

Kinetics of DPC-mediated delivery of cholesterol-conjugated RNAi triggers and their subcellular processing *in vivo*

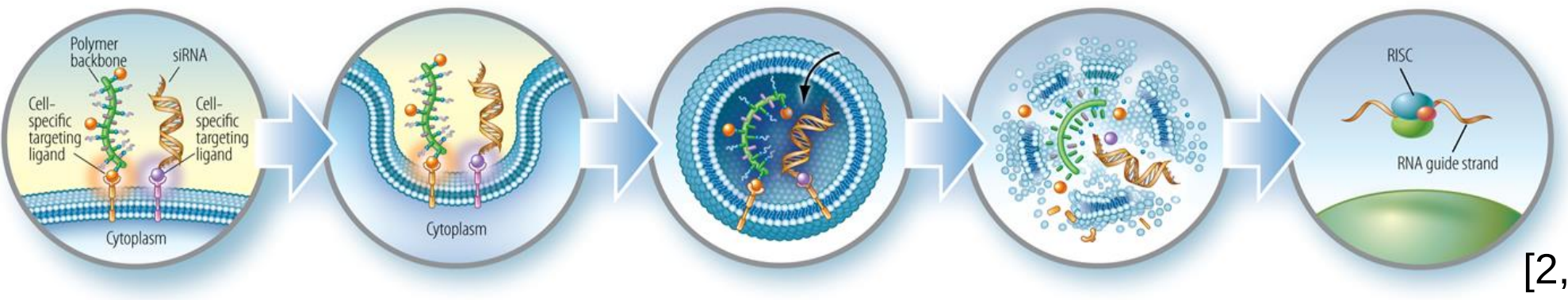
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Introduction

Dynamic Polyconjugates (DPCs) are a class of RNAi trigger (siRNA) delivery agents composed of reversibly-masked, membrane active polymers whose activity is revealed within endosomes. The DPC in the RNAi-based therapeutic ARC-520 for the treatment of chronic hepatitis B virus infection consists of a biodegradable melittin-like peptide (MLP) as the membrane-active polymer conjugated to N-acetylgalactosamine (NAG) for hepatocyte targeting. Co-injection of NAG-MLP with hepatotropic cholesterol-conjugated RNAi trigger (chol-siRNA) results in rapid, high level knockdown of HBV mRNAs. To characterize the kinetics of DPC-mediated chol-siRNA delivery and processing events that correlate with RNAi activity, we employed a fluorescent probe hybridization/AEX HPLC method that allows measurement of the siRNA guide strand (GS) and its metabolites from plasma and tissue extracts. This sensitive method allows separate measurement of full-length GS that has a 5'-phosphate (5'P FL GS) or that is 5' unphosphorylated (noP FL GS), and GS degradation products ≥ 15 nucleotides long.

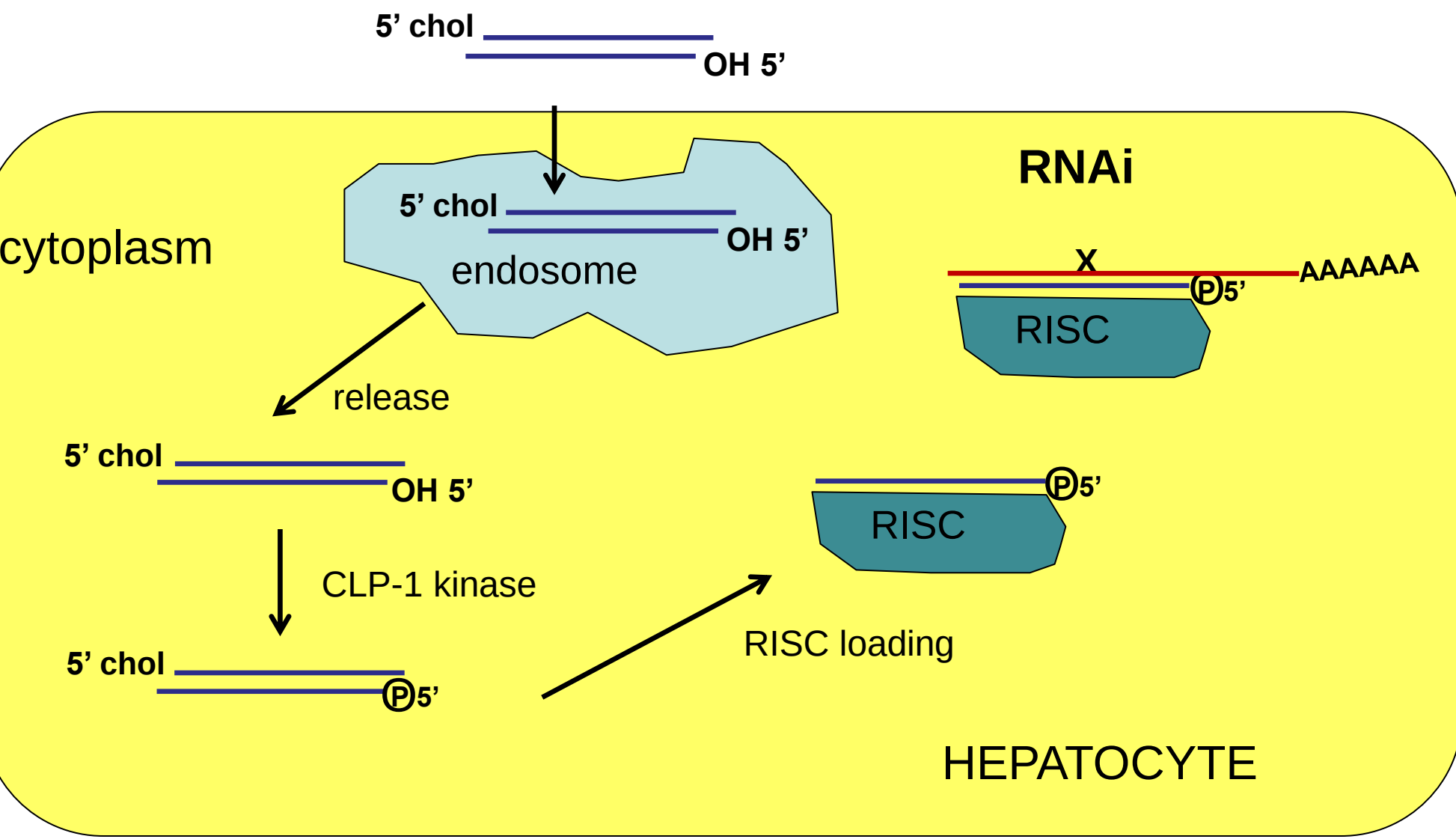
Dynamic Polyconjugates Mechanism of Action



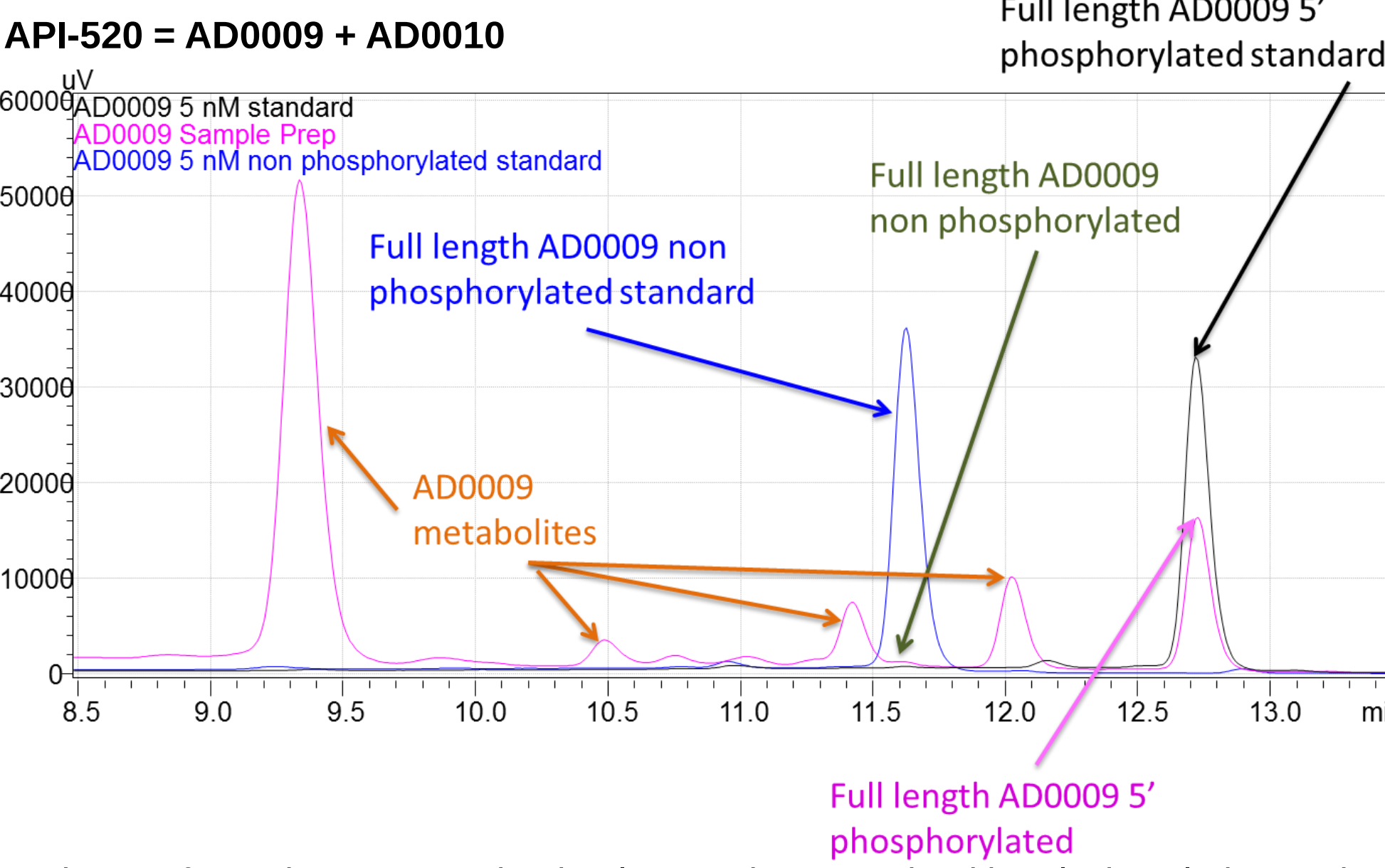
The Active Pharmaceutical Ingredients of ARC-520 (API-520) are 1:1 AD0009 + AD0010. NAG-MLP and API-520 are mixed prior to injection. All HBV genes are targeted by co-injecting NAG-MLP and API-520. These siRNAs target highly conserved HBV sequences [1].

Phosphorylation of guide strands in the cytoplasm of the hepatocyte

The chol-siRNAs were synthesized without a 5'P on the GS. NAG-MLP and chol-siRNA are taken up by endocytosis. NAG-MLP disrupts the membrane of the endosome, releasing into the cytoplasm any chol-siRNA molecules that are in the same endosomes. The GS has been shown to become 5' phosphorylated by CLP-1 kinase in the cytoplasm [4].



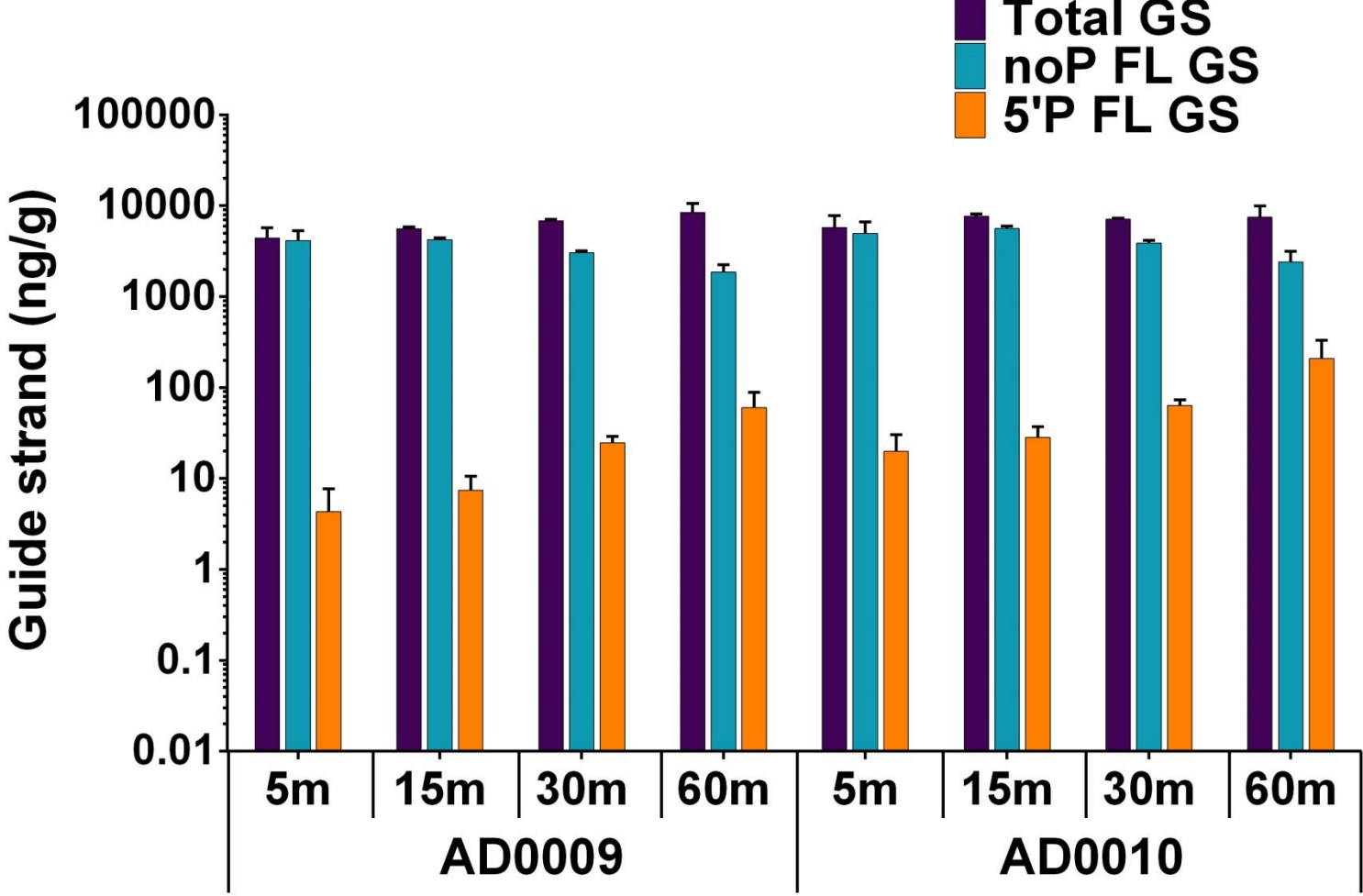
Measurement of siRNA guide strands in tissues and plasma of ARC-520-treated animals



1. Tissues from siRNA-treated animals were homogenized in a lysis solution and then centrifuged.
2. The supernatant was incubated with a fluorescent peptide-nucleic acid (PNA) probe that hybridized to the siRNA guide strand.
3. The probe-GS hybrids were analyzed by HPLC anion exchange chromatography.
4. Peaks on the chromatographs were identified by retention time relative to 5'P FL GS and noP FL GS standards. This method also distinguishes metabolites that are ≥ 15 bases long; these are included in the "total GS."

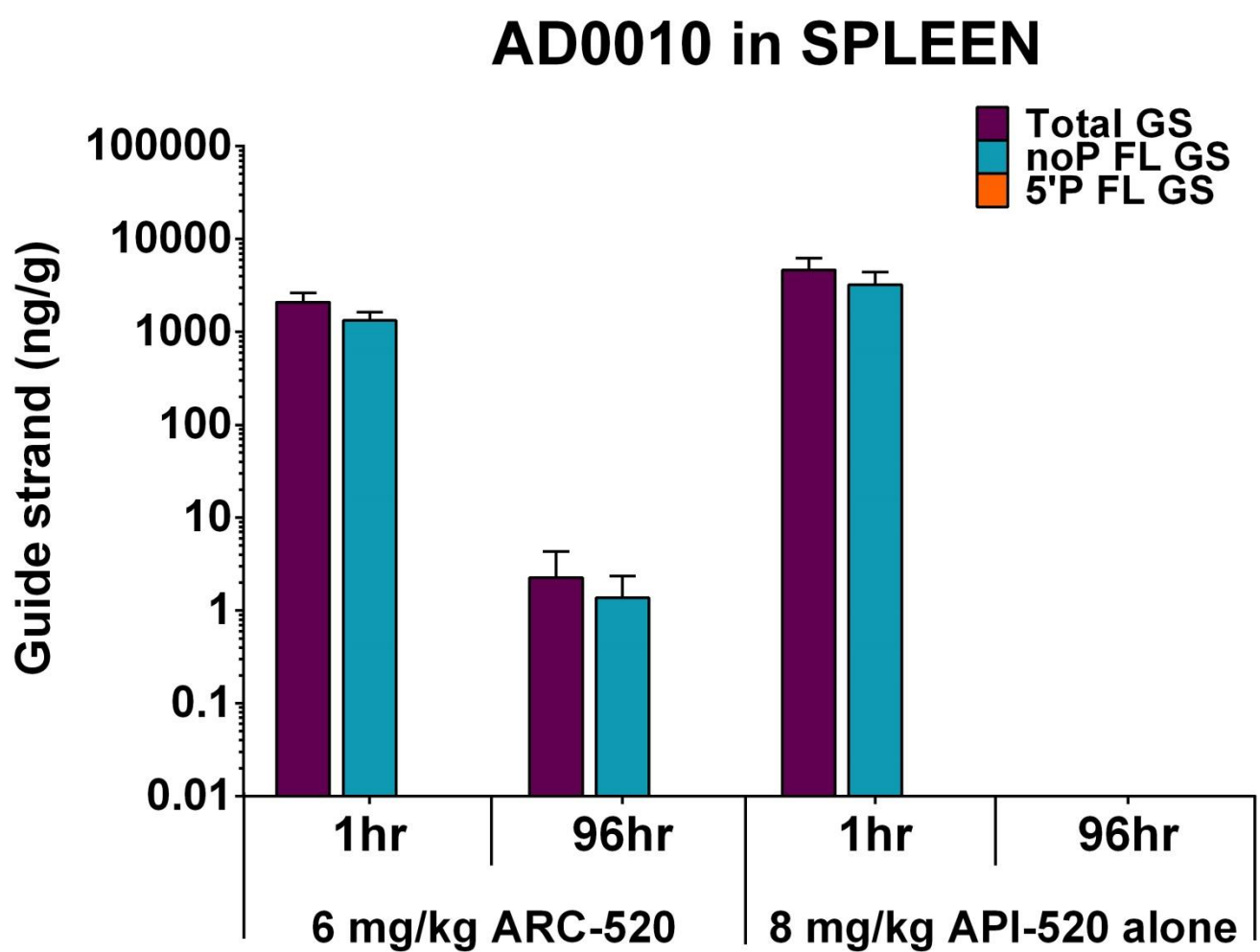
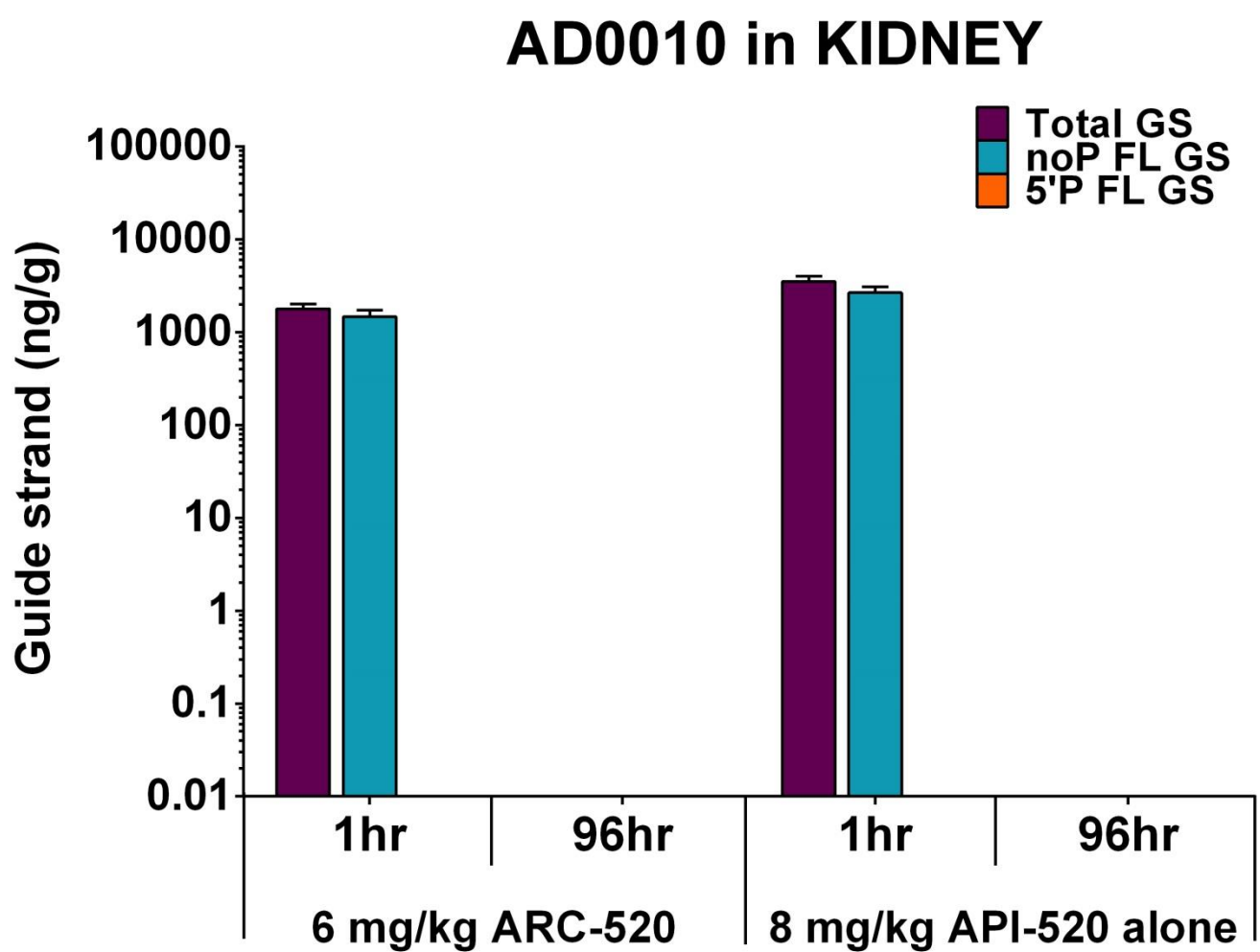
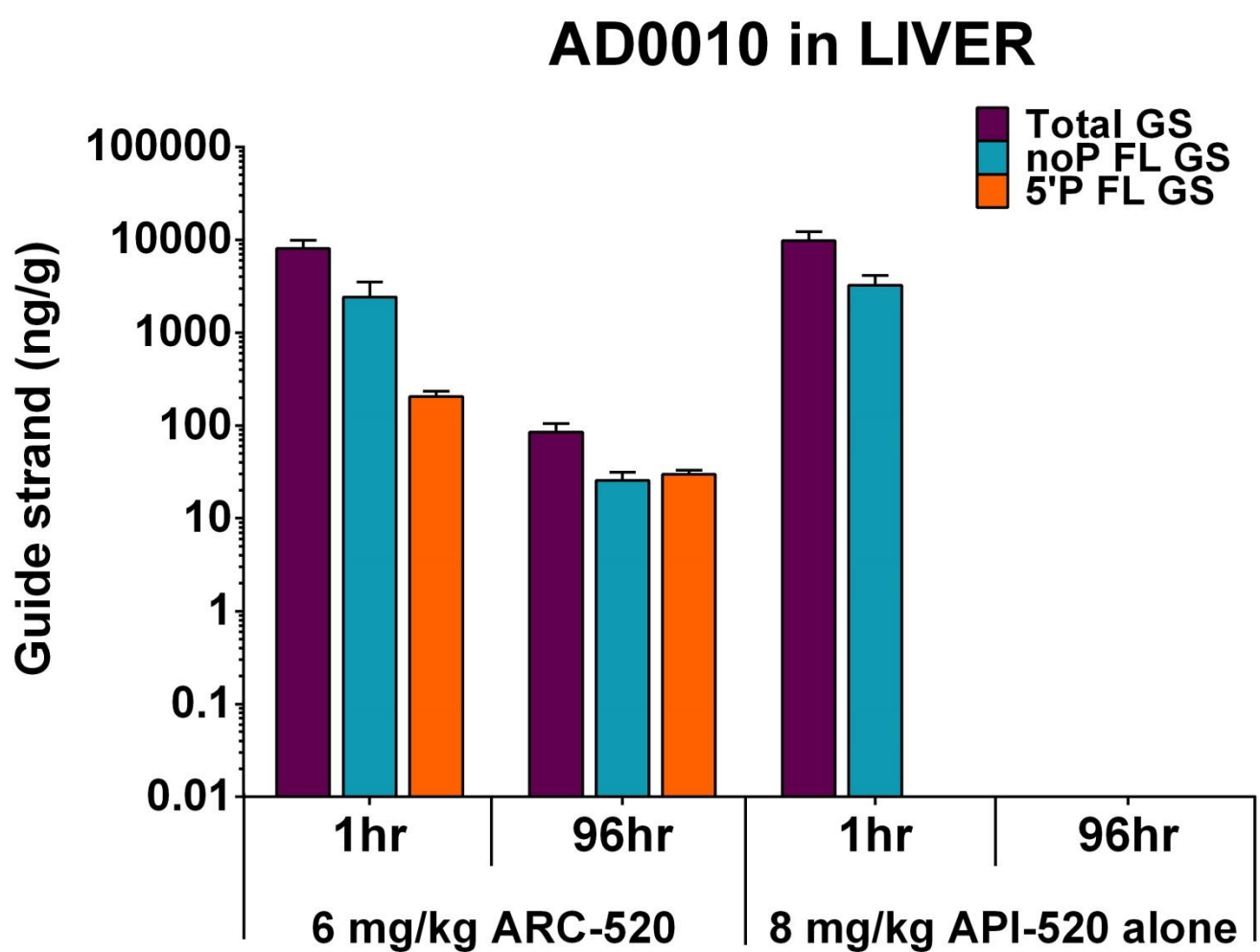
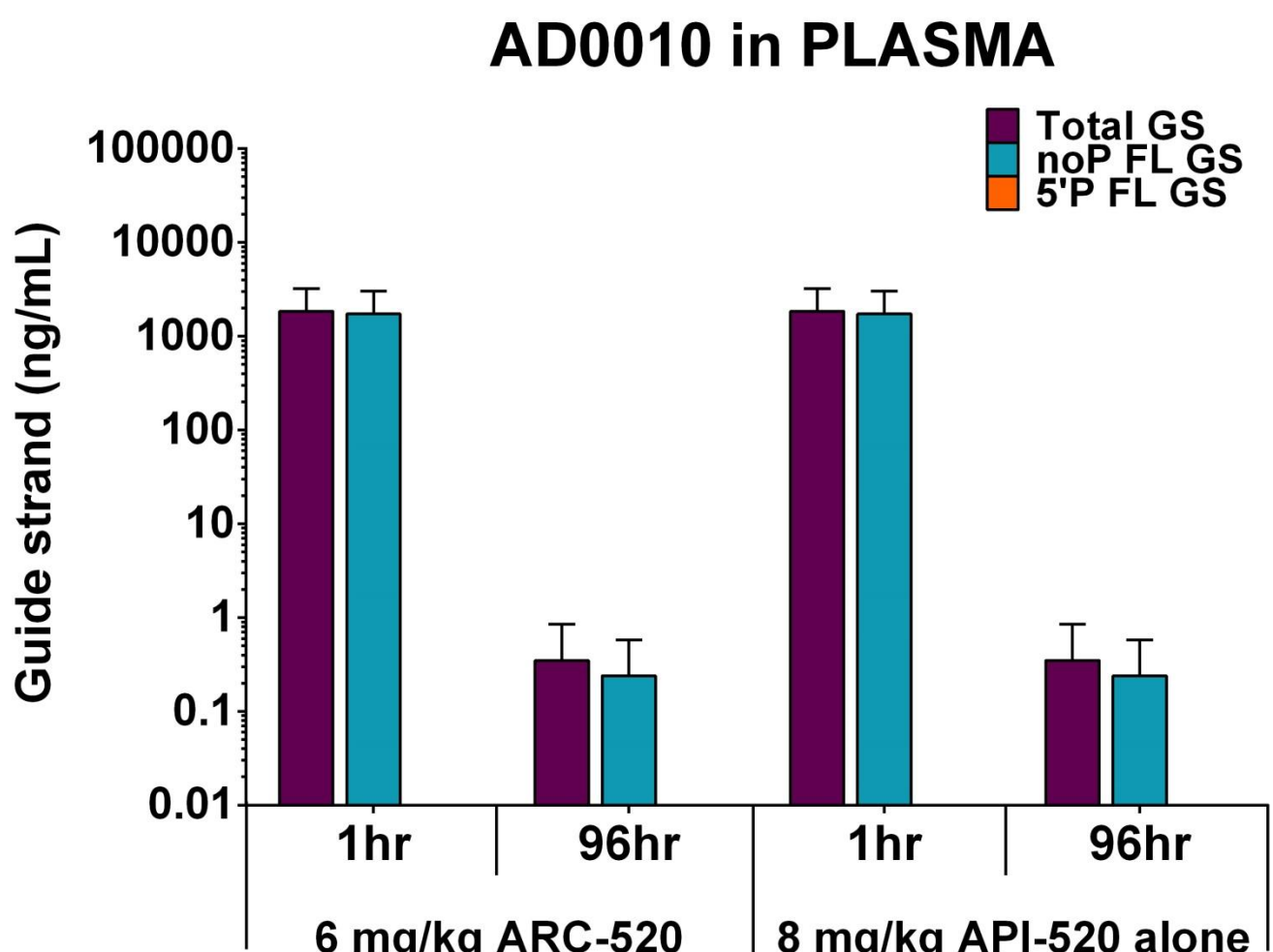
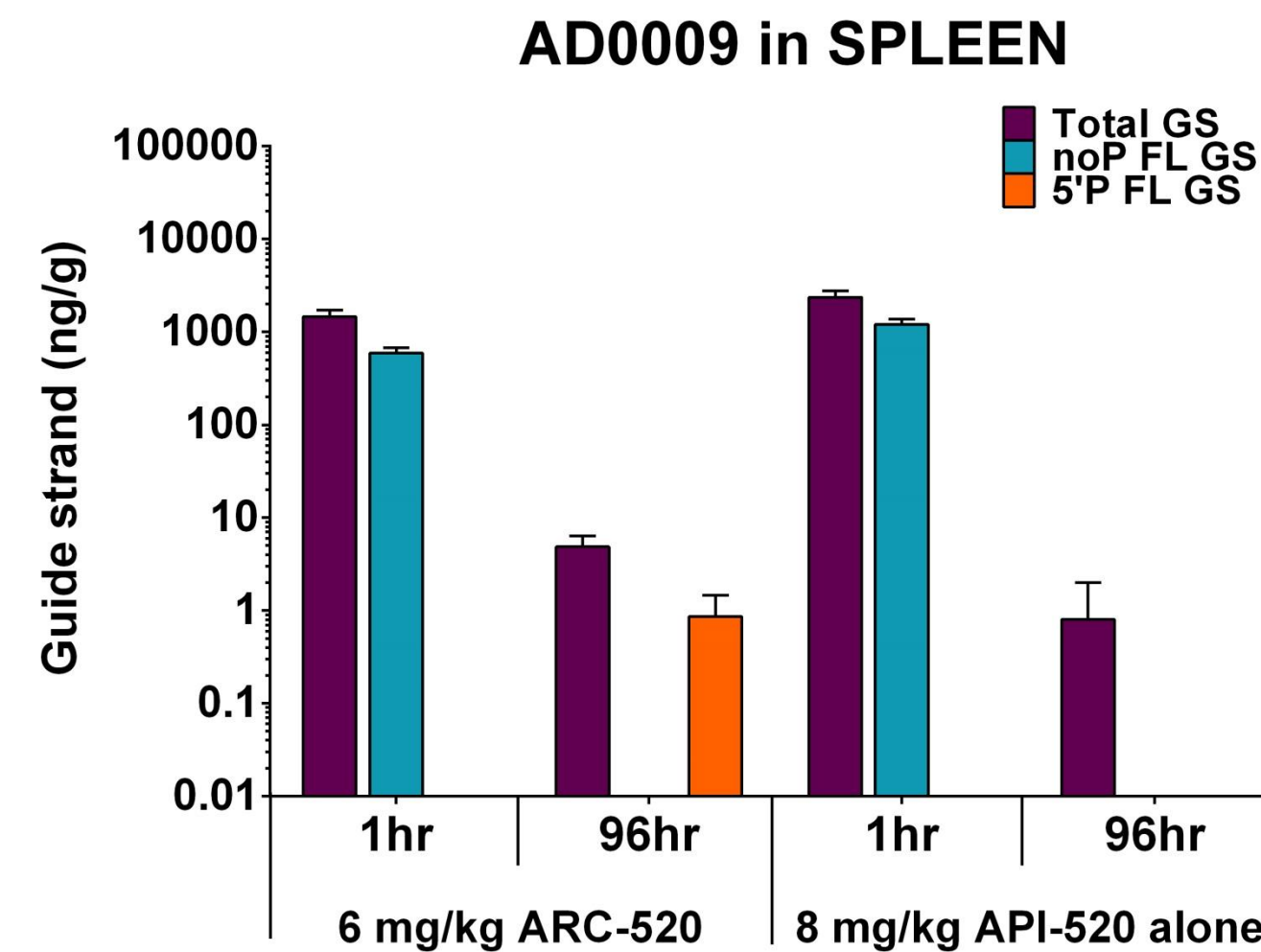
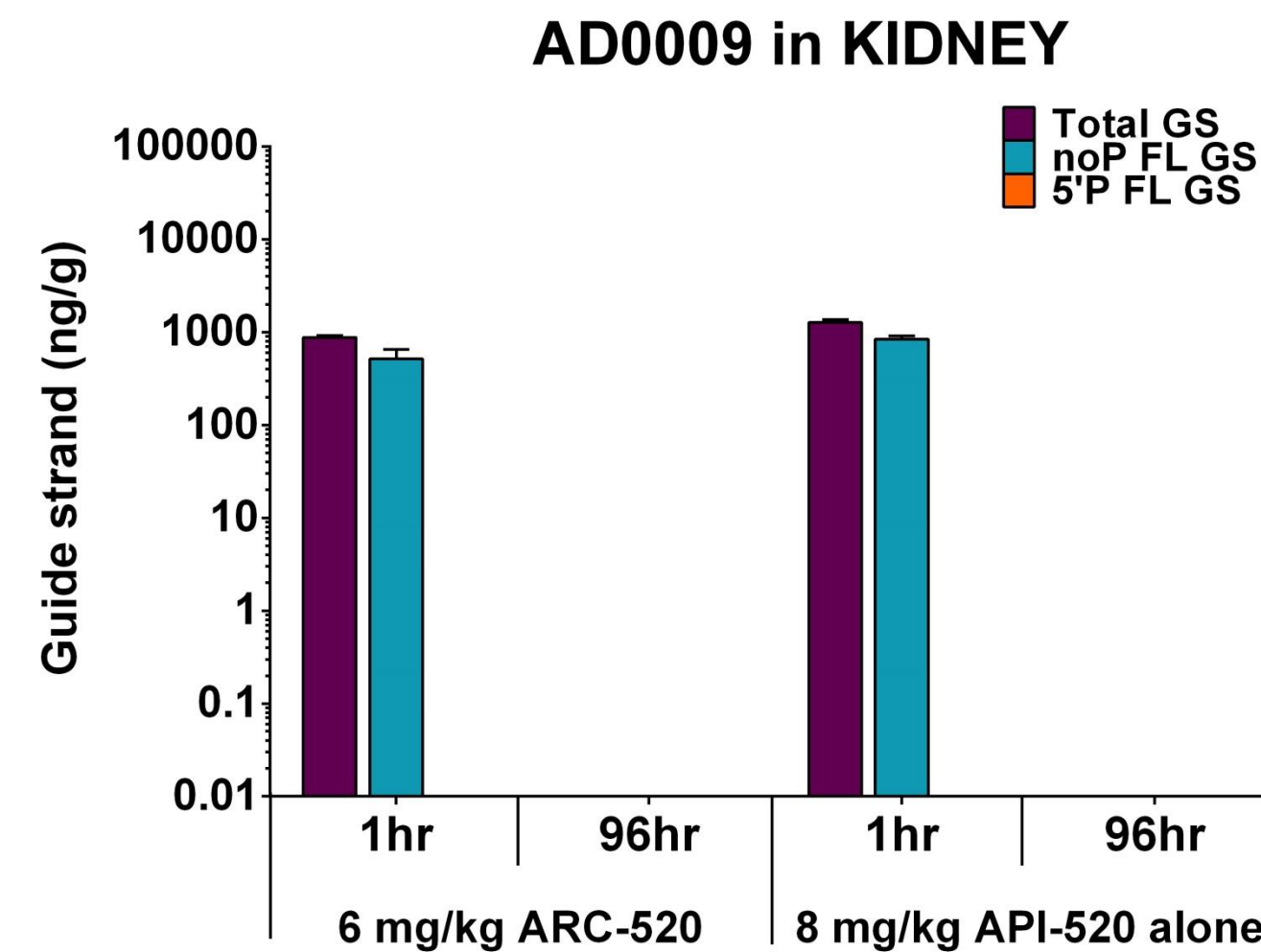
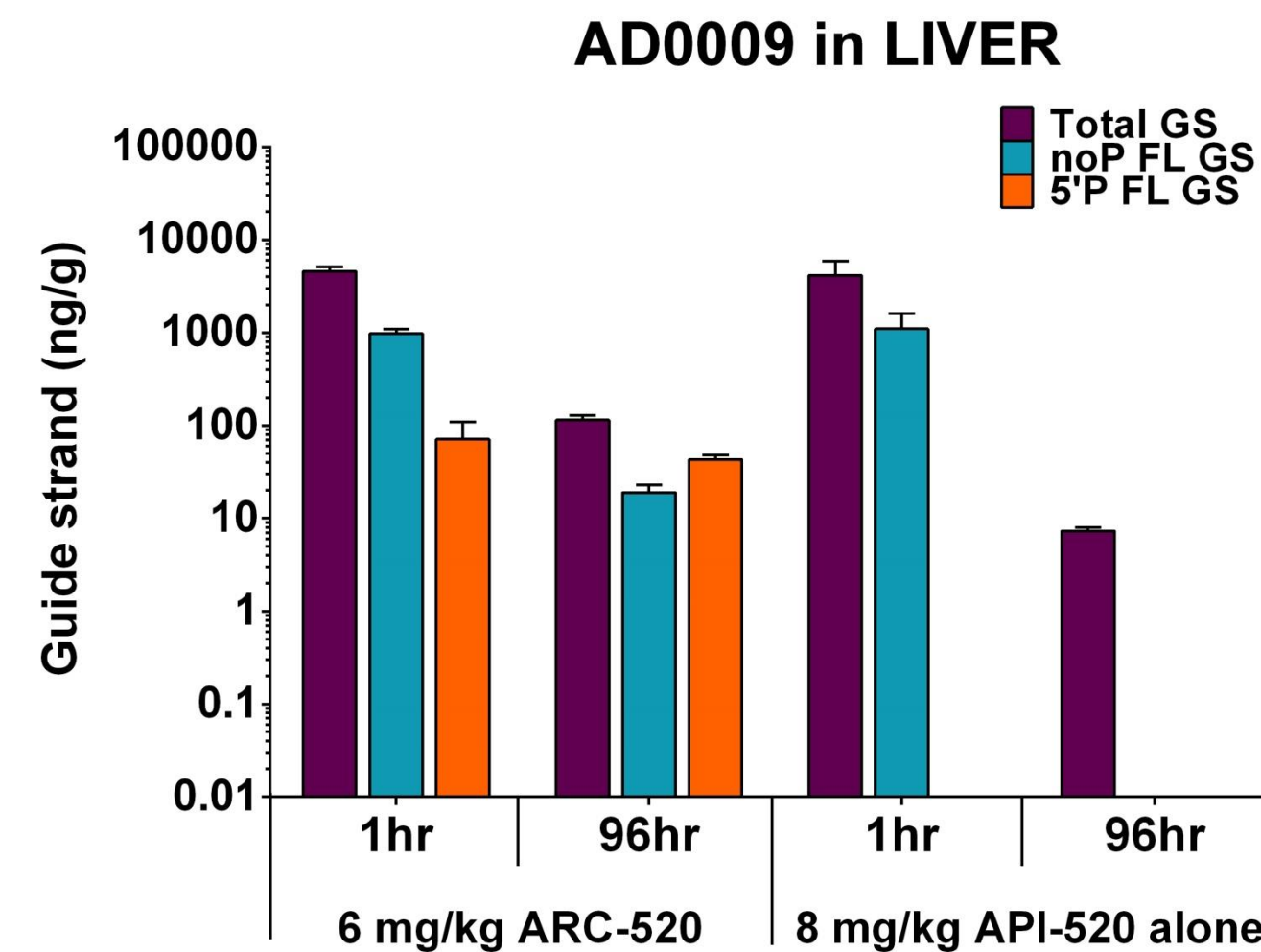
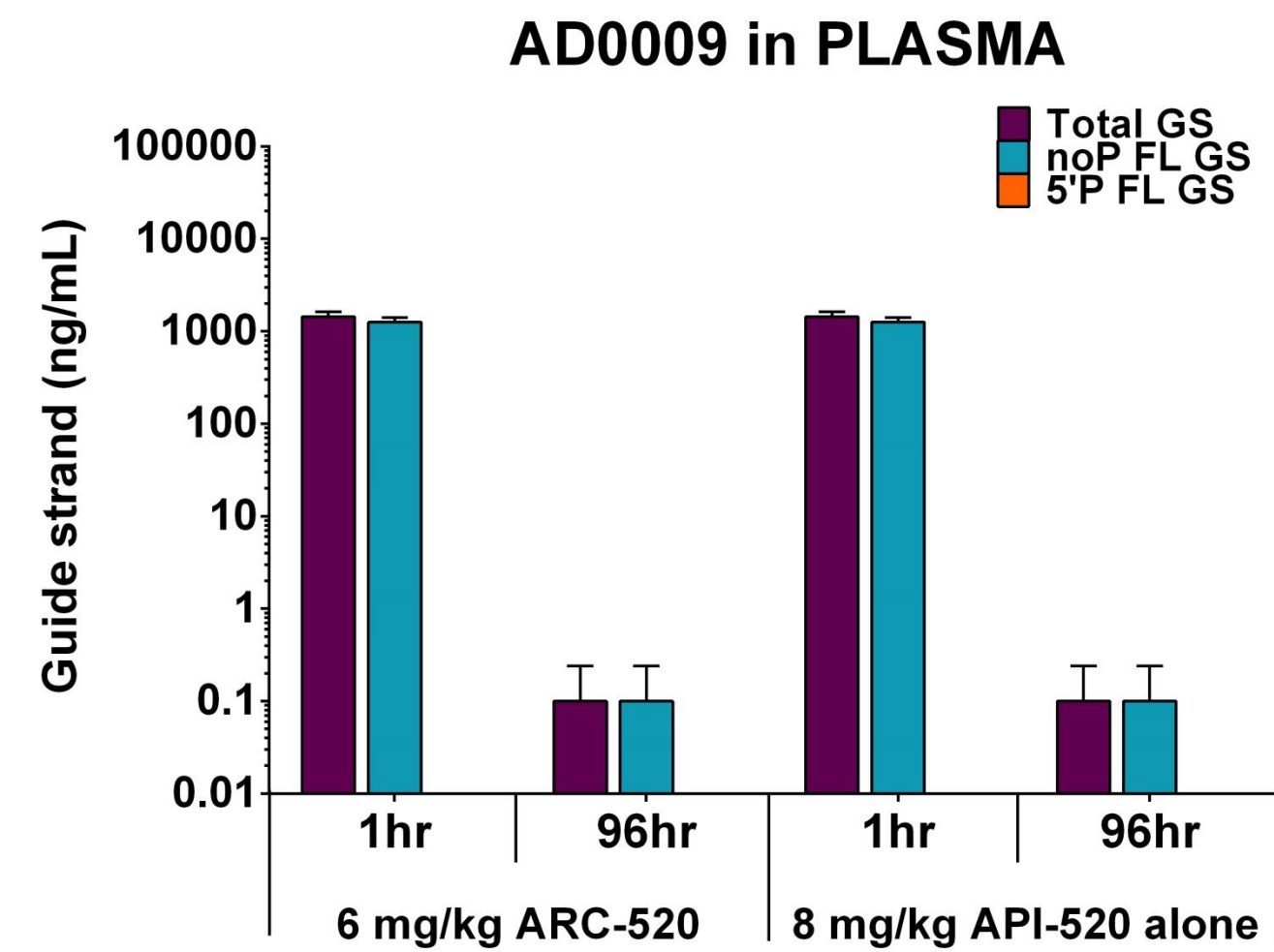
Delivery of siRNA into the cytoplasm and 5' phosphorylation of siRNA guide strand

ICR mice were given a single injection of 8 mg/kg ARC-520. Using the PNA hybridization assay, siRNA GS in the liver was measured at time points from 5 - 60 minutes after the injection.



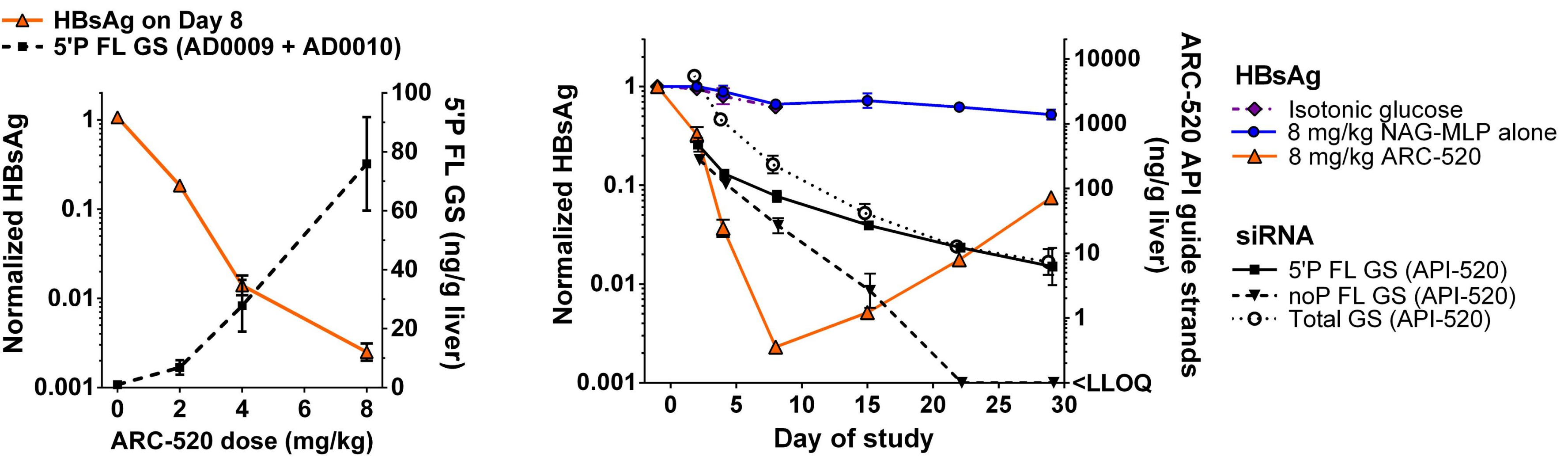
- Most of the siRNA GS in the liver was initially full-length, but not 5' phosphorylated.
- 5'P FL GS was present in the liver within 5 min of injection and increased over the course of 1 hour.
- One hour post-injection, a total of 1.7% of the total GS in the liver was 5'P FL GS.

Delivery of siRNA (API-520) into the cytoplasm requires the NAG-MLP



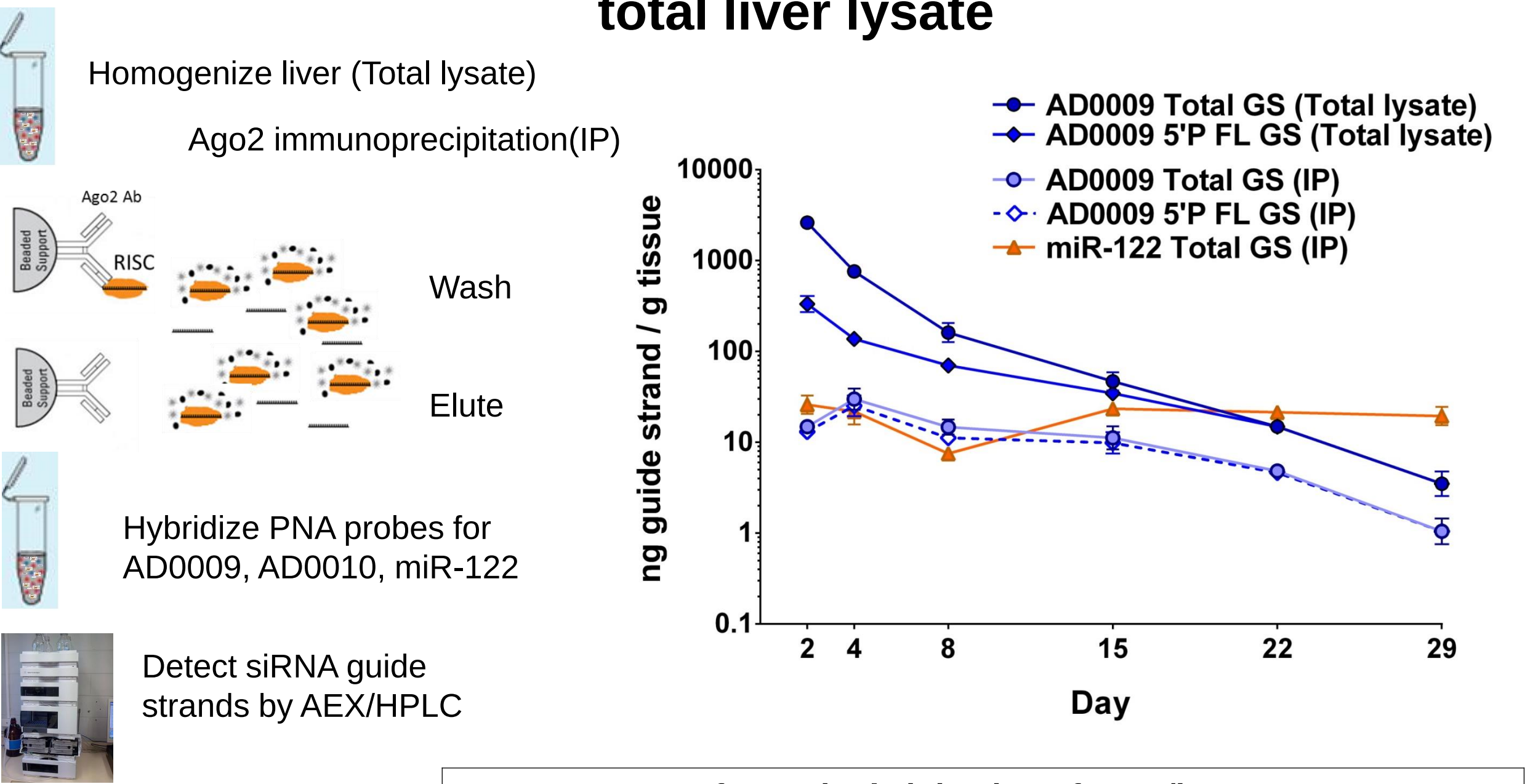
HBsAg knockdown correlates with 5'P full-length GS in the liver

Transiently transgenic NOD SCID mice expressing the entire human HBV genome in the liver were given a single injection of 2, 4 or 8 mg/kg ARC-520. HBV S antigen (HBsAg) in the serum and siRNA GS in the liver were measured at the indicated times.



- HBsAg knockdown correlated with the amount of 5'P FL GS present in the liver.
- A dose of 4 mg/kg ARC-520 resulted in 99% HBsAg knockdown on Day 8, at which time the liver contained 28 ng/g 5'P FL GS.
- A dose of 8 mg/kg ARC-520 resulted in 99% knockdown on Day 15, at which time the liver contained 27 ng/g 5'P FL GS.
- A dose of 2 mg/kg ARC-520 resulted in 82% HBsAg knockdown on Day 8, at which time the livers contained 8 ng/g 5'P FL GS.
- A dose of 8 mg/kg ARC-520 resulted in 86% knockdown on Day 29, at which time the liver contained 4 ng/g 5'P FL GS.

Quantitation of siRNA guide strands in RISC and in total liver lysate



	Days after a single injection of 8 mg/kg ARC-520					
	Day 2	Day 4	Day 8	Day 15	Day 22	Day 29
Knockdown of HBsAg	65 ± 6%	95 ± 1%	99.6 ± 0.4%	99.3 ± 1.4%	97 ± 5%	86 ± 15%
5'P FL GS in liver (ng AD0009 GS/g liver tissue)	333 ± 61	137 ± 15	70 ± 7	35 ± 6	15 ± 2	3.5 ± 1.1
5'P FL GS in RISC (ng AD0009 GS/g liver tissue)	13.1 ± 1.5	25.2 ± 7.2	11.2 ± 1.3*	9.9 ± 2.6	4.6 ± 0.6	1.0 ± 0.4
5'P FL GS in liver (ng AD0010 GS/g liver tissue)	169 ± 50	51 ± 7	17 ± 2	5 ± 1	0.5 ± 0.7	<LLOQ
5'P FL GS in RISC (ng AD0010 GS/g liver tissue)	4.3 ± 0.5	10.9 ± 2.9	5.1 ± 1.2*	1.8 ± 0.4	0.7 ± 0.1	0.1 ± 0.1

* n=2 (one failed IP sample)

Conclusions

Biodistribution studies performed using the PNA hybridization method indicated that one hour after injection the levels of FL GS were highest in liver. In contrast, levels in the other tissues and plasma were lower, with a half-life of 2-4 hours irrespective of NAG-MLP injection. Maximal levels of total GS in liver were similar whether or not NAG-MLP was co-injected with the chol-siRNA, indicating that NAG-MLP does not influence the overall amount of chol-siRNA delivered into the liver. Interestingly, although the injected RNAi trigger lacks a 5'P, a 5'P FL GS was detected in the liver within 5 minutes and levels reached a maximum at one hour, but only when NAG-MLP was co-injected. The only other tissue in which 5'P FL GS was detected was the spleen, but levels were two orders of magnitude less than in the liver. The 5'P FL GS was detected in the liver up to 4 weeks post-injection, corresponding with the duration of HBV knockdown. Others have shown that incorporation of siRNA into RISC requires a 5'P in the loaded strand [5]. We show that 5'P FL GS was present in RISC by performing Argonaute 2 immunoprecipitation followed by GS detection. These studies suggest that NAG-MLP enables endosomal escape of the chol-siRNA into the cytoplasm where it is 5' phosphorylated, possibly by the cytoplasm-localized kinase CLP1, then incorporated into RISC. Together, these studies show that the hybridization/AEX HPLC method may be used to enhance understanding of the pharmacokinetics of RNAi triggers and elucidate the processing events involved in their metabolism and activity.

References
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4. Weitzer and Martinez (2007). Cytoplasmic hC1p1 is responsible for 5'-phosphorylation of siRNA. *Nature* **447**: 222-226.
5. Nykanen, A., Haley, B., Zamore, P.D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**, 309-321.