Detection of LNA-containing antisense oligonucleotides in rat liver and kidney by immunogold transmission electron microscopy

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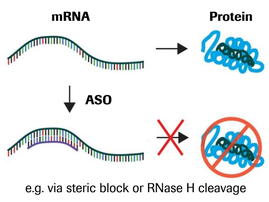
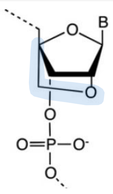
1. Introduction

Locked **N**ucleic **A**cids (**LNA**) are chemically modified RNA-like nucleotides with the potential to be used in therapeutic **a**nti**s**ense **o**ligonucleotides (**ASO**) to prevent expression of "disease-related" protein products.

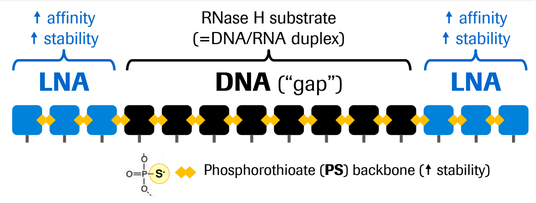
ASO mode of action



LNA structure



Gapmer LNA design



Despite their proven therapeutic potential, some LNA-containing ASO have shown **toxic** effects mainly associated with off-target effects and with their accumulation in liver and kidney.

General aim: To investigate pathogenic mechanisms of toxicity of selected tool LNA ASO with different safety profiles, comparing unconjugated (naked) LNA ASO or conjugated with *N*-acetyl galactosamine (GalNAc) for targeted delivery to hepatocytes.
Focus of this poster: To establish an immunogold methodology to investigate the accumulation of LNA in liver and kidney at subcellular level.

2. Materials and Methods

Study design

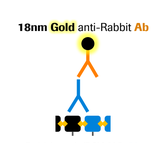
Safety profile (toxicity)	*Tool compounds*		*Target (sequence)*	*Dosing (weekly)*
	Naked	*GalNAc*		
Control	**Vehicle**			• SC administration
Non toxic	**ASO1**	Not tested	None, scramble (sequence 1)	• **Sacrificed after 1, 3 or 6 doses**
Moderately toxic	**ASO2**	**ASO3**	Human PCSK9 (sequence 2)	• Sacrificed 3 days after last dose
Highly toxic	**ASO4**	**ASO5**	Human PCSK9 (sequence 3)	• Naked LNAs: 40 mg/kg • GalNAc LNAs: 20 mg/kg

Species/Sex: **Wistar rat** (male). Special sampling of **liver & kidney**

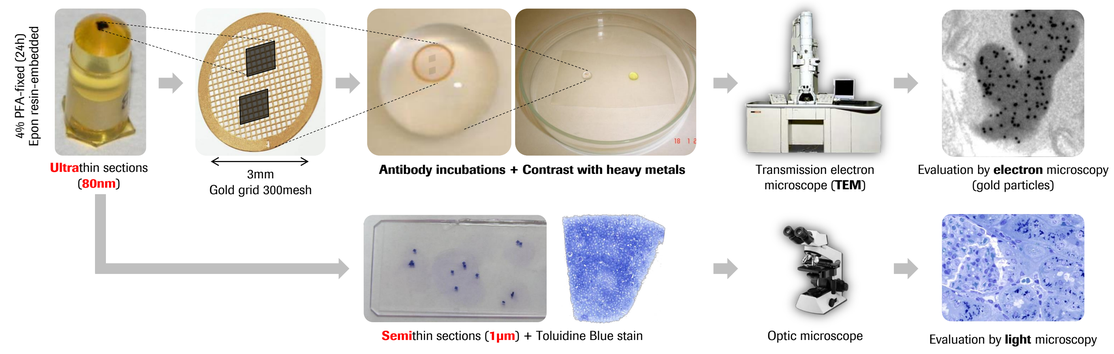
Protocol details

Immunogold labelling (main steps):

Rabbit anti-(PS)LNA Ab
Overnight 4°C
1:800 dilution
↓
Gold anti-Rabbit IgG Ab
2h room temp.
1:20 dilution
↓
Contrast 1
7min, uranyl acetate
↓
Contrast 2
5min, lead citrate

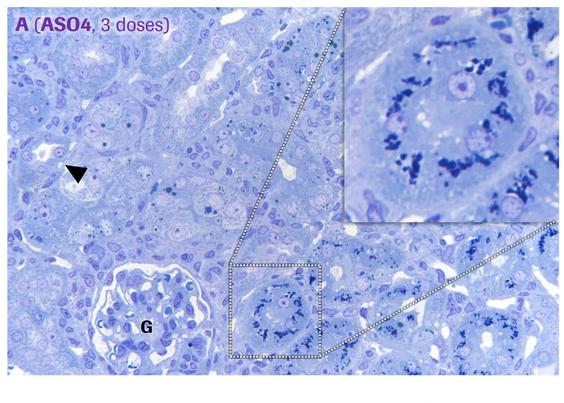


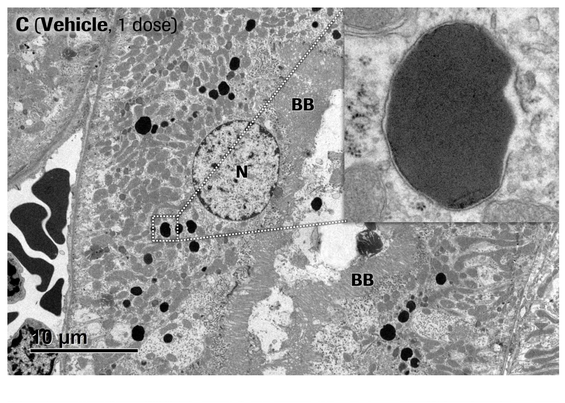
Immunogold TEM workflow

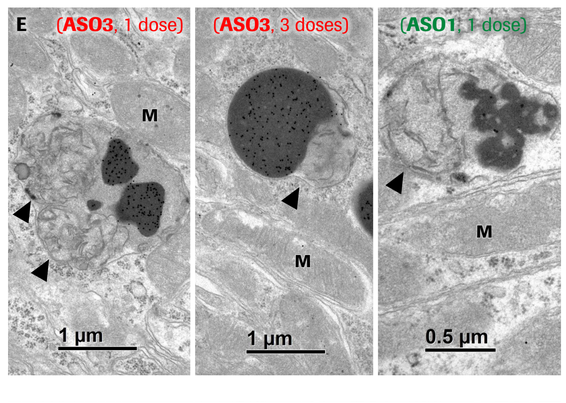


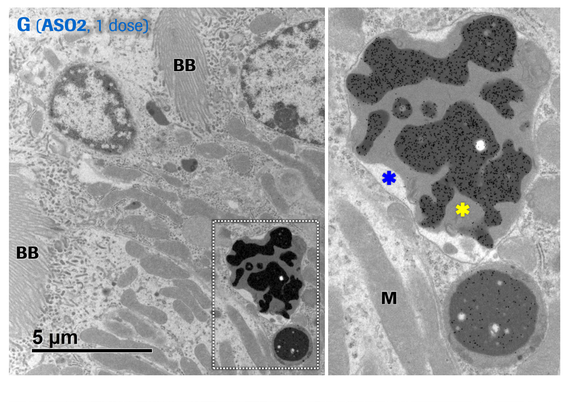
3. Results (I): KIDNEY

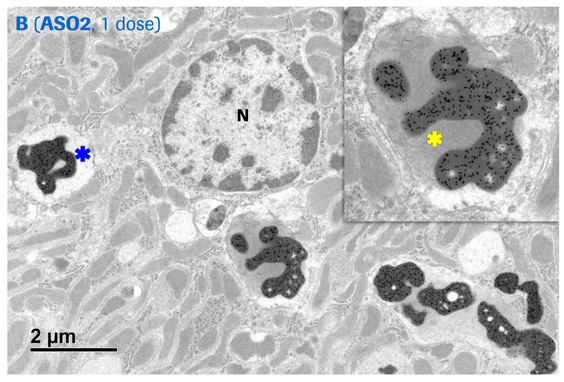
Abbreviations: **BB** (brush border), **EGPV** (electron-dense gold-positive vesicles = LNA ASO), **G** (glomerulus), **M** (mitochondrion), **N** (nucleus), **PTEC/DTEC** (proximal/distal tubular epithelial cells).

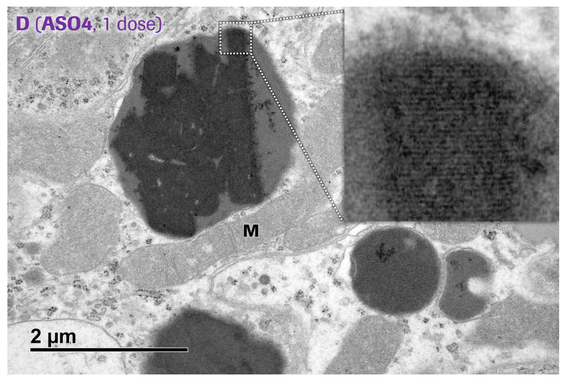


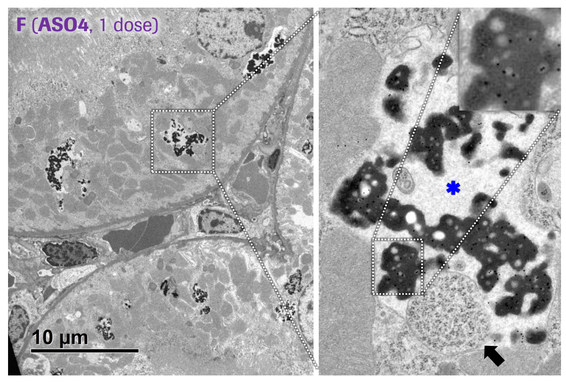


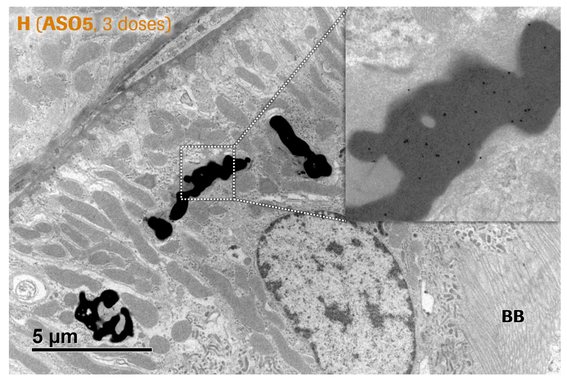












Semithin section stained with toluidine blue, showing the LNA granules in PTEC (dark blue, inset), but not in DTEC (arrowhead) or glomeruli (**A**). LNA-containing ASO are observed as electron-dense gold-positive vesicles (EGPV) with irregular shape, consistent with endosomes or secondary lysosomes (**B, E-H**) **[1,2]**.

Electron-dense protein droplets (gold negative) compatible with secondary lysosomes loaded with *alpha 2u-globulin*, are observed in both control (**C**) and dosed rats (**D**). Protein droplets can get larger and polyangular-shaped, and crystalloids can form with characteristic strial pattern (**D inset**) **[3]**.

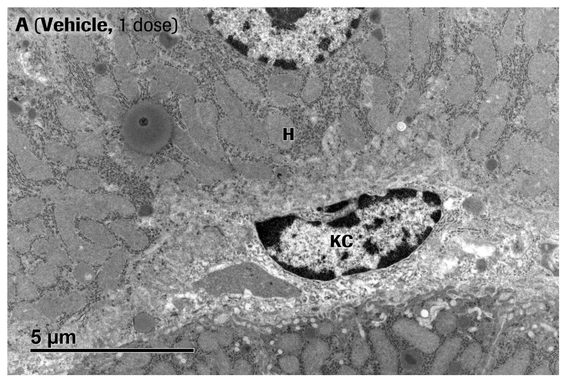
Some organelles undergoing degradation (mitochondria (**E**, arrowheads), ribosomes and RER (**F**, arrow)) were observed within the same membranes containing the LNA vesicles, suggesting (at least partially) common pathways with autophagy.

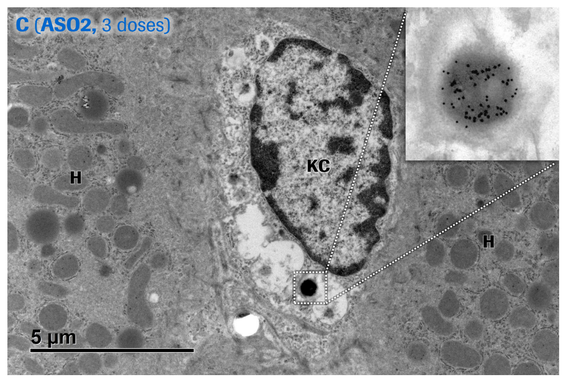
Differences in the density of gold particles were observed within the same sample (**G**, top vs bottom vesicles), and among compounds (**G** vs **H**), probably due to different stages of LNA degradation, and to differences in the antibody binding affinity to the compounds, respectively. ASO2&3 showed the strongest gold reactivity (**B, E, G**).

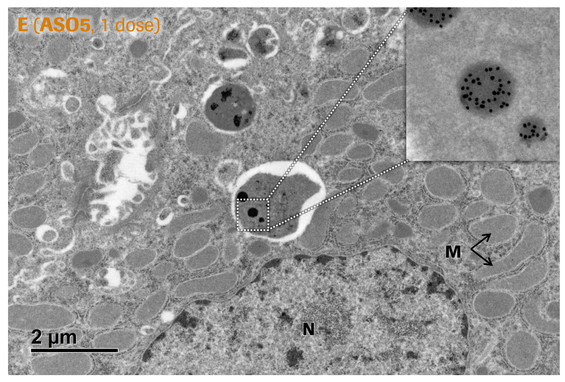
EGPV are observed surrounded by a moderately electron-dense material (grey) suggestive of lysosomal content (✱), and/or by electron-lucent material (white) suggestive of osmotic hydration (✱).

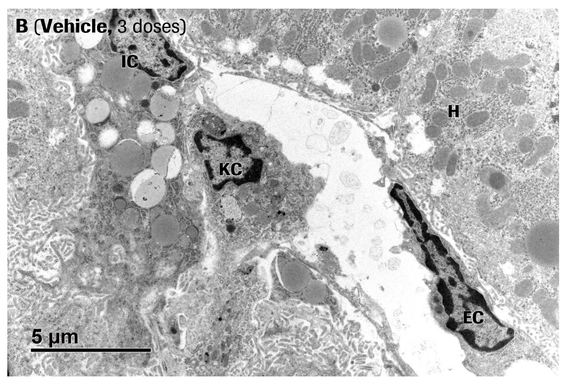
4. Results (II): LIVER

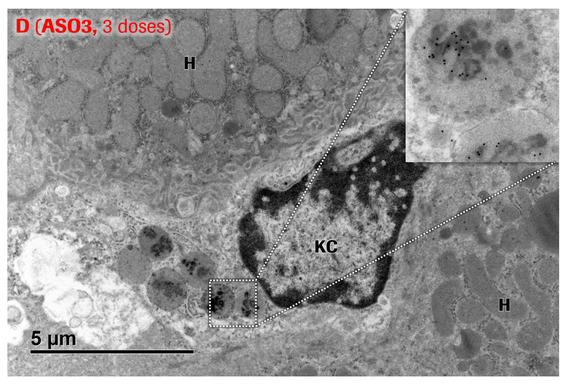
Abbreviations: **EC** (sinusoid endothelial cell), **EGPV** (electron-dense gold-positive vesicles = LNA ASO), **H** (hepatocyte), **IC** (Ito cell), **KC** (Kupffer cell) **M** (mitochondrion), **N** (nucleus).

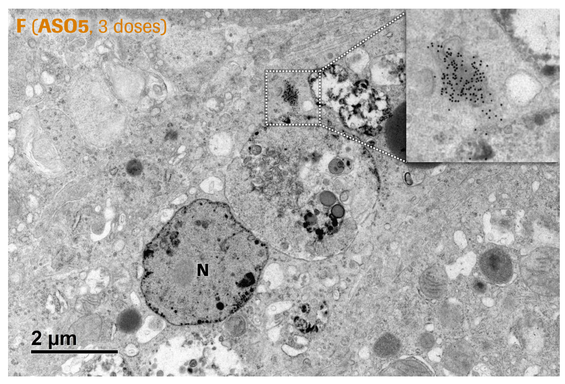












Figures A&B: No electron-dense gold-positive vesicles (EGPV) were observed in any cell type in control rats, confirming the specificity of the labelling.

Figures C&D: EGPV within **Kupffer cells** in rats dosed with both naked (**C**) and GalNAc-conjugated LNAs (**D**). LNAs in Kupffer cells show a more round morphology, reflecting phagocytic uptake (phagosomes), unlike LNAs in the kidney, where more irregular shapes are observed, most likely as a result of different uptake mechanisms (endocytic uptake, endosomes) **[1,2]**.

Figures E&F: EGPV within **hepatocytes** in rats dosed with GalNAc-conjugated LNAs. Figure **F** shows features of highly degenerated hepatocytes, due to dosing with highly toxic GalNAc-conjugated LNAs.

5. Conclusions:

Different morphologies of LNA vesicles may reflect different uptake mechanisms (endocytosis vs phagocytosis). Different stages of LNA degradation/metabolism and differences in the antibody binding affinity to the different compounds may explain the differences in the density of gold particles. EGPV were detected in kidney PTEC, as well as in Kupffer cells (naked and GalNAc-conjugated compounds) and hepatocytes (GalNAc-conjugated LNAs). This methodology provides new insights on the accumulation of LNAs at ultrastructural level.

6. References:

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[3] Read NG, 1991. The role of lysosomes in hyaline droplet nephropathy induced by a variety of pharmacological agents in the male rat. Histochem. J. 23, 436-443.