nuclei.

These methods make it possible to select appropriate targets, evaluate the efficiency of drug delivery systems and validate their concepts.

Material method

・ASO

Mouse MALAT1 ASO[1] (Fig. 1-6)
5'-GGGTmCAGCTGCCAATGmCTAG-3'
Human MALAT1-ASO[1] (Fig. 8-10)
5'-AGTACTATAGCATmCTG-3'
Fig. 8,10 5'-MALAT1 ASO-AF647-3'
Fig. 9 5'-MALAT1 ASO-AF647-3'
5'-GalNAc-MALAT1 ASO-AF647-3'
Human APOB ASO[2] (Fig. 11)
5'-GmCmCTmCAGTmCTGmCTTmCGmCAmCmC-3'
ASO has fully phosphorothioate backbone.
Underlining; 2'-O-methoxyethyl modified base ASO
mC; 5-methylcytosine
AF647; Alexa Fluor® 647.
All ASOs were purchased from GENEDESIGN, Ajinomoto Bio-Pharma Services.

・Animal study

C57BL/6J mice at 9 weeks of age were treated subcutaneously with MALAT1 ASO at 50 mg/kg or saline (n = 2/group). Mice were euthanized 24 hours after dosing and tissues were harvested. All animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Shonan Health Innovation Park.

・Histology analyses

Histological sections were stained by immunohistochemical staining (IHC) with anti-PS-ASO (phosphorothioate - antisense oligonucleotide) antibody and *in-situ* hybridization (ISH) of target mRNA.

Methods of IHC
- Staining equipment: Leica BOND RX
- Antigen retrieval: Bond ER Solution 1
- Primary anti body: AMCC-11975_1A, Anti-asO(M1-27), 1:10000
- Staining kit: BOND Polymer Refine Detection

Methods of double staining of *Malat1*-ISH and PS-ASO-IHC
- Staining equipment: Leica BOND RX
- Antigen retrieval: Bond ER Solution 2, RNAscope 2.5 LSx Protease
- Staining kit for ISH: RNAscope 2.5 LS Reagent Kit-Brown
- probe for ISH: RNAscope® LS 2.5 Probe- Mm-Malat1
- Primary anti body for IHC: AMCC-11975_1A, Anti-asO(M1-27), 1:3000
- Staining kit for IHC: BOND Polymer Refine RED Detection

・ASO treatment

ASOs with or without Lipofectamine™ 2000 Transfection Reagent were treated to Hep G2 (2 day) or A549 (1 day) cells and after incubation, cells were detached with trypsin and recovered. Nuclei were extracted from a part of cells using Nuclear/Cytosolic Fractionation Kit (Cell Biolabs).

・Measurement of ASO concentration in tissue

ASO concentration was measured by LC-MS/MS.

・Measurement of ASO concentration in cells

ASO concentrations in cells and nuclei were measured by hybridization ELISA(HELISA). We use "one-step hybridization ELISA" for 3'-fluorescein labeled ASO and "two-step hybridization-ligation ELISA" for unlabeled ASO[3]. Images were taken using a confocal microscope (Cytiva).

・quantitative RT-PCR

metastasis associated lung adenocarcinoma transcript 1 (*Malat1*) mRNA and *apolipoproteinB* (*APOB*) mRNA expression were quantified by quantitative real-time polymerase chain reaction using GAPDH as a house keeping gene.

Reference
1) G. Hung, et. Al. 2013. *Nucleic Acid Ther* **23**:6, 369-378.
2) R. S. Geary, et al. 2015. *Clin Pharmacokinet* **54**, 133-146.
3) X. Wei, et. Al. 2006. *Pharm. Res.* **23**:6, 1251-1264.

We have no financial relationships to disclose for this presentation.

Evaluation of distribution in tissue and mRNA knockdown

1. Distribution analysis of ASO by LC-MS/MS and knockdown analysis by qPCR

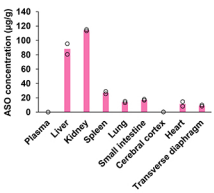


Fig. 1 ASO concentration (24 h) of plasma and tissue

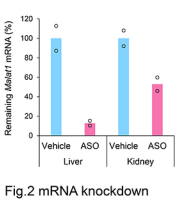


Fig.2 mRNA knockdown

2. Distribution analysis of ASO by IHC (anti-PS-ASO ab)

Liver

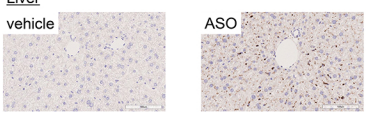


Fig. 3 Granular positive brown signals were observed in the cytoplasm of almost all the Kupffer cells and hepatocytes in ASO animals. Bar = 100 um

Kidney

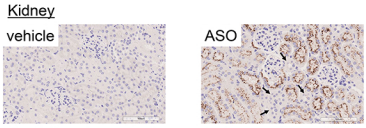


Fig. 4 Granular positive brown signals were observed in the cytoplasm of proximal tubules in ASO animals. Glomeruli and distal tubules (arrows) were almost negative. Bar = 100 um

3. Double staining of *Malat1*-ISH and PS-ASO-IHC

Liver

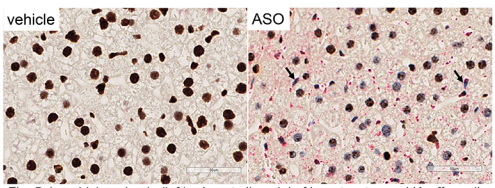


Fig. 5 In vehicle animals (left), almost all nuclei of hepatocytes and Kupffer cells showed positive (brown) for ISH for *Malat1*. In ASO animals (right), signals of PS-ASO (red) were observed in the cytoplasm of many hepatocytes and Kupffer cells. Many nuclei of the hepatocytes and Kupffer cells turned out to be negative for ISH for *Malat1* and showing bluish hematoxylin color, which suggested knockdown of *Malat1* in these cells by ASO administration. Some Kupffer cells showing knockdown clearly contained PS-ASO signals (red) in their cytoplasm (arrows). Bar = 50 um

Kidney

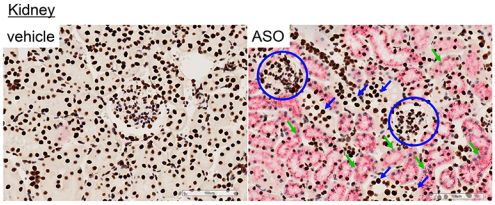


Fig. 6 In vehicle animals (left), almost all nuclei of renal tubules and glomeruli showed positive (brown) for ISH for *Malat1*. In ASO animals (right), PS-ASO signals (red) were observed almost exclusively observed in the proximal tubules. Many nuclei of the proximal tubules turned out to be negative for ISH for *Malat1* and showing bluish hematoxylin color (green arrows), which suggested knockdown of *Malat1* in these cells by ASO administration. On the contrary, nuclei of distal tubules (blue arrows) and glomeruli (blue circle) showed positive for ISH for *Malat1* (brown), which suggested knockdown by ASO administration did not occur in these cells. Bar = 100 um

Measurement of concentration of ASO in cells and nuclei

1. Extraction of nuclei from cells

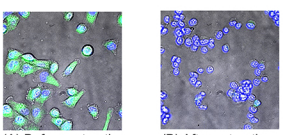
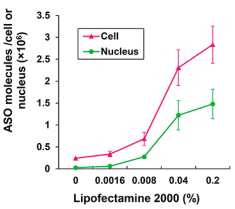


Fig. 7 (A) Before extraction of nuclei (B) After extraction of nuclei

CellMask™ Deep Red Plasma membrane Stain
Hoechst 33342

2-1. Evaluation of the transfer rate to nucleus of ASO



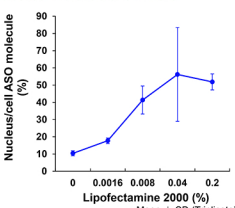


Fig.8 (A) ASO concentration (B) Transfer rate to nucleus

2-2. Cell concentration of GalNAc conjugated ASO

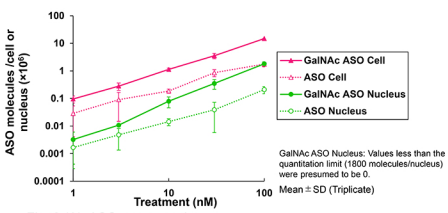
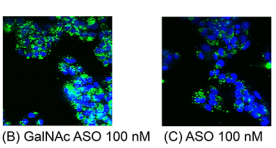


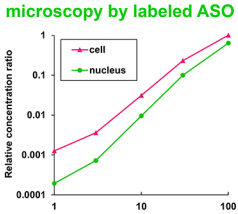
Fig. 9 (A) ASO concentration



(B) GalNAc ASO 100 nM (C) ASO 100 nM

AF647 labeled ASO
Hoechst 33342

2-3. Comparison with the results using confocal microscopy by labeled ASO



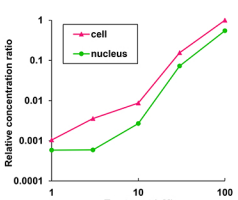


Fig. 10 (A) Relative ASO concentration by HELISA (B) Relative ASO concentration by confocal microscope

2-4. Relationship between the number of ASO molecules and KD activity

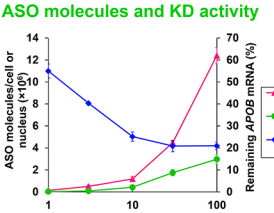


Fig. 11 ASO concentration and mRNA knockdown