Supplementary Materials for

RNAi-Based Treatment of Chronically Infected Patients and Chimpanzees Implicates Integrated Hepatitis B Virus DNA as a Source of HBsAg

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This PDF file includes:

Supplementary Materials

Supplemental Materials and Methods

Heparc-2001

Participants. Potential subjects recruited from Hong Kong underwent screening to confirm eligibility and were randomized (cohorts 1-5) during 60 days prior to the scheduled dosing date. Final confirmation of eligibility was checked on Day 1. Male or female CHB patients 18 to 65 years in age were eligible to participate. Inclusion criteria included: diagnosis of HBeAg negative (cohorts 1-4, 7) or HBeAg positive (Cohorts 5-7), immune active (cohorts 1-6) or immune active or tolerant CHB (cohort 7), HBsAg > 1,000 IU/mL (cohorts 1-4) or >500 IU/mL (cohorts 5 and 6) at screening, HBV DNA < 200 IU/mL (cohorts 1-6) and > 6 months of continuous daily, oral ETV (0.5 mg/day, cohorts 1-6). Treatment naïve patients (cohort 7) included any immune active patients who had initiated ETV therapy < 30 days before screening, immune active patients with alanine aminotransferase (ALT) > 2x ULN starting ETV on Day 1 and immune tolerant or immune active patients with ALT < 2x ULN starting ETV on Day 1. Exclusion criteria included: history of clinically relevant medical illnesses that in the Investigator's opinion might jeopardize subject safety or interfere with participation in the study, including but not limited to hematological, renal, endocrine, pulmonary, gastrointestinal, cardiovascular, hepatic, psychiatric, neurologic, or allergic disease; acute signs of hepatitis/other infection (e.g., moderate fever, jaundice, nausea, vomiting, abdominal pain) evident within 4 weeks of screening and/or at the screening examination; hepatic transaminases (ALT or AST) > 100 IU/mL; FibroScan® score > 8; use of any drugs known to induce or inhibit hepatic drug metabolism within 30 days prior to administration of study treatment; seropositive for human immunodeficiency virus, hepatitis C virus or a history of delta virus hepatitis; current use and/or a history of alcohol and/or drug abuse < 12 months from screening; use of investigational agents

or devices within 30 days prior to planned study dosing or current participation in an investigational study; a history of allergy to bee venom, history of hypersensitivity reaction requiring an emergency visit to a physician or hospital and/or requirement for treatment with steroids and/or epinephrine, or a positive reaction to the bee venom allergy skin test performed at screening. The bee venom exclusion criteria was included because the melittin-like peptide component of ARC-520 is similar to melittin, a component of honey bee venom, and therefore could theoretically cause an allergic reaction in patients with a history of bee sting allergy.

Participants were free to withdraw from the study at any time for any reason. The Principal Investigator (PI) could withdraw a subject to protect his/her health if: the need to take medication could interfere with study measurements; intolerable/unacceptable adverse experiences occurred; major violation or deviation of study protocol procedures occurred; there was non-compliance of subject with protocol; the subject was unwilling to proceed and/or consent was withdrawn; or if it was deemed in the subject's best interest.

Treatment. Arrowhead Pharmaceuticals, Inc. (Pasadena, California, USA) supplied the ARC-520 as two sterile 10-mL vials containing ARC-520 API and ARC-EX1. Prior to dosing subjects, a study pharmacist mixed one vial of ARC-EX1 with one vial of API to yield the ARC-520 injection. Each subject received a single dose of ARC-520 or placebo, administered intravenously by clinical staff at the infusion rate of 10 mg/min. There was no intra-subject dose escalation. Subjects fasted from food for at least 2 hours pre-dose and 2 hours post-dose and from water from 1 hour pre-dose to 1 hour post-dose. Subjects were confined at the clinical facility for approximately 8 hours on Day 1, and then discharged following completion of safety assessments and blood and urine sampling. Visits to the clinical facility occurred on Days 2, 3, 8, 15 (± 1), 22 (± 1), 29 (± 1), 43 (± 3), 57 (± 3), and 85 (± 3) (End-of-Study visit). Clinically

significant changes including adverse events (AEs) were followed until resolution or Grade 1 status was achieved.

Study Assessments. The safety analysis included all patients who received study medication. Safety measures included (1) AEs; (2) physical examinations; (3) vital signs (resting heart rate, semi-supine systolic/diastolic blood pressure, respiratory rate, and temperature); (4) triplicate ECG measurements at least 2 minutes apart (readings taken after the subject was supine for at least 3 minutes); (5) clinical laboratory tests (hematology, biochemistry, coagulation, urinalysis); (6) use of concomitant medications, and (7) recording reasons for treatment discontinuation due to toxicity. Abnormalities in laboratory findings or other assessments that were deemed clinically significant by the PI and were initially detected during the study or were present at baseline and significantly worsened during the study were reported as AEs, whether or not they were considered drug-related. The causality of AEs was determined by the PI. All AEs and SAEs were followed until resolution, until the condition stabilized, until the event was otherwise explained, or until the subject was lost to follow-up

Quantitative HBV serology parameters assessed were IU/mL HBV DNA (Cobas® AmpliPrep®/Cobas® TaqMan®, v2.0, Roche Diagnostics), IU/mL HBsAg (Elecsys® HBsAg II quant®, Roche Diagnostics), PEIU/mL HBeAg (Liaison® , DiaSorin), and kU/mL HBcrAg (Lumipulse® G, Fujirebo). Evaluation of additional pharmacokinetic, pharmacodynamics, and HBV serologic measures is ongoing and will be reported elsewhere. Cohort 1 to 6 patients had been on daily oral NUCs for a mean of 4.1 years prior to entering the Heparc-2001 clinical trial, minimum of 1.0 years and maximum of 7.8 years (Table S1). The twelve Cohort 7 patients were NUC-naïve prior to start of study.

Chimpanzee study

Animal care. Chimpanzees were housed and cared for at the Southwest National Primate Research Center at Texas Biomedical Research Institute or New Iberia Research Center at The University of Louisiana at Lafayette. The study design had been to dose all animals 12 times, but in anticipation of chimpanzee reclassification by the U. S. Department of Fish and Wildlife we ended the study ahead of schedule in order to monitor all animals off treatment as long as allowed. The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Institutional Animal Care and Use Committee. Both facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and operate in accordance with the NIH and U.S. Department of Agriculture guidelines and the Animal Welfare Act. Animals were housed with group members. An environmental enrichment program was provided by a staff of behavioral scientists.

Blood chemistry, coagulation and hematology. At each time point serum clinical chemistry, cholesterol and triglyceride were analyzed and a standard hematology panel was performed on whole blood. Coagulation was assessed prior to biopsies.

Analysis of HBV serum antigens. Serum samples diluted with 5% nonfat dry milk were evaluated for HBsAg using the GS HBsAg EIA 3.0 kit (Bio-Rad, Redmond, WA) as previously described (*21*) and for HBeAg using a kit from DiaSorin (ETI-EBK PLUS).

Serum HBV DNA quantitation in chimpanzees. A 100 μL serum sample was diluted to 200 μL with PBS and extracted for DNA using the QIAamp DNA mini kit (Qiagen, Cat. No. 51304) following the recommended manufacturer's protocol. Each sample was spiked with 100ng poly-

d(A) (Roche Scientific, Cat. No. 10223581001) as a carrier. After incubating with Proteinase K and lysis buffer for 10 min at 56°C, nucleic acids were precipitated with ethanol and the entire solution applied to a column. After the wash steps, the samples were eluted in 100 μL of nuclease-free water. For the quantitative PCR TaqMan assay, 10 μL were added to 40 μL of master mix consisting of core forward primer CGAGGCAGGTCCCCTAGAAG and core reverse primer TGCGACGCGGYGATTG (300 nM final concentration of each) and core probe (FAM/TAMRA dual-labeled) AGAACTCCCTCGCCTCGCAGACG (200 nM final) to a conserved region in the HBV core gene, and the TaqMan Fast Advanced DNA kit (Applied Biosystems, Cat. No. 4444557). Y represents either pyrimidine to account for HBV strain differences; mRNA-seq data revealed that chimpanzee 4x0139 had a T in this position and the rest of the chimpanzees for which biopsies were taken had a C. All TaqMan assays were performed with an Applied Biosystem (ABI) 7500 Sequence Analyzer. All primers and probes were from Integrated DNA Technologies (Coralville, IA).

Chimpanzee liver biopsies. A 16T-gauge needle was used to obtain a core size of approximately 2.5 cm from a double pass. One-half of the biopsy sample was transferred to a tube containing 1.5 mL RNAlater® Stabilization Solution (Ambion, Cat. No. AM7020), one-quarter (≥ 0.5 cm) was placed in a tube containing 10% buffered formalin solution, and one-quarter was placed into a cryovial and snap frozen in a dry ice ethanol bath.

The tissue in formalin was fixed for a minimum of 24 hours at ambient temperature, and then processed using a Shandon Excelsior ES Tissue Processor. The paraffin blocks were sectioned, stained, and examined for histopathology by a Board Certified Veterinarian Pathologist and by immunohistochemistry staining of several markers.

Liver HBV DNA quantitation and analysis of DNA form. The snap frozen liver biopsies were ground in PBS using a sterile, RNAse-free disposable pellet pestle and extracted for DNA using the QIAamp DNA mini kit (Qiagen, Cat. No. 51304) following the manufacturer's protocol. DNA concentration was determined using a Nanodrop ND-1000 and 250 ng of liver DNA was used per quantitative PCR TaqMan assay, in duplicate, using the same primers and probe as used for the serum DNA assay. The samples were analyzed utilizing a TaqMan Fast Advanced DNA kit (Applied Biosystems, Cat. No. 4444557).

Total HBV DNA includes cccDNA, replication products such as rcDNA, and integrated HBV. To evaluate the molecular form of HBV DNA species in the livers, the biopsy samples were treated with Plasmid-Safe ATP-Dependent DNase (PSD) prior to qPCR. PSD was developed to digest contaminating sheared chromosomal DNA in plasmid DNA preparations (Epicentre Technologies). PSD has its greatest activity on fragments of double-stranded DNA and to a lesser degree digests single-stranded DNA, thus is expected to be most active on integrated HBV DNA and to a lesser degree on HBV replication intermediates including rcDNA. cccDNA is expected to be resistant to digestion by PSD. The plus DNase samples were processed using the Plasmid Safe, ATP dependent DNase kit (Epicenter, Cat. No. E3105K) and 250 ng liver DNA in a 5 μL volume was mixed with 15 μL of the DNase mix, incubated for 30 min at 37°C, then heat-inactivated for 30 min at 70°C. The entire 20 μL volume was utilized in a 50 μL total volume TaqMan assay. PSD digestion decreased liver HBV DNA in HBeAg positive chimpanzee samples by 14 ± 3-fold in the pre-study samples but by only 1.8 ± 0.2-fold after NUC lead-in, suggesting a change in the number and/or form of replication products in these animals treated with NUCs. In contrast, HBV DNA in HBeAg negative chimpanzees was more sensitive to PSD digestion during NUC treatment alone (43 ± 21-fold decrease).

Targeted DNA sequencing and breakpoint analysis. Liver genomic DNA isolated from biopsy samples of eight chimpanzees was fragmented using the Covaris M220 sonicator (Covaris Inc, Woburn MA) to an average size of 500 bp. Illumina-sequencing compatible libraries were created, after which HBV-containing DNA was enriched with a SureSelectXT2 custom library (Agilent) according to the manufacturer's manual, followed by Illumina 2x150bp Hiseq2500 Rapid sequencing using version2 kits. Bcl-fastq conversion was performed using CASAVA 1.8.2. Approximately 24 million reads were generated for each sample and sequencing reads were aligned to a chimpanzee HBV sequence (AF222323.1) using CLC Genomics Workbench (version 9.0) (Qiagen) to generate consensus HBV DNA sequence for each animal. Following removal of duplicate reads marked with Picard, a second round of alignment was then performed using the chimpanzee genome sequence (panTro4) and each chimpanzee's HBV consensus DNA sequence as reference using BWA-MEM. The breakpoints at which HBV sequences were disrupted by either host sequence or rearranged HBV sequence were analyzed using a customer script generated by Basepair LLC.

The primary source of integrated HBV DNA is double-stranded linear DNA (dslDNA), an *in situ* primed, aberrant replication product that results when the cap-containing RNA primer fails to translocate to the downstream direct repeat 2 (DR2) sequence during plus strand DNA synthesis (*9*). The upstream end of dslDNA is at DR1 and the downstream end terminates in the gap region between DR2 and DR1, the majority ending proximal to DR1 (fig. S1) (*5*, *32*). Consistent with this mechanism of HBV integration, the major integration sites in our study chimpanzees were at DR1. HBeAg negative chimpanzee 88A010 (25 years old), transitional 89A008 (24 years old), and HBeAg positive 4x0139 (37 years old) had the highest number of integration events while

HBeAg positive A4A014 (the youngest at 9 years old) had the lowest, suggesting a possible increase in integrated HBV DNA with duration of infection as observed in humans (*7*).

Liver mRNA quantitation by RT-qPCR. On designated liver biopsy dates, a portion of the biopsy was put into a cryovial filled with RNAlater® Stabilization Solution. The vial was stored overnight at 4°C, and then transferred to -80°C the following day and until processed. After thawing, the biopsy material was transferred to a microcentrifuge tube and spun for 10 sec at high speed to remove residual RNAlater® Stabilization Solution. The biopsy was ground in 100 µL PBS using a sterile, RNAse-free disposable pellet pestle. Total RNA was extracted with RNA-Bee RNA isolation Reagent (Tel Test INC, Cat. No. CS105B), following the manufacturer's protocol. Purified total RNA concentration was determined using a Nanodrop ND-1000. A 5 ng sample of total RNA was used in a RT-qPCR TaqMan reaction using the RNA UltraSense One Step Quantitative RT-PCR System (Invitrogen, Cat. No. 11732927) with designed primers and probe to the HBV core and X genes. The HBV core primer probe set was the same set used in the serum and liver HBV DNA assays and was designed to detect precore transcripts and pgRNA. The X gene forward primer CCGTCTGTGCCTTCTCATCTG, reverse primer AGTCCAAGAGTYCTCTTATGYAAGACCTT and probe (FAM/TAMRA dual labeled) CCGTGTGCACTTCGCTTCACCTCTGC were designed to recognize all HBV transcripts. Y in the X reverse primer represents either pyrimidine to account for HBV strain differences. mRNA-seq analysis revealed that all of the chimpanzees had the reverse strand sequence AGTCCAAGAGTCCTCTTATGCAAGACTTT.

Short term treatment with NUCs is not expected to exert an effect on the level of transcripts, and in both groups of chimpanzees, total HBV transcripts were not significantly different at pre-study from those after NUC lead-in (Fig. 3B). Consistent with reduction of HBsAg, one week after the

second ARC-520 injection (Day 36), total HBV RNA (with X probe) was reduced 89 ± 2% in three HBeAg positive chimpanzees and 62 ± 4% in the HBeAg negative chimpanzees. HBeAg positive chimpanzee 4x0139 was omitted from this analysis because the X probe appears to be less efficient in this animal: the core probe detected approximately 100 times more transcripts than the X probe. Given the high level of HBsAg production in this chimpanzee, it is unlikely that the HBsAg transcripts comprise less than 1% of the total as assay results suggest.

Illumina mRNA-seq analysis. Total RNA was isolated from liver biopsies of each chimpanzee. Following cDNA synthesis, adapter ligation and PCR amplification, the cDNA library was subjected to 2x50bp sequencing. Approximately 40 million reads were generated for each sample and the reads were aligned to the chimpanzee reference genome sequence and the HBV consensus DNA sequence compiled for each chimpanzee, using CLC Genomics Workbench.

The total number of reads that contained HBV sequence are expected to include mRNA transcribed from cccDNA as well as any mRNA transcribed from integrated HBV genomes. Consistent with RT-qPCR results using the core probe, the HBeAg positive chimpanzees had significantly more reads that included the sequences expected to be in precore than the HBeAg negative chimpanzees.

The upstream end of dslDNA terminates at DR1 and thus lacks the precore/core promoter to drive transcription of the pgRNA and mRNA encoding HBeAg. However, integrated dslDNA is expected to contain the entire open reading frames that encode the S proteins along with their promoters. Sequences 3' of the downstream DR1, including the HBV PAS, are not present in dslDNA; and those between DR2 and DR1, such as the target sites for the siRNAs in ARC-520, may be deleted during integration (fig. S1). In HBeAg negative chimpanzees, there were less

than half as many target sites for siHBV-77, which overlaps DR1, as for siHBV-74 that is 46 bases upstream, consistent with increased deletion frequencies near the DR1 site (table S5).

A possible explanation for reduced HBsAg knockdown could be lower efficiency of RNAi in HBeAg negative chimpanzees by an unknown mechanism. This is unlikely, as further analysis of the mRNA-seq data demonstrated that transcripts containing the target site for the ARC-520 RNAi triggers were reduced by ARC-520 with similar efficiency in HBeAg positive (90.4% to 99.7%) and negative (85.7% to 97.7%) chimpanzees (table S5). Further, there was no indication of resistance to ARC-520 developing after multiple doses (table S5 and fig. S6).

PacBio SMRT sequencing and analysis. IsoSeq library was prepared according to PacBio's standard protocol. Briefly, cDNA was synthesized using the SMARTer PCR cDNA Synthesis Kit (Clontech) and was PCR-amplified. Following Blue Pippin size selection to create four size bins (<1kb, 1-2kb, 2-3kb, and 3-6kb), the cDNA was further amplified with PCR, ligated to hairpin adapters to create the SMRTbell Template, annealed to sequencing primer, and bound to polymerase for sequencing on the RSII sequencer by generation of concatamers. The sequencing reads were analyzed with the PacBio *Classify* pipeline to generate full-length nonconcatemer (FLNC) reads which were mapped to each chimpanzee's HBV consensus DNA sequence using CLC Genomics Workbench. The breakpoints at which HBV sequences were disrupted by either host sequence or rearranged HBV sequence were further analyzed using BWA-MEM. For chimpanzee 88A010, the size bins were 1-2kb, 2-3kb, 3-6kb and >6kb.

Relative to the 2,466 non-fusion HBV reads in the HBeAg positive chimpanzee A2A004 (comprising 90.5% of the total reads), a non-negligible number of HBV-chimpanzee fusion transcripts (1.3%) and HBV-HBV fusion transcripts (2%) with integration sites near DR1 were

also detected in this animal (Table 1). However, 12 of the 35 transcripts that comprised the 1.3% identified by BWA-MEM analysis as HBV-chimpanzee fusions have long stretches of poly(A) or poly(T) and appear to be artifacts misidentified as fusion transcripts. 6% of the total reads (166) from A2A004 were HBV-HBV sequences with gaps that were consistent with reported splicing events (*36*).

Most HBV transcripts in HBeAg negative 88A010 were fused to a chimpanzee sequence (66.4%), a HBV sequence (10.1%), or a sequence with unknown origin (0.9%). HBV-chimpanzee fusion transcripts in this animal utilized cryptic chimpanzee polyadenylation signals identified as the sequence AATAAA or with a single base change (table S6). Such sequences were observed within the 60 bases proximal to the poly(A) sequences. The most common PAS was AATAAA (17.7%) and the majority of transcripts (55.8%) had more than one putative PAS.

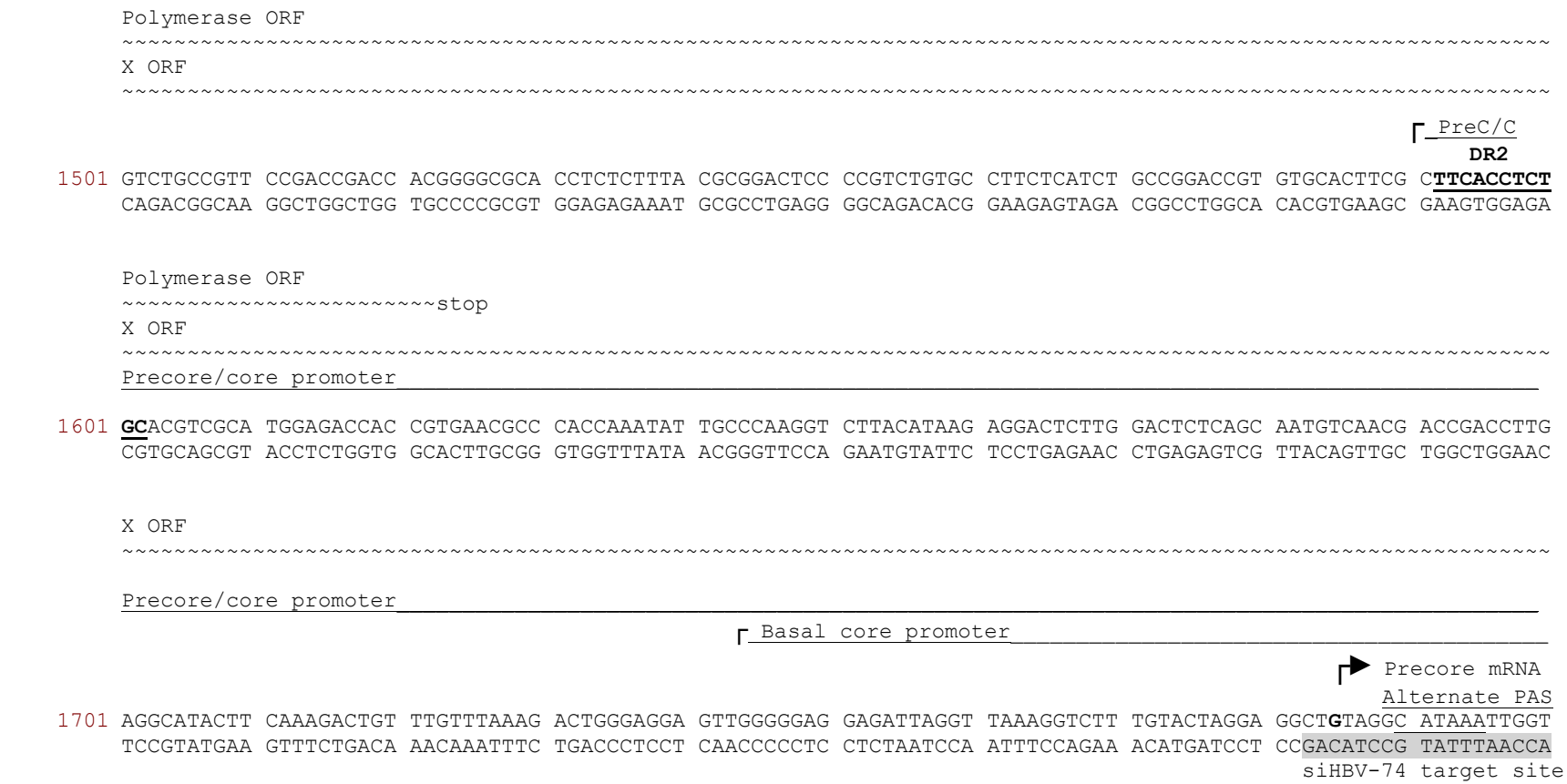
The 3' terminus of dslDNA is located between the DR2 and DR1 elements and excludes the major HBV PAS. As shown in Figure S1, an alternate PAS (sequence CATAAA) first detected in HCC tissue is located approximately 130 bases upstream of the major PAS (*33*). HBV RNA transcripts that terminate at the alternate PAS are referred to as truncated RNA (trRNA) (*34, 35*). Some integrated HBV genomes derived from dslDNA could include this alternate PAS. This alternate PAS was detected in 32.8% (42/128) of the non-fusion HBV transcripts in the HBeAg negative chimpanzee, but only in 0.3% (7/2466) of the non-fusion transcripts in the HBeAg positive chimpanzee.

If trRNA transcripts are produced from integrated HBV DNA as was previously observed in HCC, we would expect them to encode HBsAg but not HBeAg, core, nor polymerase, because the upstream and adjacent precore/core promoter is deleted in integrated dslDNA. Consistent

with this hypothesis, we observed a significant amount of trRNA encoding HBsAg in the HBeAg negative chimpanzee liver RNA along with a majority of transcripts that utilized a polyadenylation signal from the host genome.

Immunohistochemical staining of HBsAg in liver. Embedded tissues were sectioned at 4 μm. Slides were de-paraffinized in EZ-DeWax (BioGenex, Cat. No. HK 585-5K). Antigen retrieval was performed in a microwave pressure cooker for 15 min at 1000 W, and 15 min at 300 W in antigen retrieval solution (BioGenex, Cat. No. HK 086-9K). Slides were treated sequentially with peroxidase suppressor, universal block, and avidin (ThermoScientific, Cat. No. 36000). Slides were incubated sequentially at room temperature with primary antibody diluted in universal block containing a biotin block for 1 hr, biotinylated goat anti-mouse IgG for 30 min, and avidin-biotin complex for 30 min. Slides were developed with Immpact Nova Red peroxidase substrate (Vector, Cat. No. SK-4805), counterstained with Mayers (Lillie's Modification) hematoxylin (DAKO, Cat. No. S3309), dehydrated, and mounted in non-aqueous mounting media (Vector, Cat. No. H-5000). Rabbit anti-HBV surface antigen was prepared from HBV surface antigen purified from the serum of chronic carriers and used at a 1:500 dilution.

Supplementary Figure S1



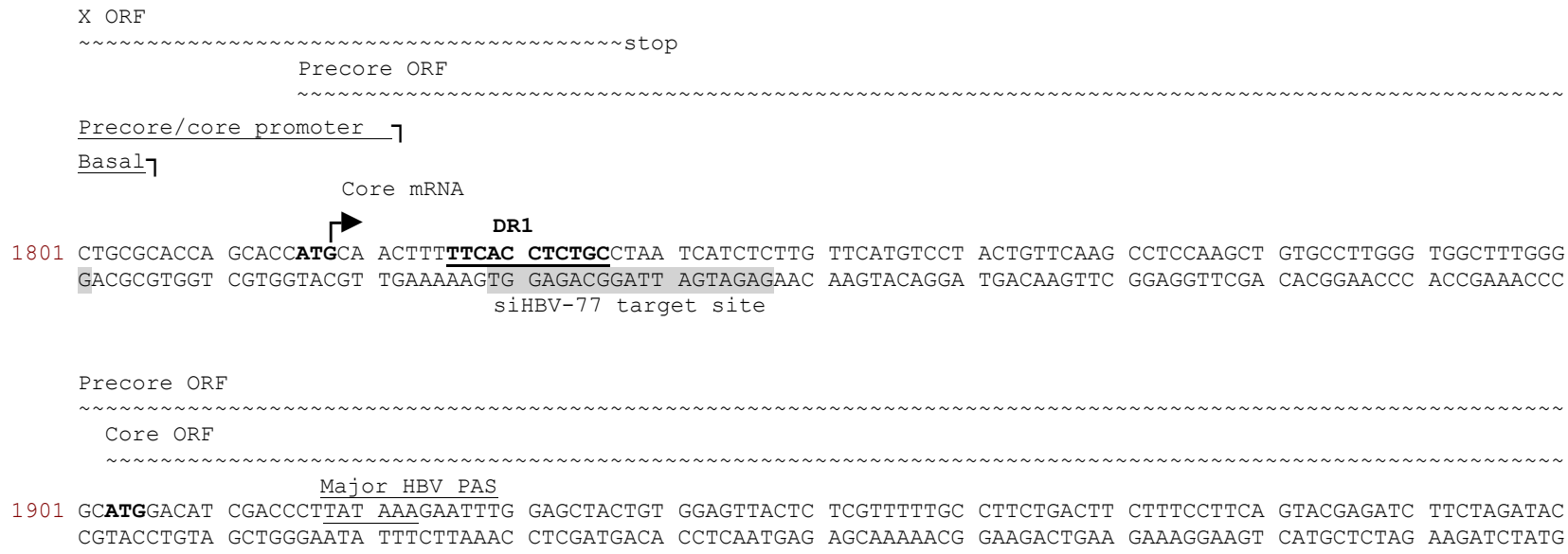


Fig. S1. HBV sequence elements near DR1 and DR2. GenBank accession number V01460 reference sequence, bases 1501 – 2000. Direct Repeats 1 and 2 (DR1, DR2) are in bold and underlined. Portions of the polymerase, X, precore and core open reading frames (ORF) are shown as wavy lines. Translation start sites (ATG) for ORFs are in bold. Transcription start sites for precore and core are marked with an arrow. The precore/core promoter is shown between brackets as a solid line. The alternate and major HBV polyadenylation signals are underlined. ARC-520 trigger sequences siHBV74 and siHBV77 are shaded.

Supplementary Figure S2

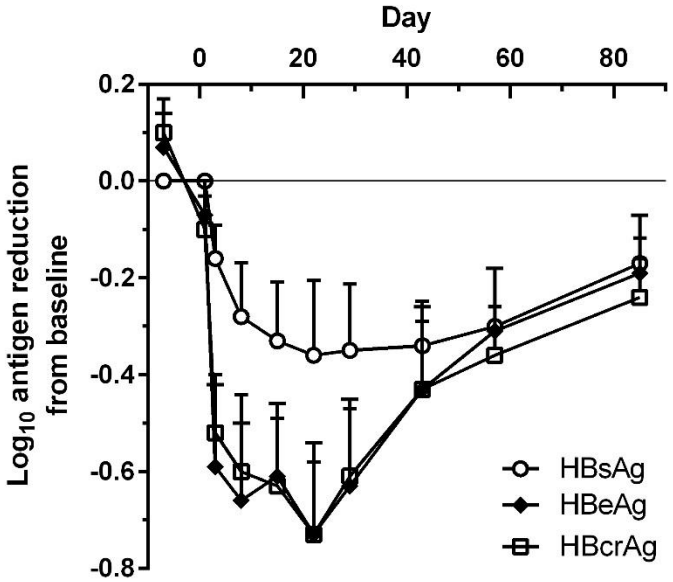


Fig. S2: Serum HBsAg, HBeAg and HBcrAg reduction in human patients treated with two doses of ARC-520 two weeks apart. CHB patients that were HBeAg positive and NUC experienced received 2.0 mg/kg doses of ARC-520 on Days 1 and 15 (cohort 6). HBeAg data from 2 patients that became HBeAg negative during therapy were excluded from the group averages. Error bars show SEM.

Supplementary Figure S3

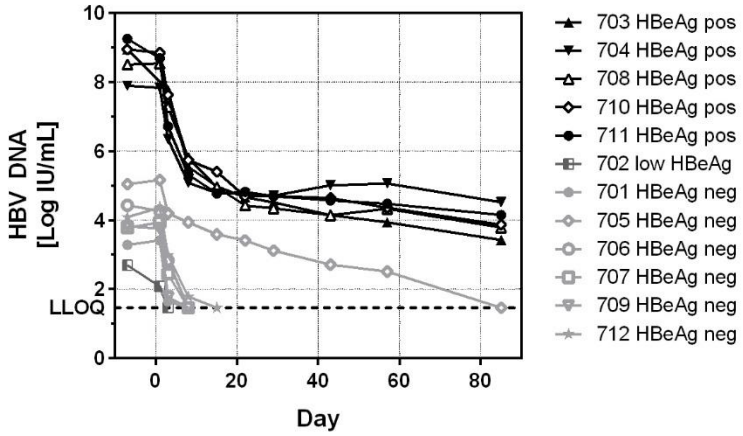


Fig. S3. Reduction of HBV serum DNA in chronically HBV infected patients treated with a single dose of ARC-520 on a background of NUCs. Clinical trial Heparc-2001 cohort 7 patients were HBeAg positive or negative (one with low HBeAg likely transitioning to HBeAg negativity) and began daily NUC treatment concomitant with a single ARC-520 injection on Day 1.

Supplementary Figure S4

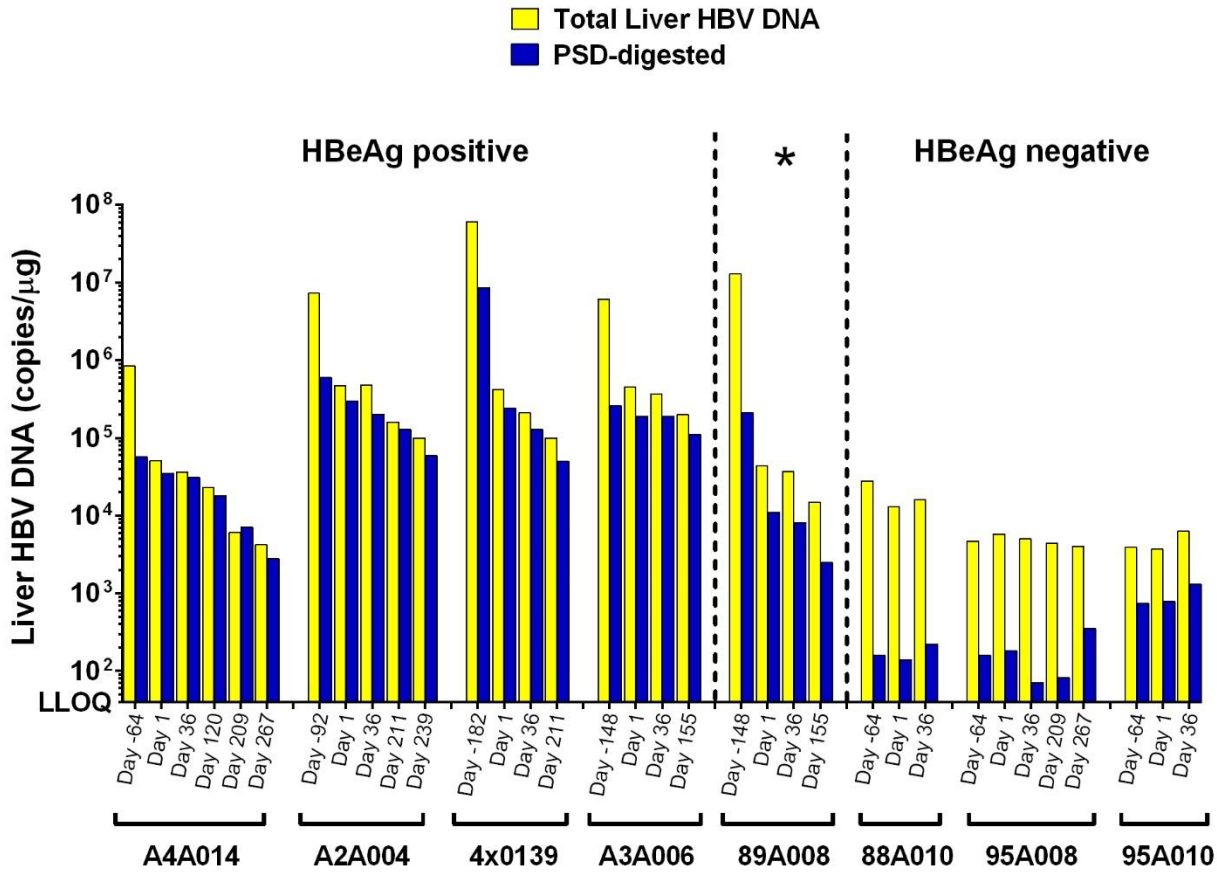


Fig. S4. Quantitation of liver HBV DNA in chimpanzees. Total DNA was isolated from liver biopsy specimens and the number of HBV genome equivalents (copies/μg total DNA) was measured by qPCR (yellow bars). HBV DNA remaining after digestion with Plasmid-safe DNase (PSD) is indicated by blue bars. Samples were collected pre-study, on Day 1 after the NUC lead-in and prior to the first dose of ARC-520, and at varied time points during dosing with ARC-520 plus NUC. Asterisk denotes data from the animal that transitioned from HBeAg positive to HBeAg negative during the study.

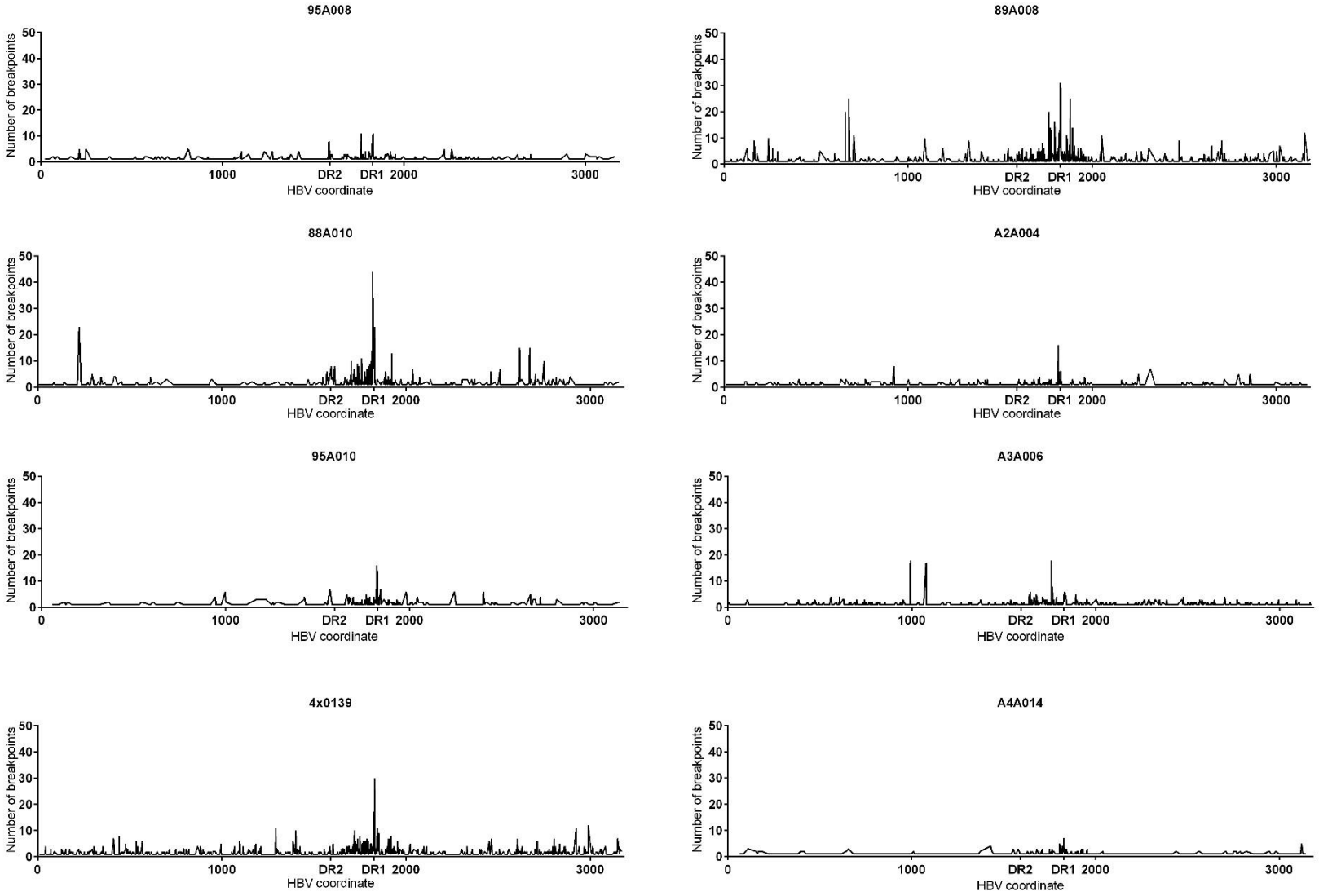
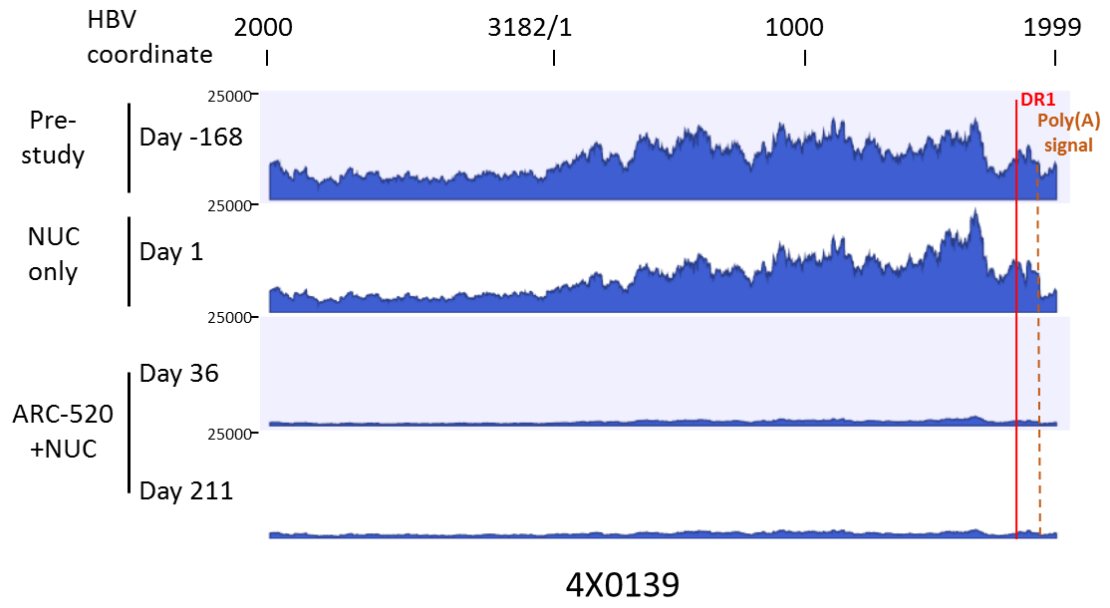


Fig. S5. Mapping of HBV DNA integration sites. Liver genomic DNA from eight chimpanzees was isolated and DNA fragments

containing HBV sequences were enriched with HBV RNA baits, followed by paired-end sequencing. Sequencing reads were aligned to the chimpanzee genome sequence and each chimpanzee's HBV consensus DNA sequence to determine the points at which HBV sequences were disrupted by host sequence.

Supplementary Figure S6

A



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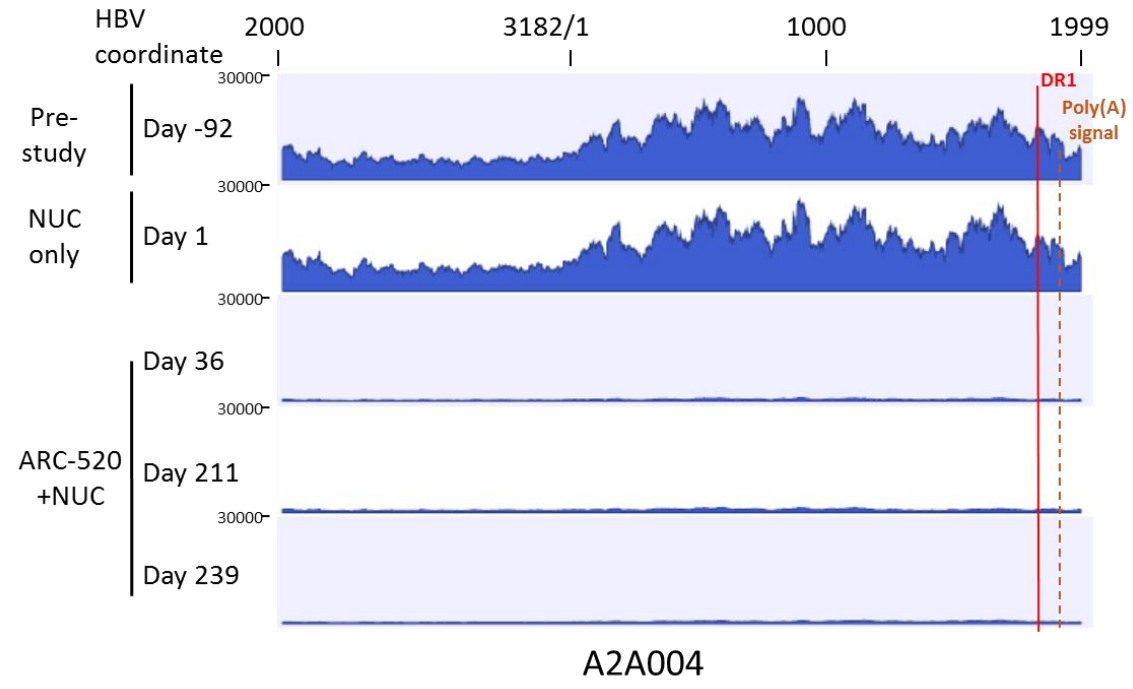

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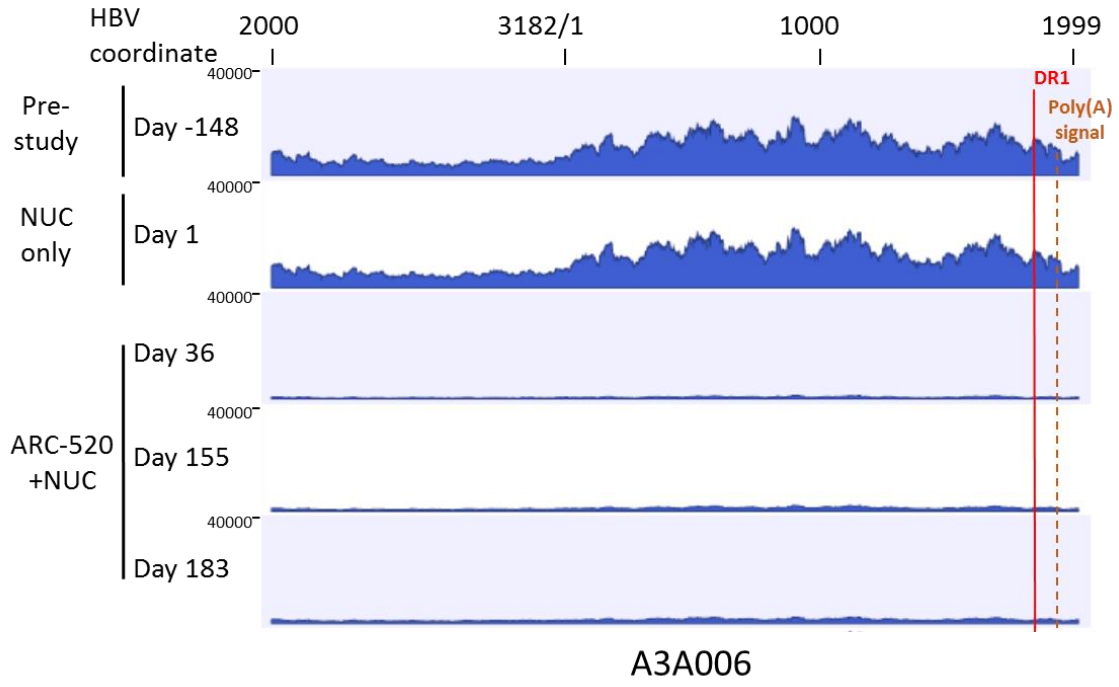
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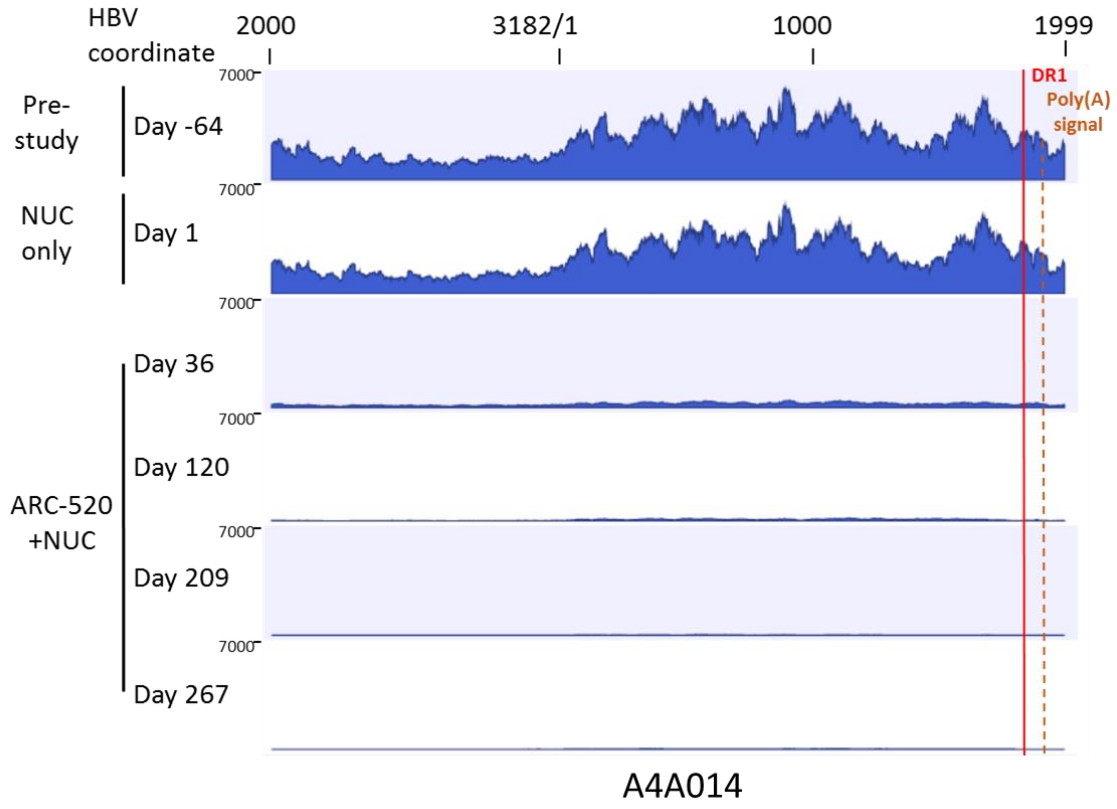
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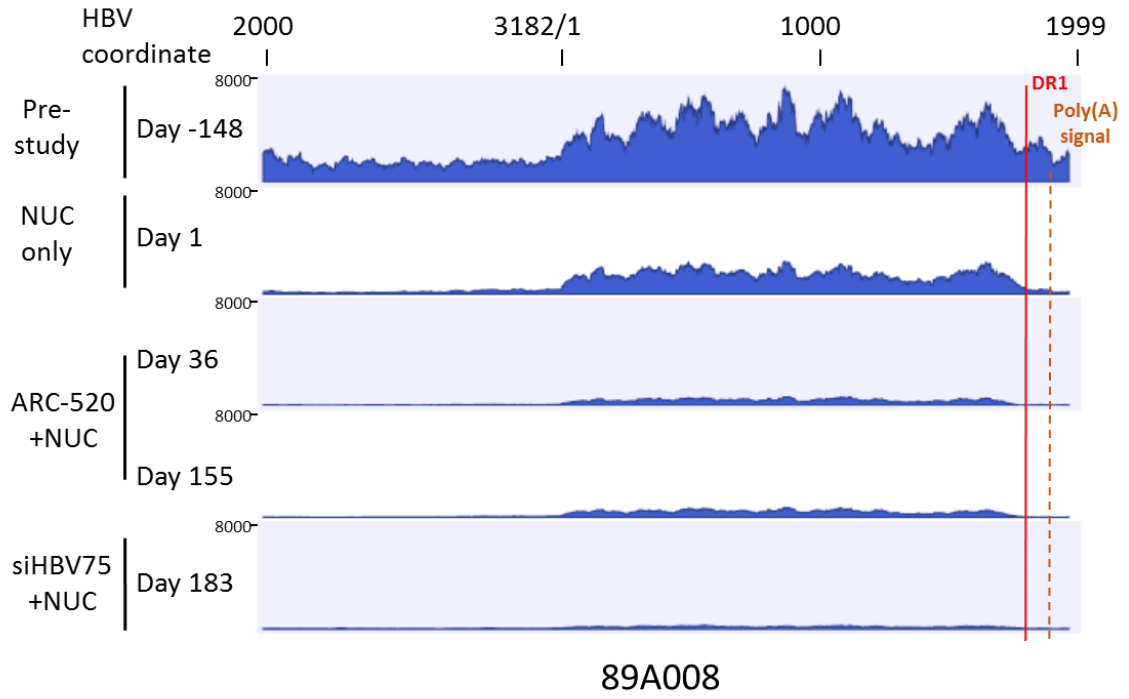

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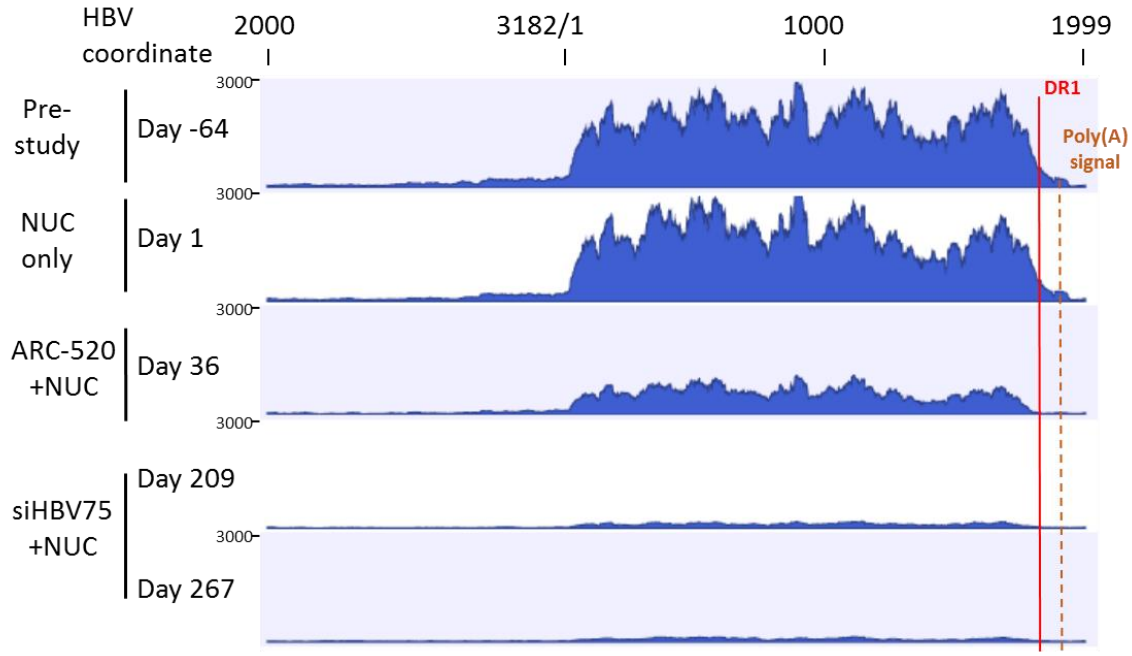

88A010

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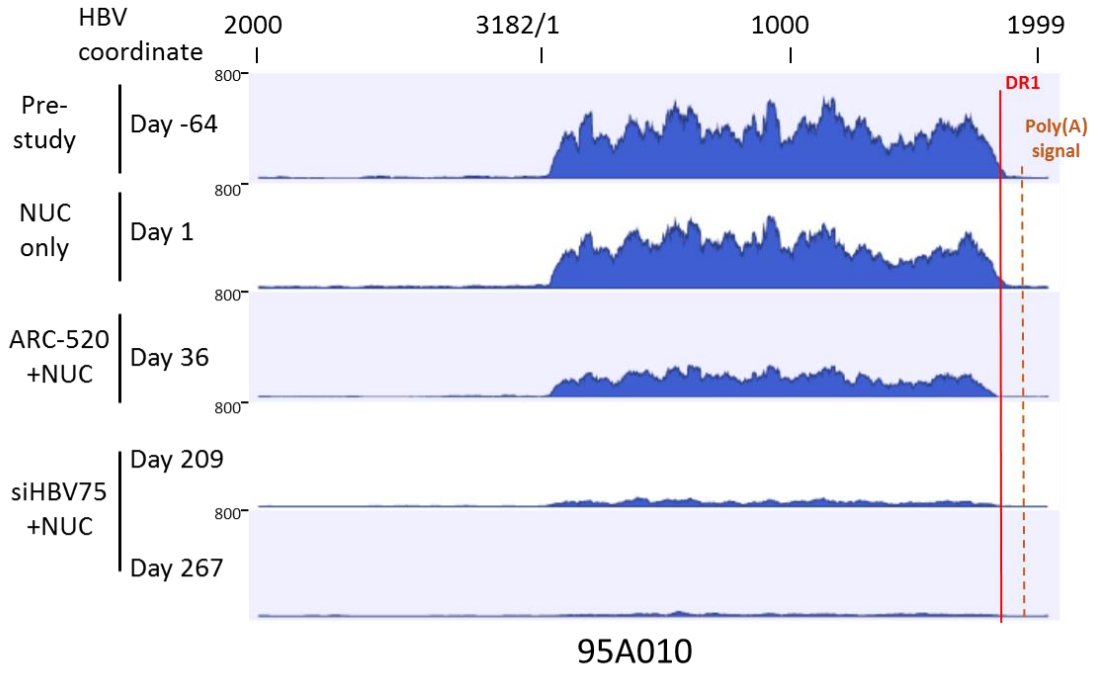
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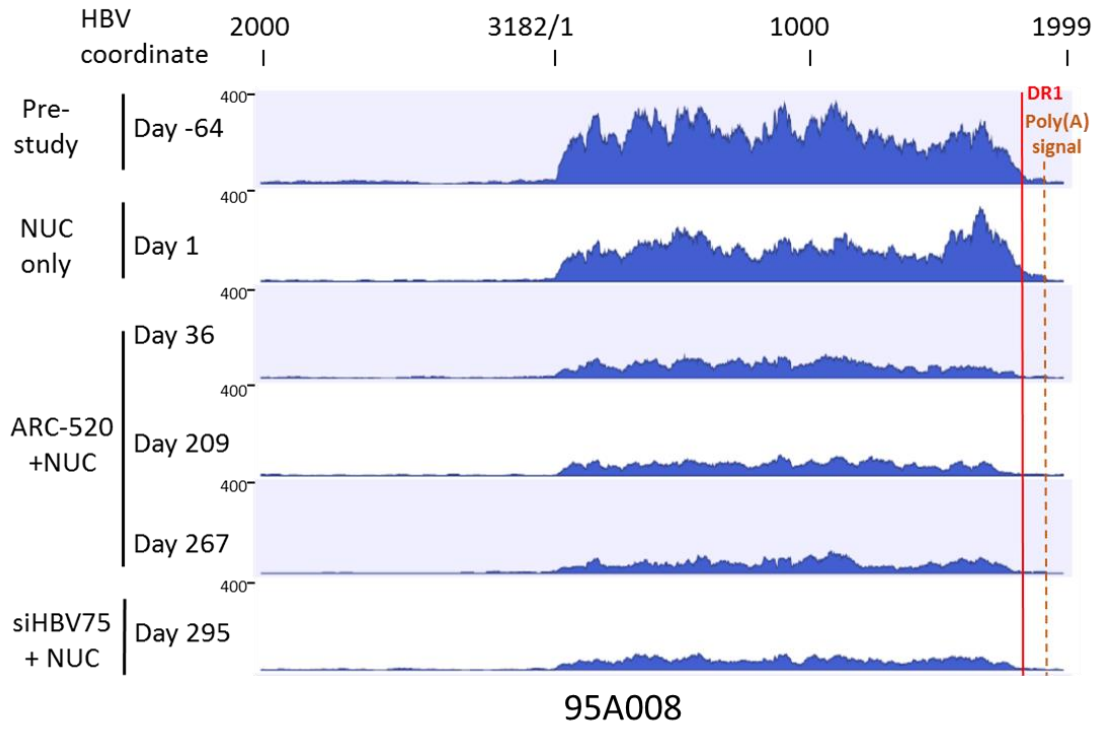
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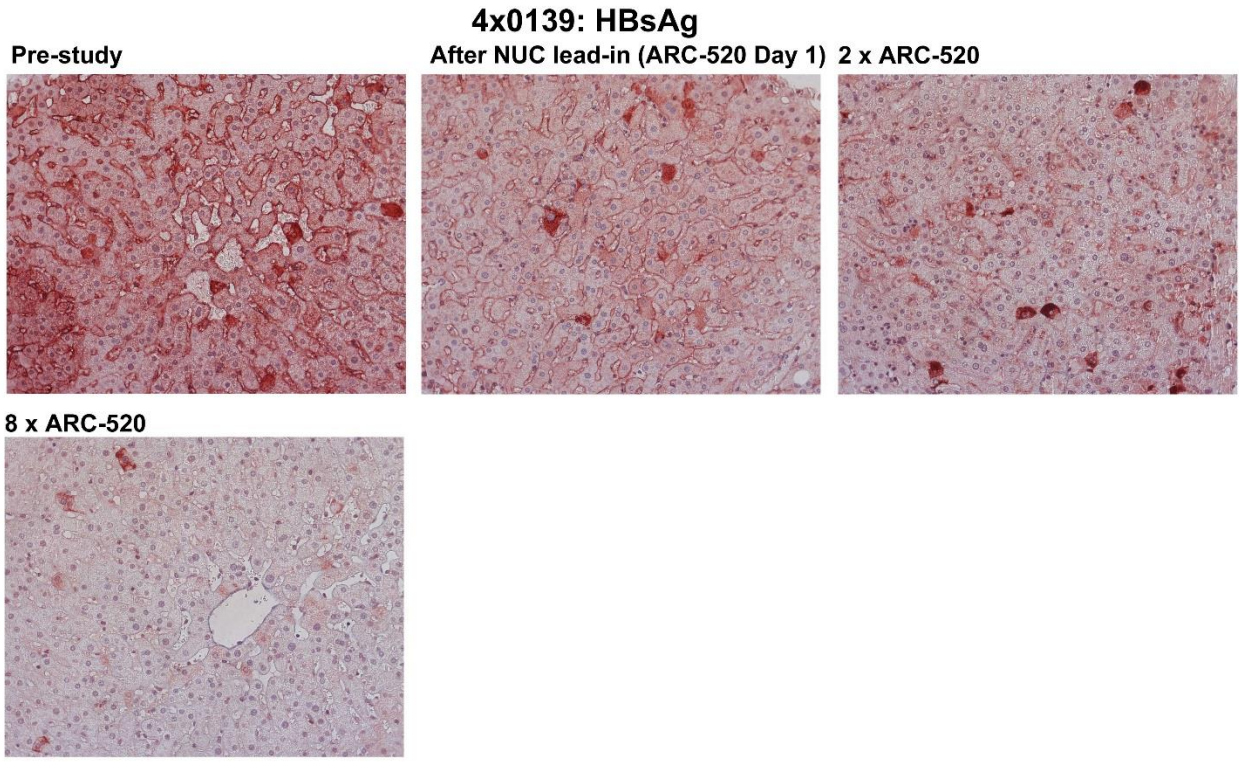
3182/1
1000
1999
Pre-study
Day -64
NUC only
Day 1
ARC-520 +NUC
Day 36
Day 209
Day 267
siHBV75 + NUC
Day 295
400
DR1
Poly(A) signal
95A008

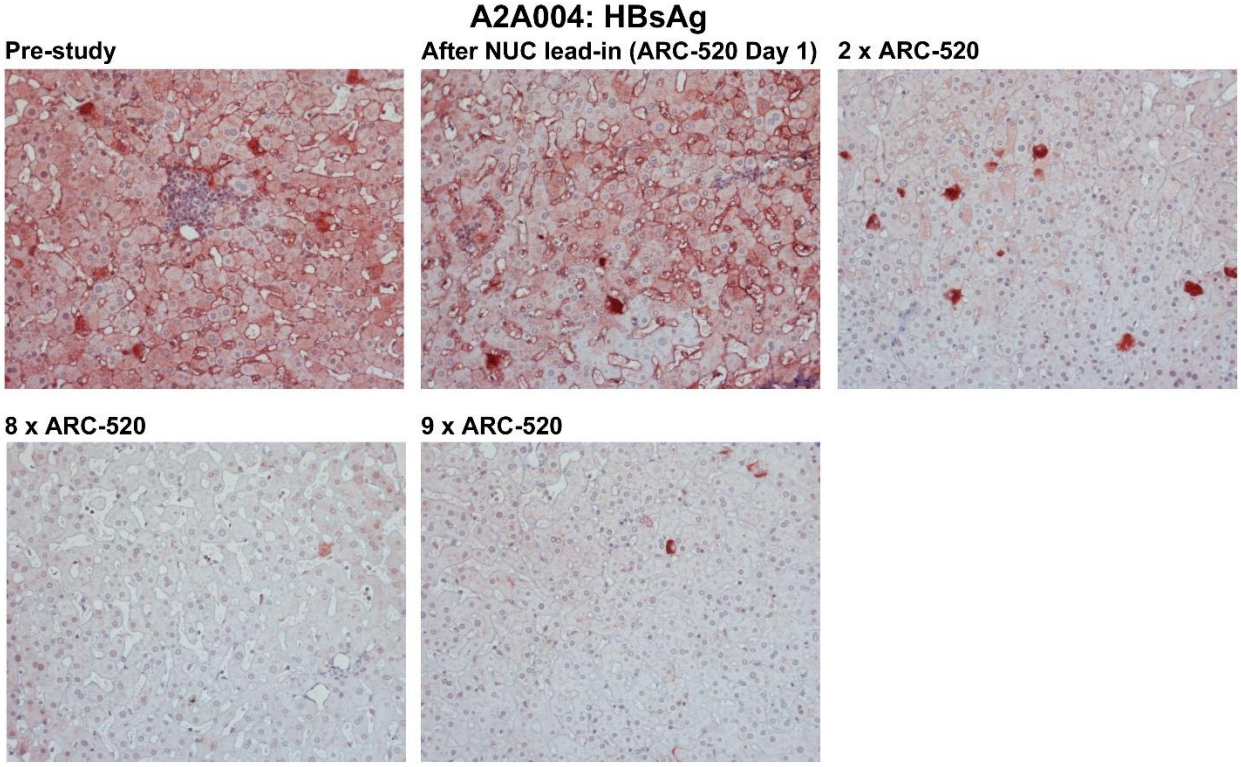
Fig. S6. Massive paired-end sequencing of chimpanzee liver mRNA and alignment to HBV reference genome. Periodic liver needle biopsies were taken from chimpanzees prior to any treatment (pre-study), following the NUC lead-in period but prior to the first ARC-520 injection (NUC only, Day 1) and during treatment with RNAi therapeutics plus NUC. HBeAg-positive chimpanzees were dosed Q4W with ARC-520: 4x0139 received eight doses (**A**); A2A004 received nine doses (**B**); A3A006 received seven doses (**C**); and A4A014 received ten doses (**D**). HBeAg transitional chimpanzee 89A008 was dosed Q4W with ARC-520 six times followed by one injection of siHBV75 (**E**). HBeAg-negative chimpanzees 88A010 (**F**) and 95A010 (**G**) were dosed Q4W seven times with ARC-520 and three times with siHBV75; and 95A008 was dosed Q4W ten times with ARC-520 and once with siHBV-75 (**H**). The sequencing reads were aligned to each chimpanzee's own consensus HBV DNA sequence.

Supplementary Figure S7

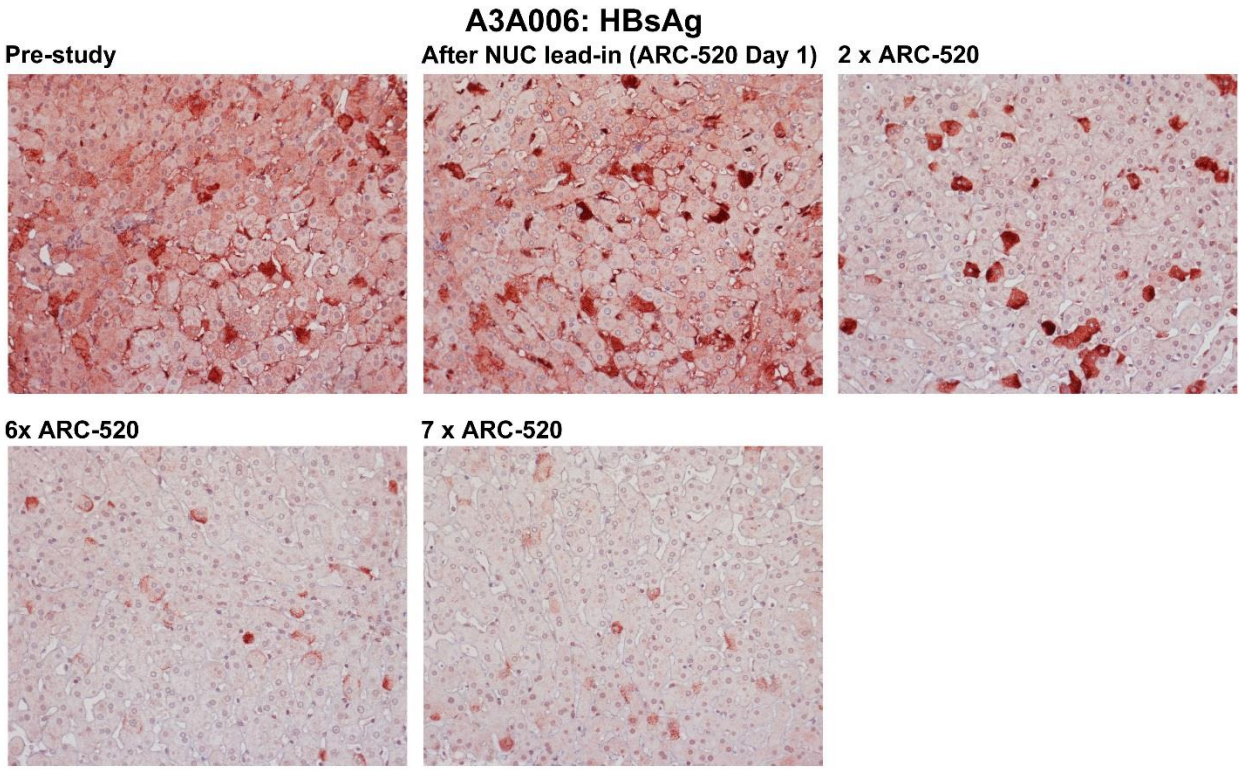
A



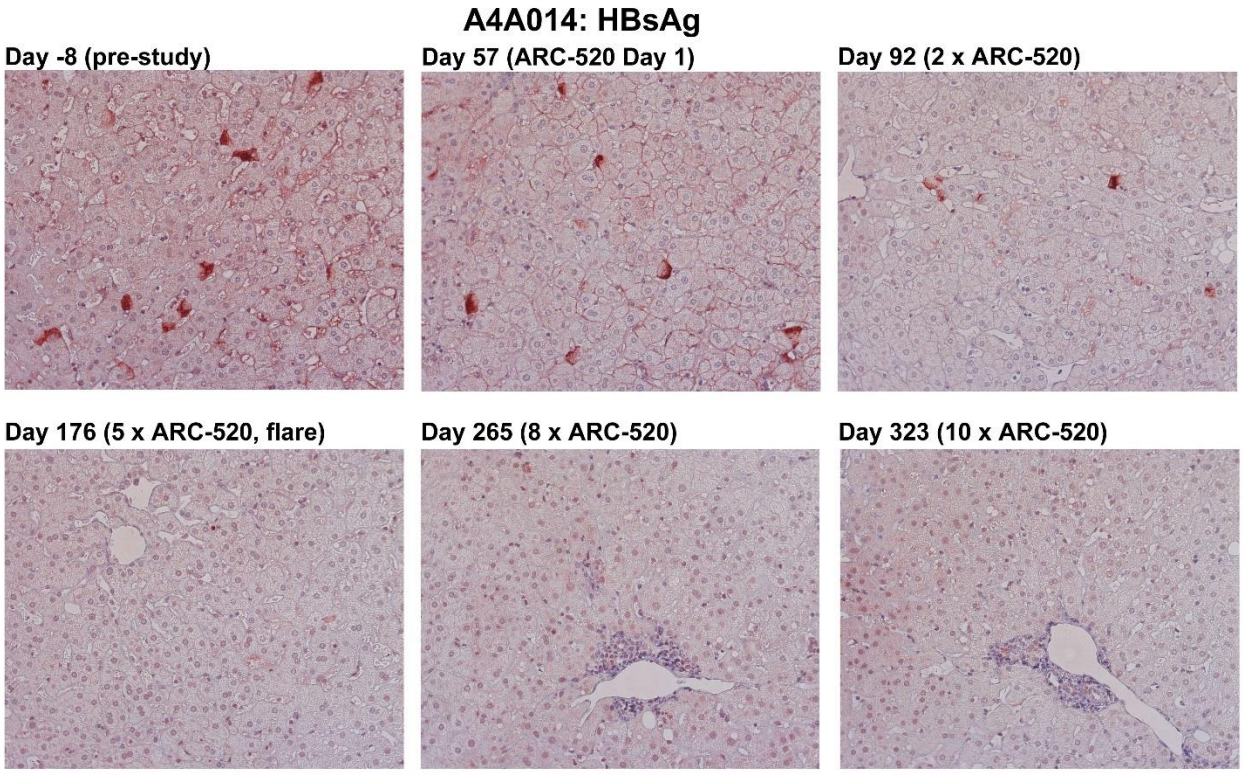
B


A2A004: HBsAg
Pre-study
After NUC lead-in (ARC-520 Day 1)
2 x ARC-520
8 x ARC-520
9 x ARC-520

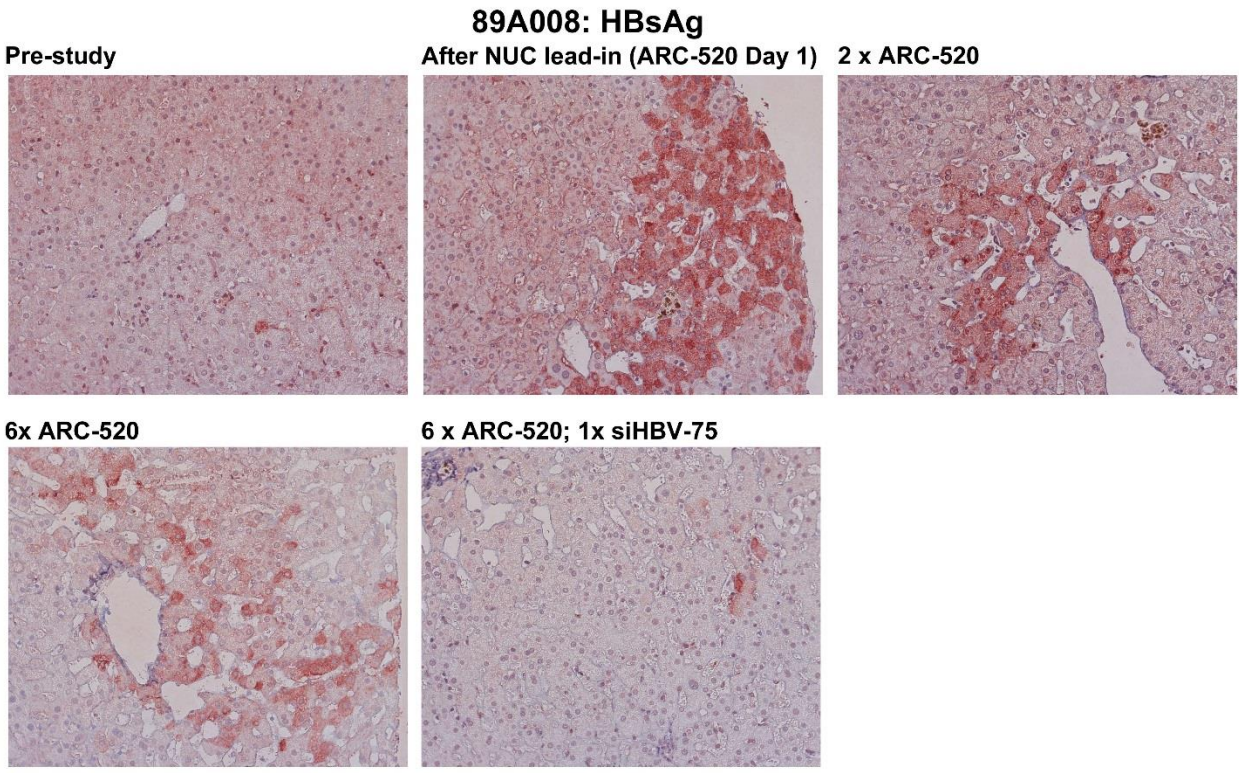
C


A3A006: HBsAg
Pre-study
After NUC lead-in (ARC-520 Day 1)
2 x ARC-520
6x ARC-520
7 x ARC-520

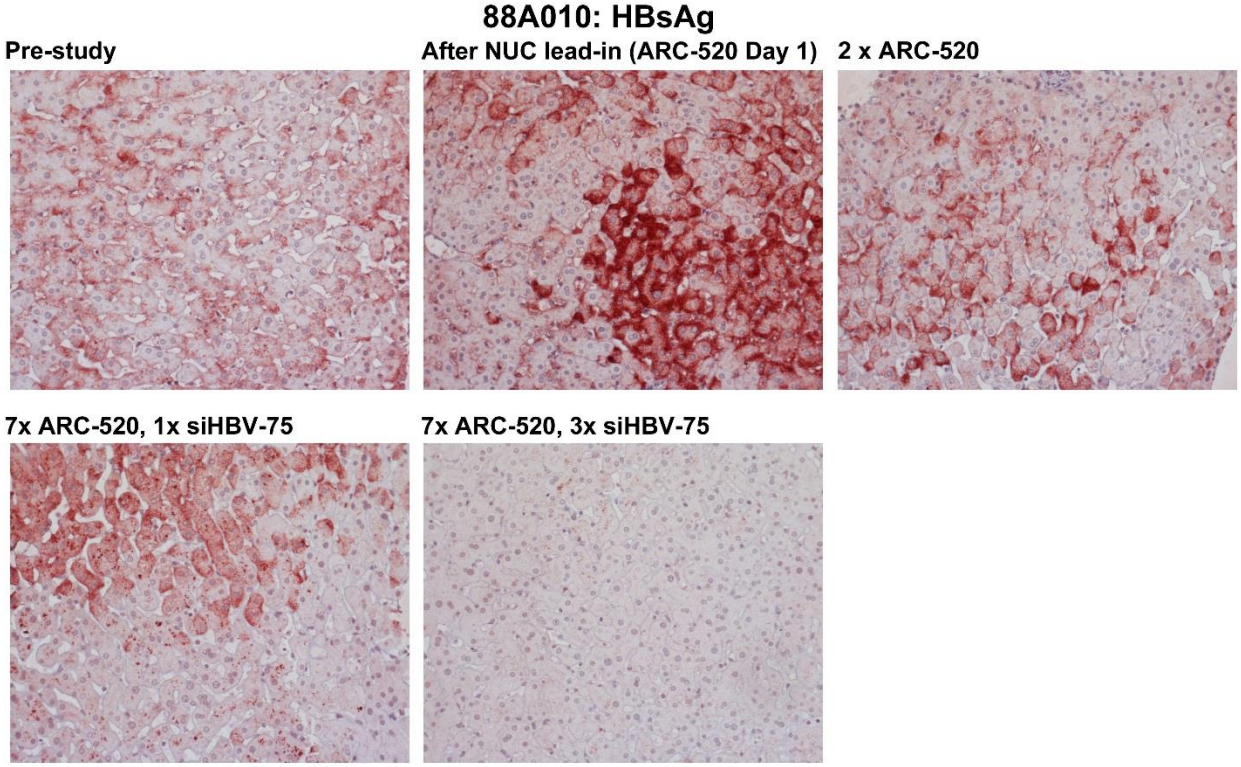
D


A4A014: HBsAg
Day -8 (pre-study)
Day 57 (ARC-520 Day 1)
Day 92 (2 x ARC-520)
Day 176 (5 x ARC-520, flare)
Day 265 (8 x ARC-520)
Day 323 (10 x ARC-520)

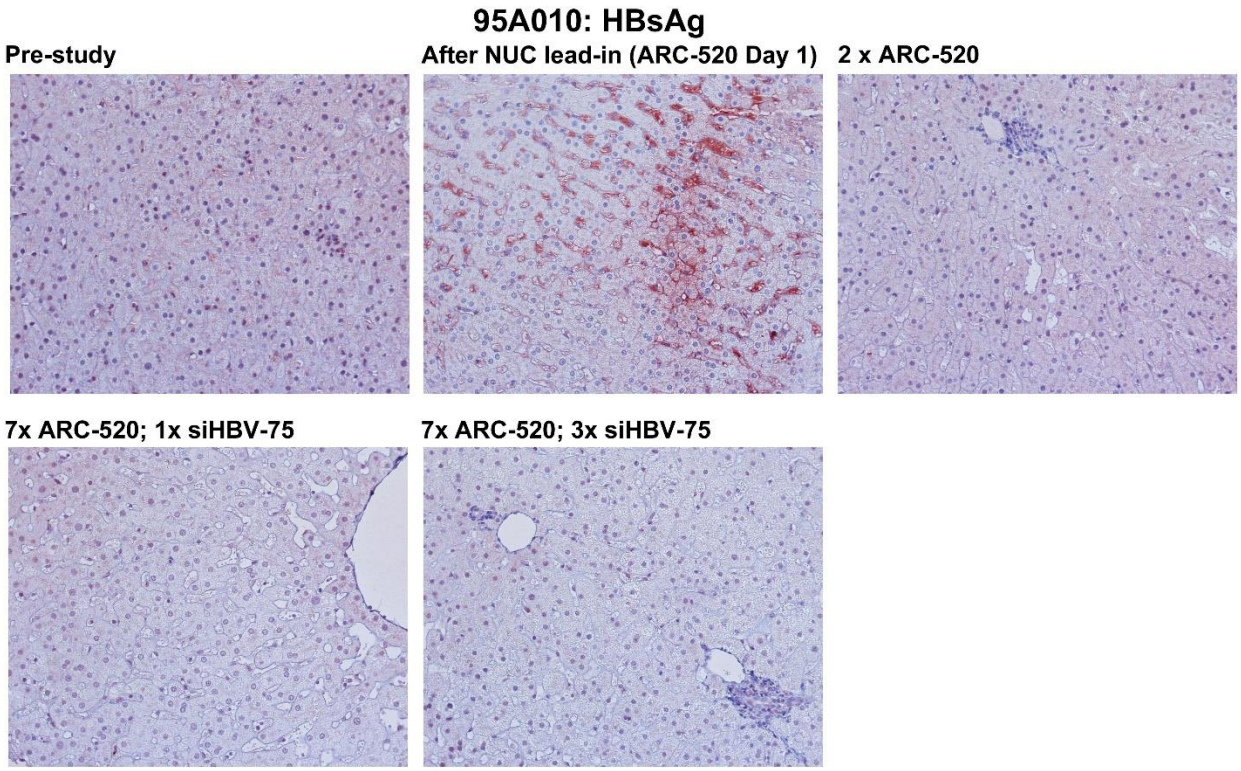
E


89A008: HBsAg
Pre-study
After NUC lead-in (ARC-520 Day 1)
2 x ARC-520
6x ARC-520
6 x ARC-520; 1x siHBV-75

F


88A010: HBsAg
Pre-study
After NUC lead-in (ARC-520 Day 1)
2 x ARC-520
7x ARC-520, 1x siHBV-75
7x ARC-520, 3x siHBV-75

G


95A010: HBsAg
Pre-study
After NUC lead-in (ARC-520 Day 1)
2 x ARC-520
7x ARC-520; 1x siHBV-75
7x ARC-520; 3x siHBV-75

H

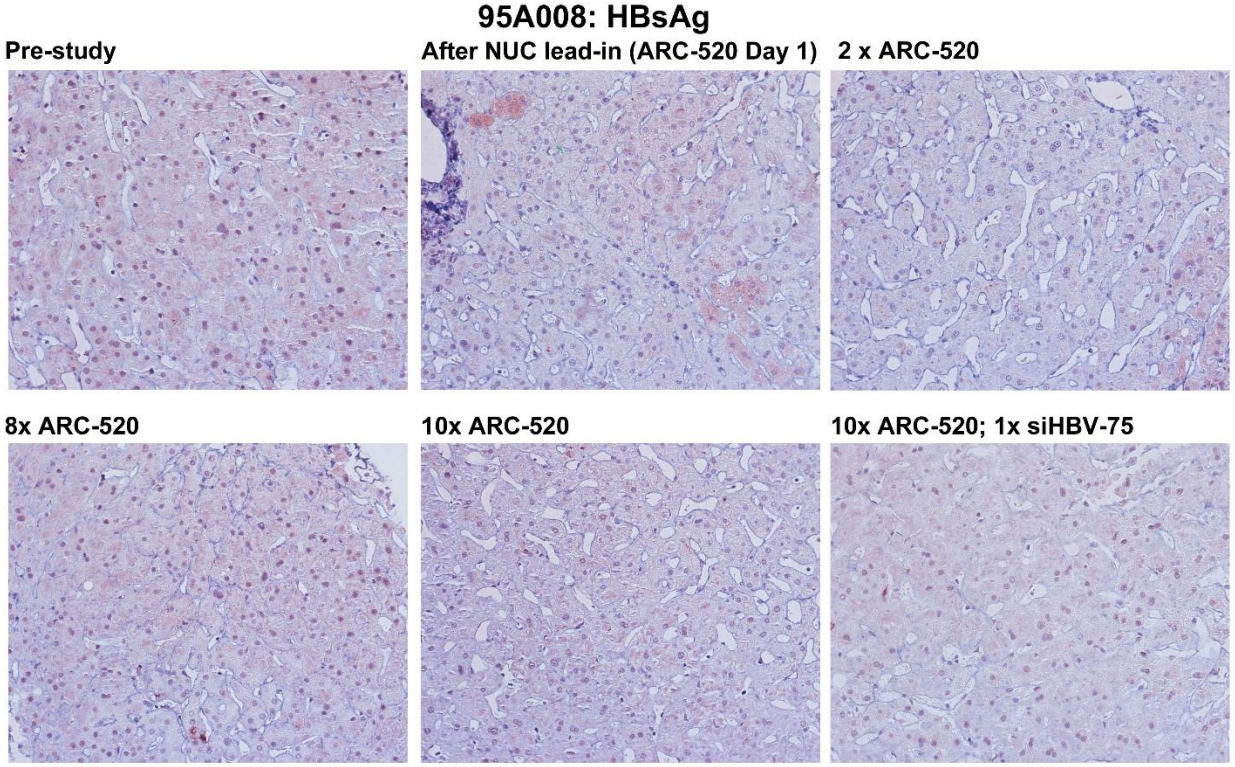


Fig. S7. Immunohistochemical staining of HBsAg in livers of HBeAg positive and HBeAg negative chimpanzees.

Liver tissue samples from needle biopsies collected prior to (pre-study), after the NUC lead-in (Day 1), and at various times after Q4W RNAi dosing and concomitant with daily NUCs were stained for HBsAg by immunohistochemistry. (**A**) The NUC lead-in was 169 days for HBeAg positive 4x0139 after which she was given 8 doses of ARC-520. (**B**) The NUC lead-in was 85 days for HBeAg positive A2A004 after which he was given 9 doses of ARC-520. (**C**) The NUC lead-in was 141 days for HBeAg positive A3A004 after which he was given 7 doses of ARC-520. (**D**) The NUC lead-in was 57 days in A4A014 after which she was given 10 doses of ARC-520. (**E**) The NUC lead-in was 141 days in HBeAg transitional 89A008 after which he was given 6 doses of ARC-520 followed by one dose of siHBV-75. (**F**) The NUC lead-in was 57 days in

HBeAg negative 88A010 after which he was given 7 doses of ARC-520 followed by 3 doses of siHBV-75. (**G**) The NUC lead-in was 57 days in HBeAg negative 95A010 after which she was given 7 doses of ARC-520 followed by 3 doses of siHBV-75. (**H**) The NUC lead-in was 57 days in HBeAg negative 95A008 after which he was given 10 doses of ARC-520 followed by one dose of siHBV-75.

Table S1. Individual CHB patient demographics at screening in clinical trial Heparc-2001

Subject	Sex	Age (years)	Weight (kg)	HBeAg status	Years on NUCS	Serum HBV DNA (Log_{10} IU/mL)	Serum HBsAg (Log_{10} IU/mL)
104 PBO	Female	57	49.9	Negative	5.0	ND	3.5
107 PBO	Male	56	80.4	Negative	5.1	ND	3.1
201 PBO	Male	56	80.4	Negative	3.8	<1.46	3.2
208 PBO	Female	56	62.4	Negative	4.7	ND	3.0
303 PBO	Male	41	78.7	Negative	3.3	<1.46	3.1
307 PBO	Male	40	56.9	Negative	6.9	<1.46	3.3
401 PBO	Male	41	72.0	Negative	6.0	<1.46	3.2
408 PBO	Male	42	62.8	Negative	3.8	ND	3.6
503 PBO	Male	39	61.2	Positive	7.8	<1.46	3.0
507 PBO	Male	29	57.2	Positive	3.9	<1.46	3.1
101	Female	55	56.6	Negative	6.5	ND	3.6
102	Male	48	63.4	Negative	5.9	<1.46	3.1
103	Male	38	54.4	Negative	3.9	ND	4.3
105	Male	41	58.9	Negative	2.5	<1.46	3.4
106	Male	59	77.8	Negative	6.1	<1.46	3.1
108	Female	53	47.2	Negative	6.2	ND	3.0
202	Female	45	64.4	Negative	2.5	<1.46	3.2
203	Female	43	56.5	Negative	1.0	<1.46	3.4
204	Male	44	57.3	Negative	6.3	<1.46	3.3
205	Male	49	61	Negative	4.8	ND	3.5
206	Male	40	79.6	Negative	1.4	<1.46	3.4
207	Male	37	59.6	Negative	3.2	ND	4.4
301	Male	40	64.7	Negative	3.3	ND	3.0
302	Male	41	73.4	Negative	3.8	<1.46	4.0
304	Female	41	63.3	Negative	3.8	<1.46	3.9
305	Female	45	53.5	Negative	6.0	<1.46	3.3
306	Male	43	72.2	Negative	2.7	<1.46	3.4

308	Female	42	47.1	Negative	3.2	ND	3.8
402	Male	39	76.7	Negative	3.4	<1.46	3.2
403	Male	36	69.2	Negative	6.7	<1.46	4.2
404	Female	41	47.4	Negative	2.8	<1.46	4.1
405	Male	41	68.3	Negative	4.1	<1.46	3.7
406	Male	41	64.1	Negative	4.6	<1.46	3.3
407	Male	41	76.6	Negative	1.2	ND	3.3
501	Male	44	80.6	Positive	4.0	<1.46	3.5
502	Male	48	66.9	Positive	7.6	ND	3.4
504	Male	37	78.8	Positive	1.4	ND	3.0
505	Female	30	58.4	Positive	5.8	ND	4.3
506	Female	33	57.1	Positive	3.3	<1.46	4.2
508	Male	45	57.5	Positive	3.2	ND	3.3
601	Male	33	59.3	Positive	1.9	<1.46	3.7
602	Male	27	63.2	Positive	2.1	<1.46	3.4
603	Female	39	87.0	Positive	4.1	<1.46	3.7
604	Male	32	103.0	Positive	2.8	<1.46	3.3
605	Female	32	57.4	Positive	1.4	<1.46	3.1
606	Female	37	44.4	Positive	2.6	ND	3.0
701	Male	56	65.1	Negative	0	3.28	0.9
702	Male	36	71.6	Positive*	0	2.69	3.7
703	Female	35	57.0	Positive	0	8.95	3.1
704	Female	51	54.2	Positive	0	7.90	4.9
705	Male	56	69.8	Negative	0	5.04	4.8
706	Male	36	73.8	Negative	0	4.43	4.9
707	Male	35	56.3	Negative	0	3.78	1.2
708	Female	31	50.9	Positive	0	8.51	3.5
709	Male	44	112.5	Negative	0	3.82	4.7
710	Male	23	63.1	Positive	0	8.95	3.1
711	Female	35	53.2	Positive	0	9.25	4.0
712	Female	39	58.5	Negative	0	4.08	3.3

ND: not detected. *low HBeAg

Table S2. Mean CHB patient demographics at screening in clinical trial Heparc-2001

Cohort	Prior ETV	ARC-520 Dose	HBeAg status	N (Male / Female))	Age Mean years (range)	Baseline HBV DNA Log_{10} IU/mL Mean (range)	Baseline HBsAg Log_{10} IU/mL Mean (range)
PBO	Yes	0 mg/kg	Negative/Positive	10 (8/2)	44 (29-57)	BLOQ (ND - <1.46)	3.21 (3.00 – 3.60)
1	Yes	1 mg/kg	Negative	6 (4/2)	49 (38-59)	BLOQ (ND - <1.46)	3.41 (2.98 – 4.270
2	Yes	2 mg/kg	Negative	6 (4/2)	43 (37 – 49)	BLOQ (ND - <1.46)	3.55 (3.23 – 4.39)
3	Yes	3 mg/kg	Negative	6 (3/3)	42 (40 – 45)	BLOQ (ND - <1.46)	3.58 (3.04 – 4.01)
4	Yes	4 mg/kg	Negative	6 (5/1)	40 (36-41)	BLOQ (ND - <1.46)	3.63 (3.15 – 4.24)
5	Yes	4 mg/kg	Positive	6 (4/2)	40 (30-48)	BLOQ (ND - <1.46)	3.61 (3.03 – 4.24)
6	Yes	2 x 2 mg/kg	Positive	6 (3/3)	33 (27-39)	BLOQ (ND - <1.46)	3.36 (2.98 – 3.70)
7	No	4 mg/kg	Positive	6 (2/4)	35 (23-52)	7.78 (2.69 – 9.25)	3.70 (3.07 – 4.94)
7	No	4 mg/kg	Negative	6 (5/1)	44 (35-56)	4.07 (3.28 - 5.04)	3.28 (0.90 – 4.89)

BLOQ, below limit of quantitation (<1.46 Log IU/mL); ND, not detected

Table S3: Treatment emergent adverse events in cohorts 1-7 of the Heparc-2001 study

TEAE	Treatment Group											
	1.0 mg/kg (cohort 1)		2.0 mg/kg (cohort 2)		3.0 mg/kg (cohort 3)		4.0 mg/kg (cohorts 4,5,7)		2 mg/kg X 2 (cohort 6)		Placebo	
	Mild	Mod	Mild	Mod	Mild	Mod	Mild	Mod	Mild	Mod	Mild	Mod
Extravasation			1(2%)									
Malaise				1(2%)								
Influenza	1(2%)											
Blood CK increase			1(2%)									
Diabetes Mellitus			1(2%)									
Pain in extremity					1(2%)							
Presyncope				1(2%)								
Headache							1(2%)					
Dizziness							1(2%)					
Fever									1(2%)			

TEAE = treatment-emergent adverse event; CK = creatinine kinase; mod = moderate

Table S4. Characteristics of chronically HBV-infected chimpanzees and treatment regimens.

Age, weight, serum HBV DNA, HBsAg and ALT are shown as the values on study day 1. NUC pre-treatment indicates the number of days that animals were given daily oral ETV prior to the first dose of ARC-520 (ARC-520 Day 1). ARC-520 was given Q4W after the NUC lead in period. Chimpanzee 04x0139 was in addition to ETV given daily oral tenofovir during the 61 days prior to first ARC-520 injection and throughout ARC-520 treatment.

Animal ID	Sex	Age (years)	Weight (kg)	HBeAg status	Serum HBV DNA (copies/mL)	Serum HBsAg (µg/mL)	ALT (U/L)	Days NUC pre-treatment prior to 1st ARC-520 injection	Number ARC-520 injections	Doses ARC-520 (mg/kg x number of injections)
04x0139	Female	37	48	Positive	9.2 $\log_{10}$	924	33	169	8	3 mg/kg x 4; 4 mg/kg x 4
A2A004	Male	12	77	Positive	8.5 $\log_{10}$	3,188	70	85	9	3 mg/kg x 5; 4 mg/kg x 4
A3A006	Male	10	51	Positive	8.3 $\log_{10}$	2,104	64	141	7	4 mg/kg x 7
A4A014	Female	9	54	Positive	7.7 $\log_{10}$	253	114	57	10	2 mg/kg x 2; 3 mg/kg x 4; 4 mg/kg x 4
89A008	Male	24	63	Positive/negative (Transitional)	8.6 $\log_{10}$	245	281	141	6	4 mg/kg x 6
04x0506	Female	34	70	Negative	2.5 $\log_{10}$	1.3	26	85	11	2 mg/kg x 2; 3 mg/kg x 5; 4 mg/kg x 4
88A010	Male	25	65	Negative	3.0 $\log_{10}$	199	102	57	7	2 mg/kg x 2; 3 mg/kg x 4; 4 mg/kg x 1
95A008	Male	18	74	Negative	2.9 $\log_{10}$	1.2	67	57	10	2 mg/kg x 2; 3 mg/kg x 4; 4 mg/kg x 4
95A010	Female	18	65	Negative	3.5 $\log_{10}$	86	47	57	7	2 mg/kg x 2; 3 mg/kg x 4; 4 mg/kg x 1

Table S5. mRNA-seq data obtained from liver biopsies of chimpanzees chronically infected with HBV.

Chimp ID	HBeAg status	Time point of biopsy specimen (relative to ARC-520 injections)	HBsAg (µg/mL) at time of biopsy	Total HBV reads	Reads containing siHBV-74 target site		Reads containing siHBV-77 target site(s)		Ratio siHBV-77: siHBV-74 target sites	Percentage of target sites containing dominant sequence	
					Number reads	Reduction following ARC-520 (%)	Number reads	Reduction following ARC-520 (%)		siHBV-74 target site	siHBV-77 target site
4x0139	Positive	Pre-dose	358	498,566	4,511	93.9%	4,763	93.9%	1.1	97.8%	64.1%
		After 8th injection*	18.5	47,353	275		292		1.1	97.1%	61.3%
A2A004	Positive	Pre-dose	2,866	795,621	5,471	96.3%	11,111	96.7%	2.0	98.5%	97.4%
		After 9th injection*	12.9	28,396	201		370		1.8	98.4%	98.3%
A3A006	Positive	Pre-dose	1,640	664,329	4,661	90.8%	9,656	90.4%	2.1	98.5%	97.8%
		After 7th injection*	19.9	80,865	427		924		2.2	98.1%	97.4%
A4A014	Positive	Pre-dose	187	161,348	1,145	99.7%	2,409	ND	2.1	98.8%	98.4%
		After 10th injection*	2.3	589	4		ND		ND	100.0%	NA
89A008	Transitional	Pre-dose	151	60,451	475	88.0%	215	89.3%	0.5	96.6%	95.1%
		After 6th injection*	19.1	18,045	57		23		0.4	100.0%	100.0%
88A010	Negative	Pre-dose	195	76,061	643	93.2%	283	93.6%	0.4	98.2%	95.7%
		After 2nd injection*	78	24,545	44		18		0.4	100.0%	90.0%
95A010	Negative	Pre-dose	72	12,255	60	96.7%	15	86.7%	0.3	96.7%	100.0%
		After 2nd injection*	30	5,670	2		2		1.0	100.0%	100.0%
95A008	Negative	Pre-dose	1.2	5,815	87	97.7%	28	85.7%	0.3	98.8%	87.1%
		After 10th injection*	0.23	1,725	2		4		2.0	100.0%	100.0%

*Last biopsy following ARC-520 injection; ND, not determined

Table S6. Polyadenylation signals in HBV-chimpanzee fusion transcripts of HBeAg negative chimpanzee 88A010.

Putative polyadenylation signal	Number of transcripts	Percentage
TATAAA	6	1.7%
CATAAA	1	0.3%
AATGAA	0	0.0%
AAAAAA	4	1.1%
AACAAA	2	0.6%
AAGAAA	4	1.1%
AATAAA	64	17.7%
AATAAC	1	0.3%
AATAAG	1	0.3%
AATAAT	1	0.3%
AATACA	12	3.3%
AATAGA	1	0.3%
AATATA	3	0.8%
AATCAA	1	0.3%
AATTAA	2	0.6%
ACTAAA	1	0.3%
AGTAAA	2	0.6%
ATTAAA	16	4.4%
CATGAA	1	0.3%
GATAAA	2	0.6%
GATGAA	1	0.3%
TATGAA	1	0.3%
>1 PAS	202	55.8%
None	33	9.1%
Total	362	100%