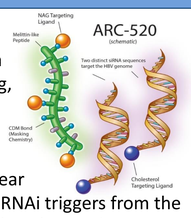
Gene expression analysis during and after RNAi treatment of chronically HBV infected chimpanzees

Zhao Xu[1], Deborah Chavez[2], Jason E. Goetzmann[3], Robert E. Lanford[2] and Christine I. Wooddell[1]

[1] Arrowhead Pharmaceutics, Madison, WI; [2] Texas Biomedical Research Institute, San Antonio, TX; [3] New Iberia Research Center, University of Louisiana at Lafayette, New Iberia, LA

Background and study design



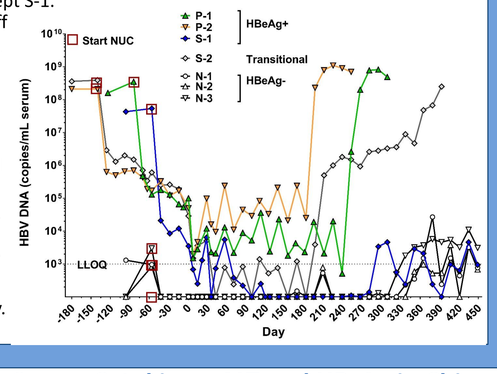
ARC-520 was designed to decrease all cccDNA-derived viral transcripts via RNA interference (RNAi) and thus reduce all viral products - HBsAg, HBeAg, HBV replication products, core, polymerase and X protein – with the idea that reducing viral products may allow de-repression of the host immune system to control HBV infection. ARC-520 consists of two cholesterol-conjugated synthetic RNAi trigger molecules that target HBV sequences near DR1 plus a hepatocyte-targeted peptide (EX1) for efficient delivery of the RNAi triggers from the endosome to the cytoplasm where the RNAi machinery resides (Wooddell et al., 2013).

Following a NUC only lead-in of 8-24 weeks, 9 chronically HBV-infected chimpanzees were treated with daily NUCS plus monthly intravenous infusions of RNAi trigger(s) + EX1, then were taken off all treatment. All chimps received at least 6 doses of ARC-520. Some received an RNAi trigger (siHBV75) designed to target transcripts from integrated HBV. Safety and efficacy were monitored with regular blood collection and periodic liver biopsies. 7 chimps received multiple liver biopsies, including after end of therapy. Liver mRNA was subjected to RNA-seq, differential gene expression (DGE) and Ingenuity Pathway Analysis to gain insight into animal responses.

Off-treatment serum HBV DNA

Off all treatment, HBV DNA and HBsAg rebounded to pre-study levels in all chimps except S-1.

At 31 weeks off treatment, S-1 was HBeAg-neg., anti-HBeAg pos., ALT normal, and controlled HBV infection with HBV DNA 5 $\log_{10}$ lower and HBsAg 1.7 $\log_{10}$ lower than pre-study.



Minimal cytokine response of human whole blood exposed to ARC-520

ARC-520 RNAi triggers were fully chemically modified to prevent cytokine induction. Fresh human whole blood was exposed to ARC-520 at plasma equivalent exposures to intravenous doses of 2-12 mg/kg ARC-520 (20-120 µg/mL). The observed elevation of cytokine levels, typically by a few-fold, was quite small compared to the increase in cytokine levels by 2-3 orders of magnitude in positive controls (LPS and R848).

ARC-520 (µg/ml)	GM-CSF	IFNα2	IL-1ß	IL-6	IL-8	MCP-1	MIP-1α	TNFα
120	3.0 (1)	<2	4.5-**12.7** (2)	2.0-2.5 (3)	2.1-8.3 (2)	2.3-3.3 (2)	2.9-**18.8** (4)	3.7 (1)
100	2.1 (1)	<2	2.3-9.0 (3)	<2	2.0-2.8 (2)	2.8 (1)	3.1-5.1(3)	2.3 (1)
80	<2	<2	8.3 (1)	<2	<2	2.4 (1)	2.2-2.3 (2)	<2
60	<2	<2	2.6-9.6 (2)	<2	<2	<2	<2	<2
40	<2	<2	2.0 (1)	<2	<2	<2	<2	<2
20	<2	<2	7.7 (1)	<2	<2	<2	<2	<2
R848 max	8.2-**20.5** (5)	**17.9-126.2** (3)*	**68.7-219,804** (5)	**135.9-10,010** (5)	4.7-**72.5** (5)	9.4-**19.1** (5)	37.5-**2041**(5)	**88.6-1081** (5)
LPS max	6.4-**33.2** (5)	2.5-8.6 (3)*	**588.8-219,804** (5)	**135.9-10,010** (5)	5.2-**601** (5)	4.2-9.4 (5)	**73.5-2041** (5)	**109.2-1185** (5)

Fold-increase in cytokine levels exceeding the baseline values by more than an order of magnitude are shown in bold. The number in parenthesis indicates the number of subjects (out of 5) showing positive response. *IFNα2 signal could not be normalized in 2 donors because of the very low negative control signal.

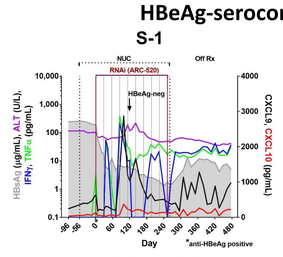
HBV RNAi efficacy in chimpanzees

3 high viremia HBeAg positive chimps (**P-1**, **P-2** and **S-1** that sero-converted for HBeAg during ARC-520 treatment) reduced serum HBV DNA by 5.6 ± 0.4 $\log_{10}$ copies/mL and HBsAg by 2.3 ± 0.4 $\log_{10}$ µg/mL during 7-10 monthly ARC-520 injections. 3 HBeAg-negative chimps (**N-1**, **N-2** and **N-3**) with ~3 $\log_{10}$ copies/mL HBV DNA and 0.1-2.3 $\log_{10}$ µg/mL HBsAg at start had mostly undetectable HBV DNA on NUCs ± RNAi. In these, HBsAg was reduced 0.5-0.9 $\log_{10}$ on ARC-520 but 2.1-3.2 $\log_{10}$ µg/mL with addition of siHBV-75 (Wooddell et al., 2017). A transitional chimp (**S-2**) became HBeAg-negative with reductions of 6.2 $\log_{10}$ HBV DNA and 0.9 $\log_{10}$ HBsAg on ARC-520, and 2.2 $\log_{10}$ HBsAg on siHBV-75.

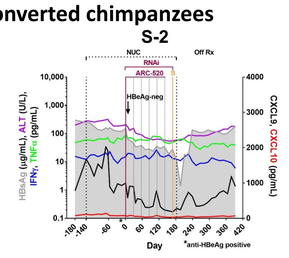
Serum Cytokines, ALT and HBsAg in Chimpanzees Before, During and After Treatment with RNAi Agents

S = siHBV-75; X = siHBV-74 (Wooddell et al, 2013)

HBeAg-seroconverted chimpanzees

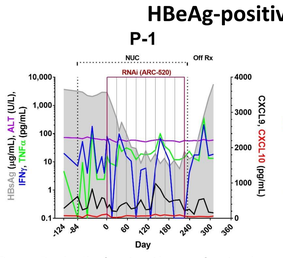


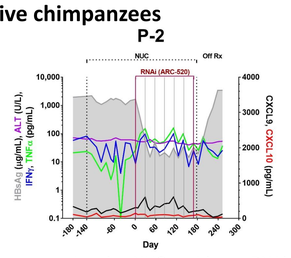
IFNɣ, TNFα, CXCL9 and CXCL10 induced during RNAi, peak prior to ALT flare, and when HBV was controlled off treatment.



ALT and CXCL9 elevated during NUC lead-in and off treatment, decreased during RNAi. IFNɣ relatively low.

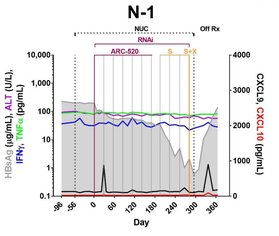
HBeAg-positive chimpanzees



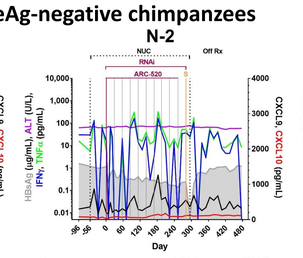


Periodic fluctuations of cytokines occurred during NUC lead-in, on RNAi treatment, and off all treatment. TNFα higher during RNAi and during viral rebound, relative to the NUC lead-in. Minimal change in ALT.

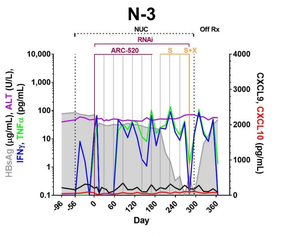
HBeAg-negative chimpanzees



TNFα higher than IFNγ. Minimal changes in these cytokines and ALT. HBV DNA near LLOQ.



Cyclically elevated IFNγ and TNFα (coincide) and CXCL9; steady ALT; very low HBsAg. HBV DNA near LLOQ.

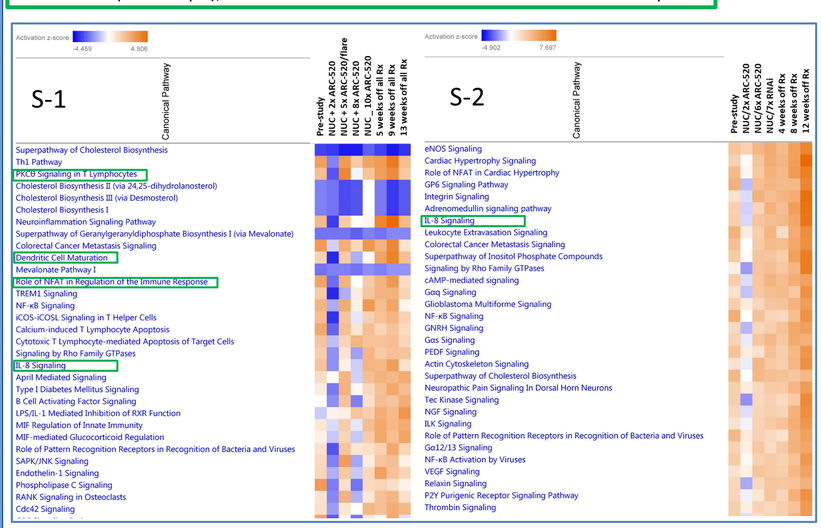


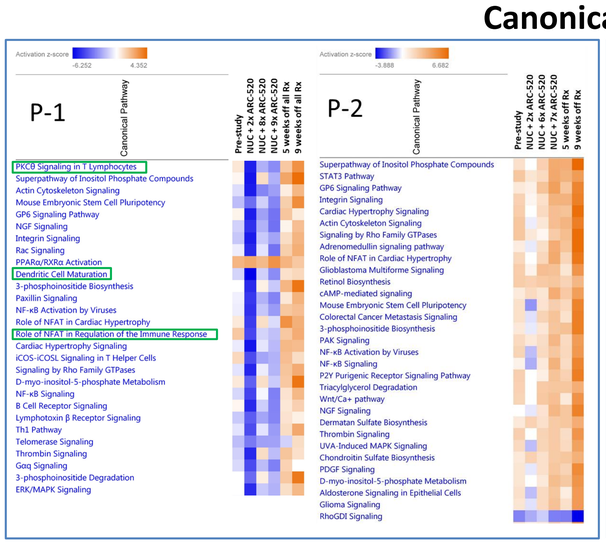
Cyclically elevated IFNγ and TNFα (coincide) and CXCL9. Serum HBV DNA near LLOQ.

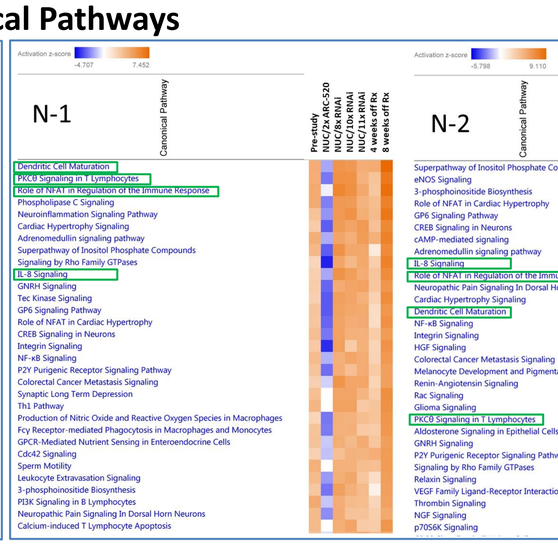
DGE with 1.5-fold change, FPKM > 0.2, all time points relative to pre-RNAi but after NUC lead-in (RNAi Day 1), z-score indicates activation or inhibition at each time point

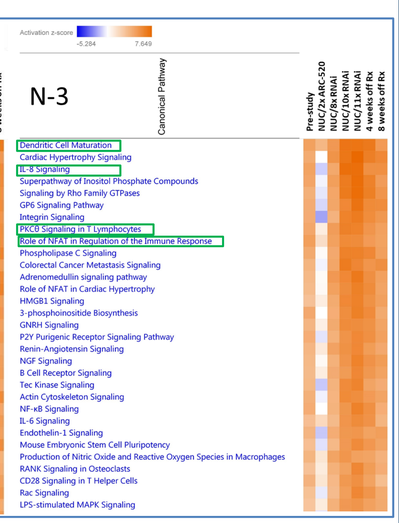
Differential Gene Expression Analysis of Liver Biopsy mRNA (Ingenuity Pathway Analysis)

Canonical Pathways

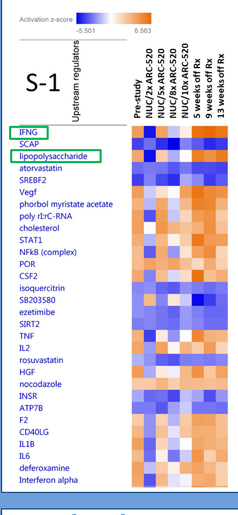


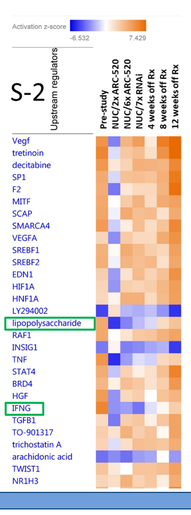




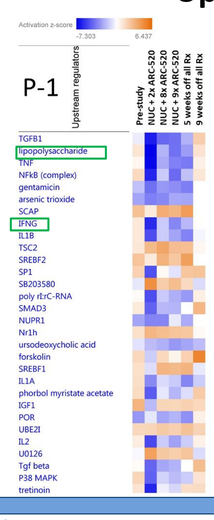


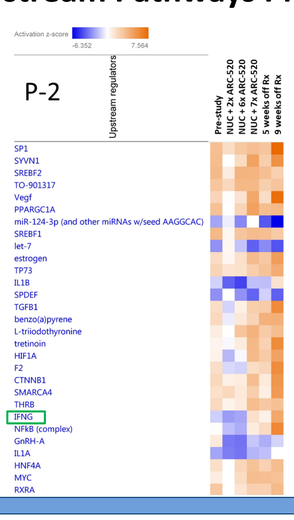
Upstream Pathways Predicted to Lead to Expression of Canonical Pathways



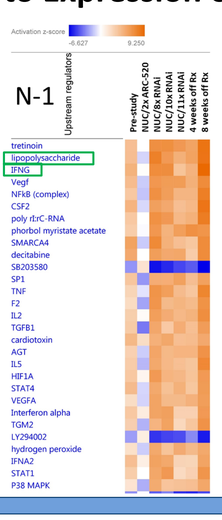


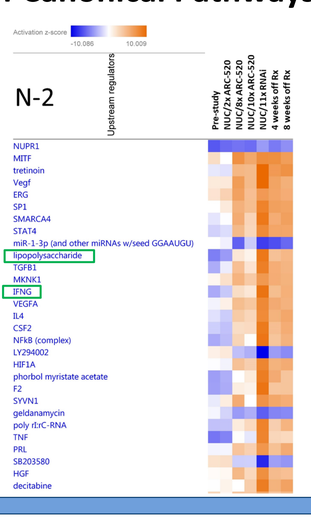
HBeAg-seroconverted : S-1 with sustained host control of HBV - many immune-related pathways cyclically activated during ALT flare and off treatment, less so during RNAi treatment. Canonical pathways are similar to HBeAg-negatives. Top predicted upstream pathways were IFNɣ and lipopolysaccharide (LPS) . S-2 without sustained response – minimal immune-related canonical pathways. Modest upstream activation of IFNɣ and LPS only during high replication.

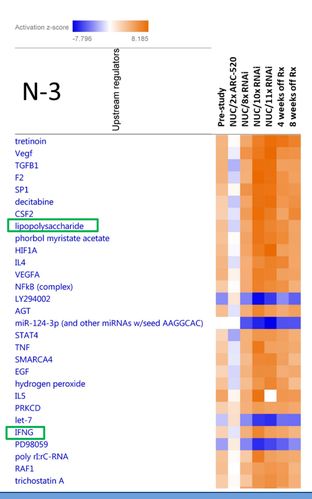




HBeAg-positive: More immune-related pathways activated in P-1 than P-2, modestly upregulated during periods of high replication and generally down-regulated during RNAi. LPS and IFNɣ predicted to be upstream activator pre- and post-treatment when replication was high.







HBeAg-negative: Canonical pathways activated during RNAi and during rebound replication: dendritic cell maturation, PKCΘ signaling in T lymphocytes, role of NFAT in regulation of the immune response and IL-8 signaling. Common upstream regulators are LPS, IFNɣ, and Vegf, among others.

Methods

NUC: Oral entecavir given daily in drink or fruit during lead-in and concomitant with RNAi treatment until 1-2 weeks after final RNAi dose.
Dosing: Monthly IV infusion of RNAi triggers (2-4 mg/kg) + EX1.
Cytokines (Luminex panel of 25 human cytokines/chemokines) measured every 1-2 weeks throughout study. Among chimpanzees, none were consistently elevated during RNAi treatment. IFNɣ, IFNɣ–responsive cytokines CLCL9 and CXCL10, and TNFα in serum were graphed along with HBsAg and alanine aminotransferase (ALT).
Differential gene expression (DGE) performed with mRNA-seq reads aligned to human genome; DGE relative to end of NUC lead-in; and expression analysis with Ingenuity Pathway Analysis (Qiagen).

Conclusions

The RNAi therapeutic ARC-520 caused negligible cytokine response in human whole blood and healthy volunteers (Schluep et al., 2017). Chimpanzees chronically infected with HBV had periodic serum elevations of IFNɣ, TNFα, CXCL9, and in some animals CXCL10, throughout the study. These elevations did not coincide with injection of RNAi treatment (half-life in plasma is <8 hrs). RNAi treatment appeared to generally reduce cytokine response but in some animals and at some times perhaps facilitated it, such as in chimp S-1 with repeatedly decreasing replication off treatment.

Pathway Analysis compares mRNA-seq gene expression data across time points to identify pathways that change by specified parameters, thereby allowing the generation of hypotheses. Dendritic cell maturation, PKCΘ signaling in T lymphocytes, role of NFAT in regulation of the immune response and IL-8 signaling were canonical pathways activated in the 3 HBeAg-negative chimps and the HBeAg-seroconverted chimp S-1, especially when HBsAg was deeply reduced and during cyclical, apparently-controlled HBV replication off treatment. Upstream regulators associated with the activated canonical pathways were IFNɣ and LPS, among others. Some of the same pathways were very modestly activated during high viral replication before and after treatment in HBeAg-positive chimps P-1 and P-2 and HBeAg-seroconverted chimp S-2, all of which had high viral replication prior to and following RNAi treatment. In conclusion, one chimpanzee seroclared HBeAg on therapy and demonstrated persistent viral control off therapy that resembled inactive carrier state as in the HBeAg-negative chimpanzees. The remaining chimps, while responsive to therapy, did not appear to have lasting immune changes and were unable to demonstrate altered viral control upon therapy discontinuation.

REFERENCES

Schluep et al. Safety, Tolerability, and Pharmacokinetics of ARC-520 Injection, an RNA Interference-Based Therapeutic for the Treatment of Chronic Hepatitis B Virus Infection, in Healthy Volunteers. *Clin Pharmacol Drug Dev*. 2017; 6(4):350-62.

Wooddell et al. Hepatocyte-targeted RNAi therapeutics for the treatment of chronic hepatitis B virus infection. *Mol Ther.* 2013 May; 21(5):973-85.

Wooddell et al. RNAi-based treatment of chronically infected patients and chimpanzees reveals that integrated hepatitis B virus DNA is a source of HBsAg. *Sci Transl Med*. 2017 Sep; 27(9):409.