



Blockage of HBV Virus Replication and Inhibition of cccDNA Establishment by Core Protein Allosteric Modifiers (CpAMs)

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Background

Approximately 240 million people worldwide are chronically infected with Hepatitis B virus (HBV) and are at risk of developing chronic liver diseases, such as hepatitis, fibrosis, cirrhosis and hepatocellular carcinoma (HCC). Currently approved treatments for chronic HBV include nucleos(t)ide analogs (Nucs) such as entecavir (ETV) and tenofovir, and interferons (IFN and pegylated-interferon (PEG-IFN)). While Nucs are very effective at inhibiting HBV replication for prolonged periods, cure rates occur in <10% of treated patients. The deficiency of these therapies appears to be their limited effect on cccDNA formation. Hence, new classes of anti-HBV molecules are needed to significantly improve cure rates in chronic HBV patients. HBV core protein (Cp) is involved in multiple steps of the HBV life cycle, including the formation, amplification and maintenance of cccDNA. A novel class of direct acting HBV antivirals, Core Protein Allosteric Modifiers (CpAMs), have been discovered to target HBV core protein and reduce HBV replication and cccDNA levels.^{1,2} Here we report on the mechanism of action of a series of proprietary CpAMs using a variety of preclinical assays.

Materials and Methods

Inhibitors: Assembly Biosciences' CpAMs were synthesized at Sai Life Sciences. GLS4 (CpAM) was synthesized by published procedures.^{3,4} ETV⁵ (Nuc) was purchased from ACME Bioscience.

Cell culture and compound treatment: Huh7 cells were transfected with plasmids expressing HBV constructs in 96-well plates and treated with compounds. Supernatants and cells were harvested at 5 days post-infection. Primary Human Hepatocytes (PHH; Lonza) were infected with HBV from HepAD38 at an MOI of 50-250 and treated with inhibitors at the indicated time points and concentrations. Supernatants and cells were harvested at indicated time points.

Molecular profiling of CpAMs: HepAD38 cells⁶ were treated with inhibitors and induced simultaneously. Cells were retreated 3 days later and harvested 6 days post induction. HBV total RNA/encapsidated pgRNA, Cp, capsid, and capsid associated core DNA were detected by Northern Blot, Western Blot, Enzyme Immunoassay (EIA), and Southern Blot, respectively, as previously described.⁷

HBcAg quenching assay: Huh7 cells were transfected with a plasmid expressing the complete Cp open reading frame under the control of a CMV promoter. Supernatants were harvested 4 days post transfection and subjected to a custom in-house electrochemiluminescent (ECL) ELISA. This assay detects an HBc epitope that gets masked upon addition of CpAMs.

HBV DNA quantification in transiently transfected cells: HBV DNA levels in DNase I-treated supernatants were quantified by Taqman quantitative PCR (qPCR) using primers and a probe specific to the HBV core gene.

HBV DNA/pgRNA quantification in PHH: HBV DNA/pgRNA levels were quantified using bDNA probes (Affimetrix) specific to the relevant HBV sequences.

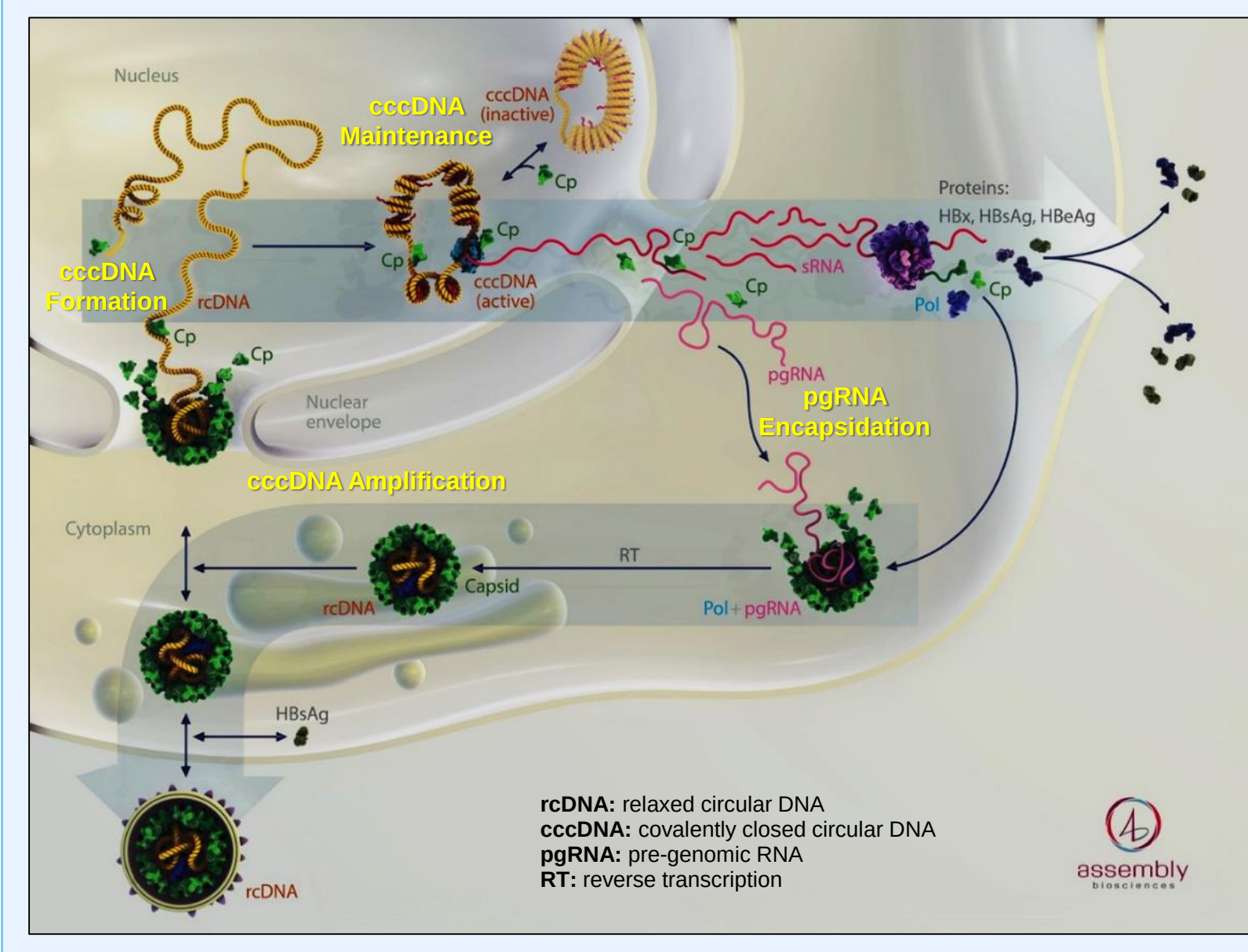
HBsAg/HBeAg levels quantification: HBe and HBs antigens were quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits (Autobio Diagnostics and Beijing Wantai Biological Pharmacy Enterprise, respectively) as well as custom in-house ECL ELISAs.

HBV RNA staining: Total HBV RNA levels were visualized using ViewRNA technology (Affymetrix) and a set of bDNA probes designed to detect all species of HBV mRNAs.

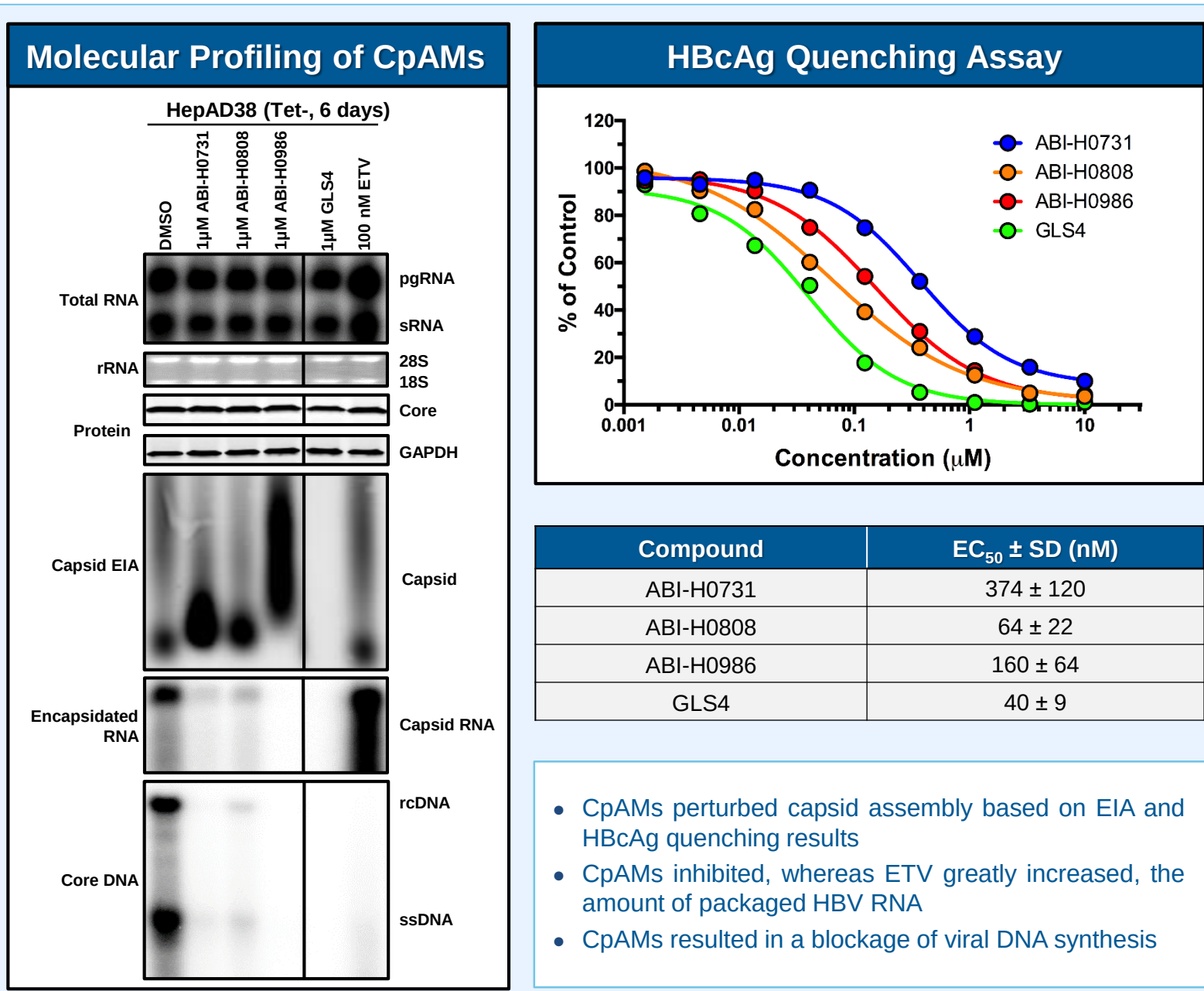
cccDNA Southern Blots: Cells infected with HBV from HepAD38 were treated with inhibitors. Four days post infection, cells were harvested and extrachromosomal DNA extracted by a Hirt-modified method.⁸ Non-supercoiled DNA was removed via T5 exonuclease (NEB) digestion. High-fidelity EcoRI endonuclease (NEB) was then used to linearize supercoiled HBV cccDNA.

EC₅₀ calculations: EC₅₀ values were calculated using GraphPad Prism software.

Cp Involved in Multiple Steps of HBV Lifecycle



CpAMs Perturb Cp Assembly and HBV Replication



CpAMs Exhibit Broad Genotypic Activity

HBV Genotype	EC ₅₀ ± SD (nM)	
	ABI-H0731	ETV
A	113 ± 22	1.9 ± 0.5
B	86 ± 11	2.3 ± 0.5
C	88 ± 27	1.2 ± 0.1
D	142 ± 48	5.0 ± 1.9

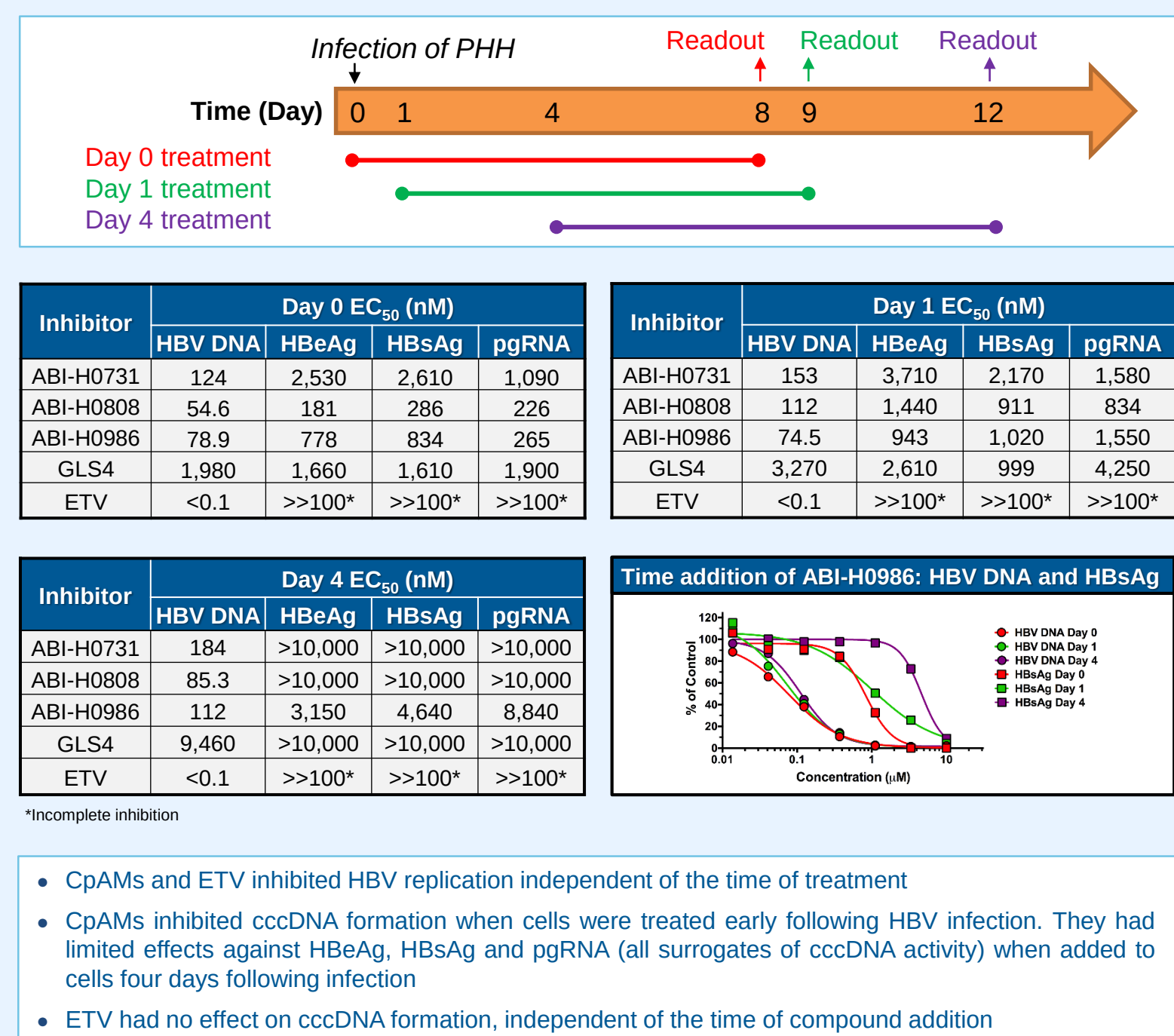
- CpAM ABI-H0731 exhibited mean EC₅₀s ranging from 86 to 142 nM in reducing viral replication of the four major HBV genotypes (A-D)
- Assembly CpAMs possess broad genotypic antiviral activity

CpAMs Active Against Nuc Resistant Mutant

HBV Construct	EC ₅₀ ± SD (nM)	
	ABI-H0731	ETV
Wild type	183 ± 14	13 ± 7
L180M+M204V	194 ± 1	> 100

- CpAM ABI-H0731 retained antiviral potency against the Nuc resistant variant L180M+M204V (Genotype D)
- CpAM activity should be retained in patients harboring Nuc-resistant variants

Time Dependent Inhibition of cccDNA Formation



CpAMs Impair cccDNA Formation in HepG2-NTCP and PHH Infection Models

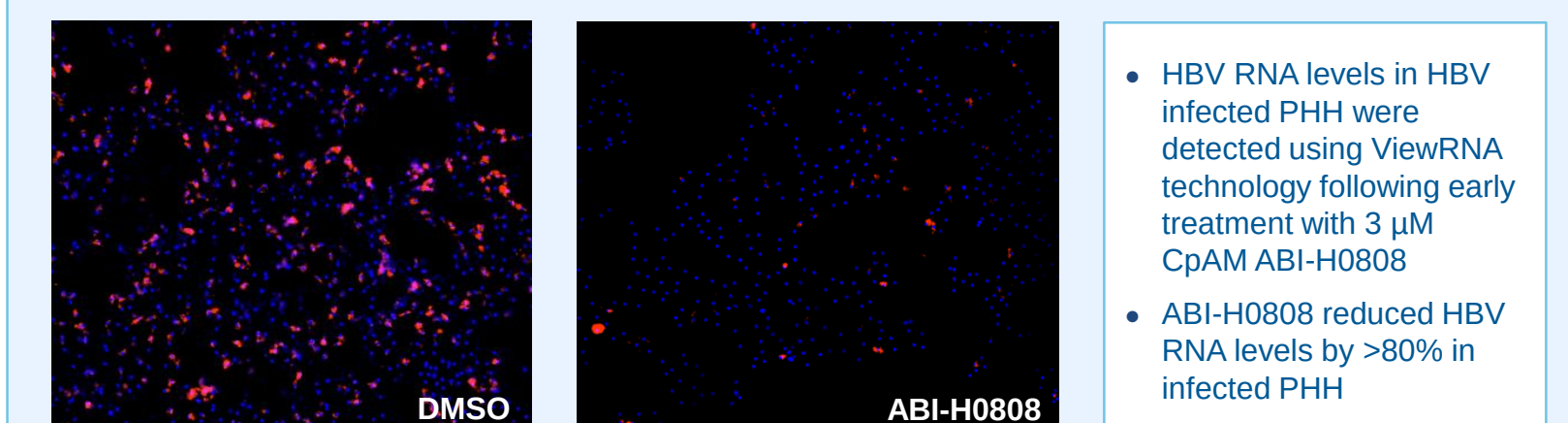
Parameter	HepG2-NTCP EC ₅₀ ± SD (nM)				
	ABI-H0731	ABI-H0808	ABI-H0986	GLS4	ETV
Viral DNA	307 ± 41	95 ± 36	57 ± 9	58 ± 6	0.8 ± 0.2
HBeAg	4,950 ± 520	372 ± 27	920 ± 126	549 ± 48	>>100
HBsAg	7,300 ± 770	945 ± 82	1,990 ± 170	673 ± 41	>>100
pgRNA	2,680 ± 580	419 ± 31	1,050 ± 100	279 ± 43	>>100

Parameter	PHH EC ₅₀ ± SD (nM)				
	ABI-H0731	ABI-H0808	ABI-H0986	GLS4	ETV
Viral DNA	154 ± 18	80 ± 17	70 ± 7	1,940 ± 40	<0.1
HBeAg	2,210 ± 310	196 ± 63	677 ± 102	1,480 ± 170	>>100*
HBsAg	3,000 ± 270	310 ± 64	892 ± 133	1,860 ± 430	>>100*
pgRNA	1,840 ± 380	305 ± 67	611 ± 233	1,930 ± 30	>>100*

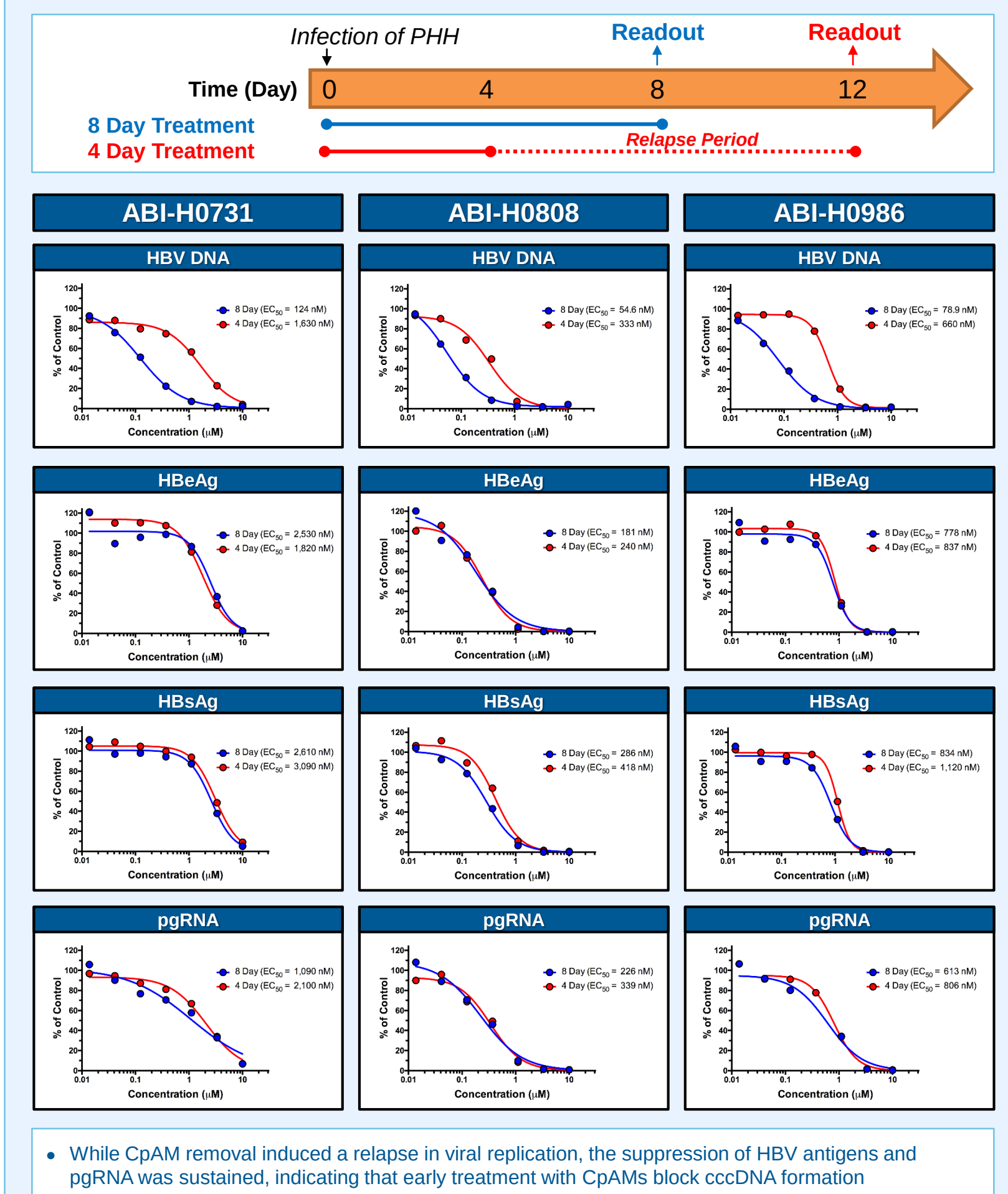
*Incomplete inhibition

- CpAMs reduced HBeAg, HBsAg and pgRNA expression (markers of cccDNA formation)
- Assembly CpAMs exhibited improved antiviral activities, unlike other CpAMs such as GLS4, which lost potency in PHH
- ETV showed minimal inhibition of antigen production in PHH when tested up to 100 nM (>1,000x EC₅₀)

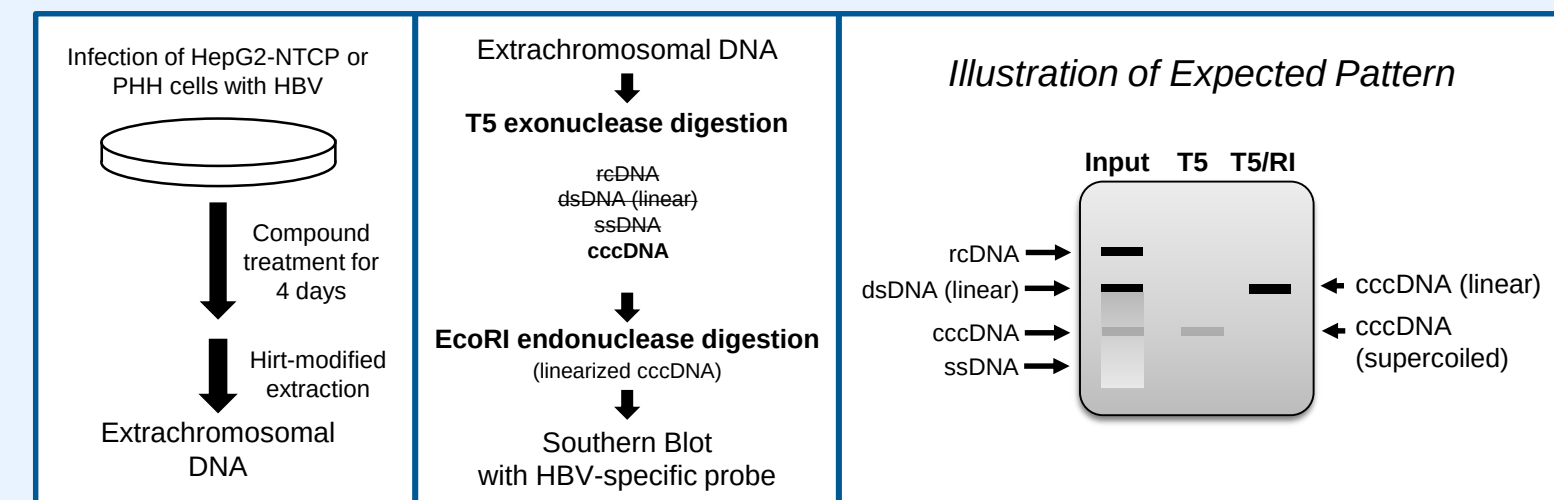
Detection of HBV RNAs by ViewRNA in PHH



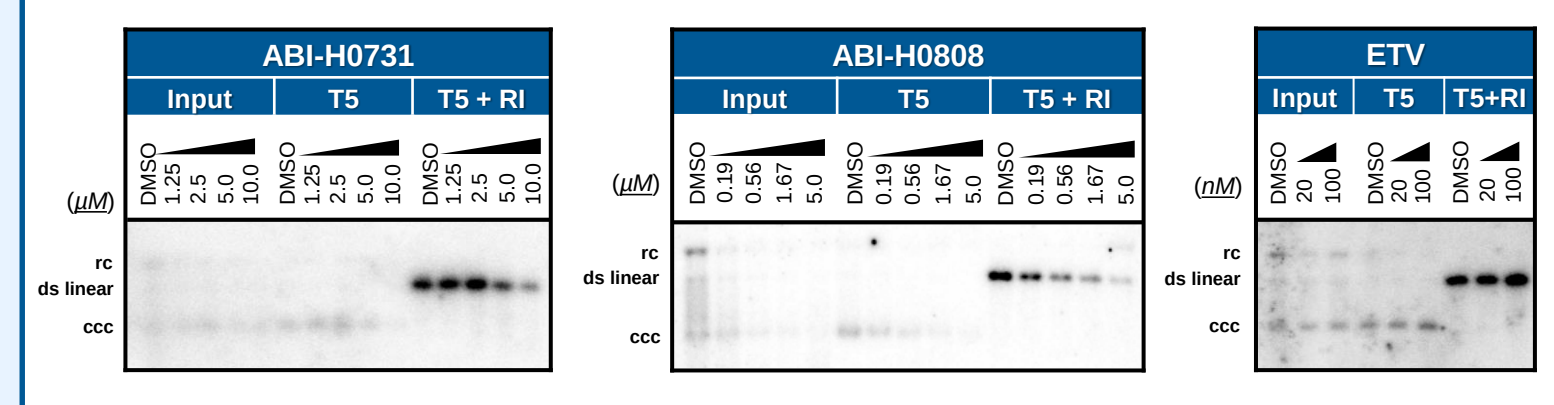
CpAM Effect on cccDNA is Sustainable



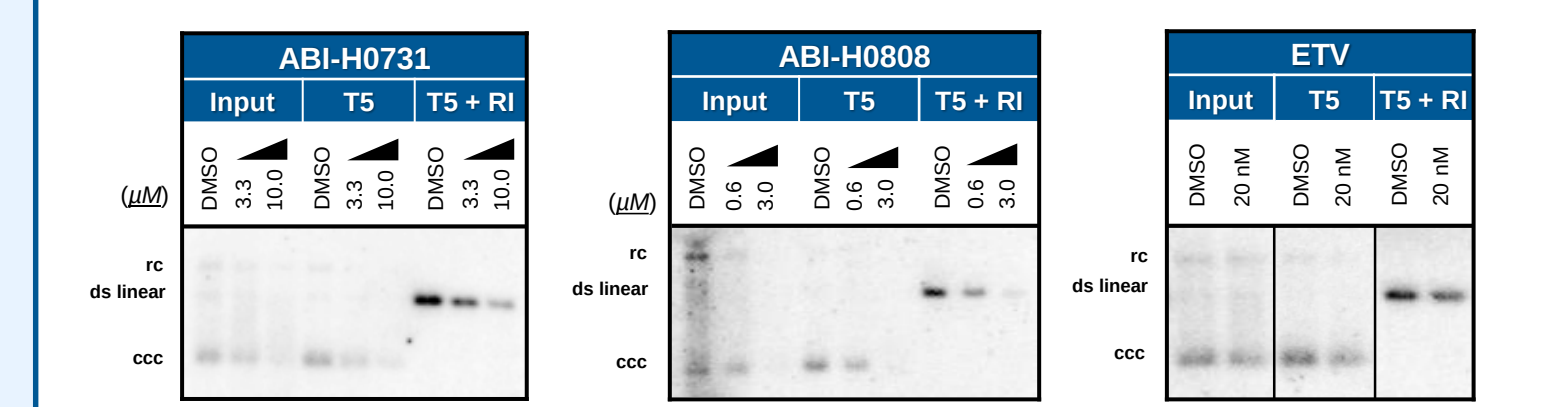
HBV cccDNA Detection by Southern Blot



HepG2-NTCP



PHH



- CpAMs reduced HBV cccDNA formation in both NTCP-HepG2 cells and PHH
- ETV (EC₅₀ concentrations 125-200x) had minimal effect on cccDNA levels

Summary

- Assembly Biosciences has discovered a proprietary series of selective and potent CpAMs
- CpAMs target core protein and interfere with multiple steps in the viral cycle
- CpAMs exhibit broad genotypic activity and retain potency against a Nuc resistant variant
- Treatment with CpAMs early after HBV infection inhibits the formation of cccDNA based on surrogate markers HBeAg, HBsAg and pgRNA levels, and direct cccDNA detection (Southern Blot)
- The suppression of HBV antigens and pgRNA expression is sustainable following CpAM withdrawal
- CpAMs represent a novel class of HBV antivirals and Assembly Biosciences' first candidate is progressing into clinical development

References

- Bourne, C. et al. Small-molecule effectors of hepatitis B virus capsid assembly give insight into virus life cycle. *J. Virol.* (2008)
- Stray, S. J. et al. A heteroaryl dihydropyrimidine activates and can misdirect HBV capsid assembly. *Proc. Natl. Acad. Sci. USA* (2005)
- Siegfried G, et al. Bromo-phenyl substituted thiazolyl dihydropyrimidines. US patent application publication no. US 2012/0282221 A1 (2012)
- Wang et al. In vitro inhibition of HBV replication by a novel compound, GLS4, and its efficacy against adenofovir-dipivoxil-resistant HBV mutations. *Antivir. Ther.* (2012)
- Innaïmo, S. F. et al. Identification of BMS-200475 as a Potent and Selective Inhibitor of Hepatitis B Virus. *J. Antimicrob. Chemother.* (1997)
- Ladner, S. K. et al. Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication. *Antimicrob. Agents Chemother.* (1997)
- Cai, D. et al. Establishment of an Inducible HBV Stable Cell Line that Expresses cccDNA-dependent Epitope-tagged HBeAg for Screening of cccDNA Modulators. *Antivir. Res.* (2016)
- Cai et al. A southern blot assay for detection of hepatitis B virus covalently closed circular DNA from cell cultures. *Methods Mol. Bio.* (2013)