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PEVIFOSCORVIR SODIUM (CAM-E) IS SUPERIOR TO NUCLEOS(T)IDE ANALOGS IN SUPPRESSING HEPATITIS B VIRUS DOUBLE-STRANDED LINEAR DNA IN VITRO

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BACKGROUND

Circulating hepatitis B virus (HBV) DNA consists mostly of relaxed circular DNA (rcDNA) and double-stranded linear DNA (dslDNA). dslDNA is the precursor form for HBV DNA integration into the host genome, which contributes to disease progression and development of hepatocellular carcinoma.[1] It remains unclear to what extent nucleos(t)ide analogs (NAs), such as tenofovir disoproxil fumarate (TDF) and entecavir (ETV), affect rcDNA vs. dslDNA production. Additionally, the HBV capsid assembly process has emerged as a key target for the treatment of chronic HBV infection.[2] In a Phase 1 clinical trial, the capsid assembly modulator (CAM-E, inducing empty capsids) pevifoscorvir sodium (pevy) induced reductions of HBV DNA, RNA, HBsAg, HBeAg and HBcrAg in participants with chronic HBV infection, suggesting best-in-class potential.[3-5] Here, we investigated the effects of NAs and CAMs on production and secretion of HBV rcDNA and dslDNA using an in-house digital PCR (dPCR) assay across HBV cell models and serum samples.

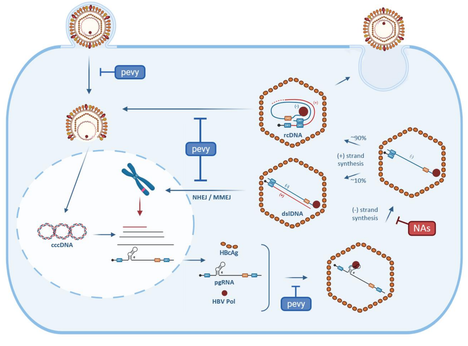
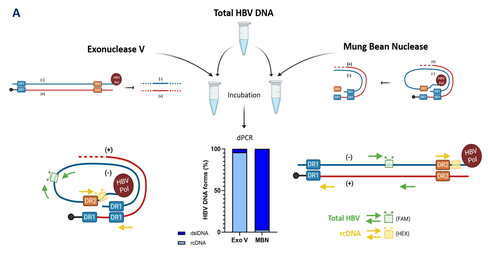


Figure 1. HBV replication cycle and mechanisms of action of nucleos(t)ide analogs and pevifoscorvir sodium. Following viral entry, rcDNA is delivered to the nucleus, where it can be converted to cccDNA. Reverse transcription of pgRNA generates mainly rcDNA and, to a lesser extent, dslDNA, both of which can be secreted in viral particles. While rcDNA can recycle to the nucleus to replenish cccDNA, dslDNA can contribute to HBV DNA integration into the host genome. NAs inhibit HBV DNA synthesis, whereas pevy, a capsid assembly modulator, blocks pgRNA encapsidation and can also interfere with capsid disassembly, thereby preventing cccDNA establishment and HBV DNA integration. Figure created with BioRender.

METHODS

A duplex dPCR assay was established to distinguish and quantify HBV rcDNA and dslDNA. The assay combines a total HBV DNA primer-probe set with an rcDNA-specific set. Assay specificity was evaluated by selective nuclease digestion of either DNA form before analysis. The effects of TDF, ETV and ALG-001075, the active parent of pevy, on intracellular and secreted HBV DNA were assessed in HepG2.117, HepAD38, HBV-infected HepG2-NTCP cells and primary human hepatocytes (PHH). The assay was further applied to serum samples from HBV-infected chimeric human liver mice and treatment-naive participants with chronic HBV infection. Antiviral EC_{50} values were determined by four-parameter nonlinear regression and compared using an extra sum-of-squares F test in GraphPad Prism.



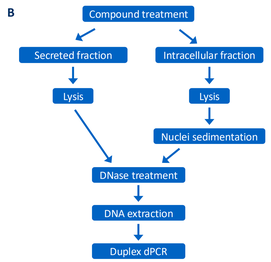


Figure 2. Duplex dPCR assay principle, validation and sample preparation workflow. (A) Principle and validation of the duplex dPCR assay. A total HBV DNA primer-probe set was combined with an rcDNA-specific set to distinguish rcDNA from dslDNA. Exonuclease V selectively digested dslDNA, whereas Mung Bean Nuclease selectively digested rcDNA, confirming assay specificity. **(B)** Workflow for purification of encapsidated HBV DNA. Following compound treatment, culture medium was collected as the secreted fraction, and NP-40 was used to lyse the cells and disrupt the viral envelope. Nuclei were removed by centrifugation to obtain the intracellular cytoplasmic fraction. Both fractions were then treated with DNase to remove non-encapsidated DNA, followed by QuickExtract DNA extraction and duplex dPCR.

RESULTS

ALG-001075 REDUCES dslDNA AND rcDNA EQUIPOTENTLY, WHILE NAs ARE LESS POTENT AGAINST dslDNA

Production of intracellular and secreted rcDNA and dslDNA was assessed in HepG2.117 cells following treatment with TDF, ETV and ALG-001075. All compounds inhibited both DNA forms in a dose-dependent manner. However, the NAs TDF and ETV consistently showed higher EC_{50} values for dslDNA than for rcDNA. In contrast, CAM-E ALG-001075 inhibited both DNA forms with similar potency (Figure 3). This difference was also observed in HepAD38 and HBV-infected HepG2-NTCP cells (Table 1).

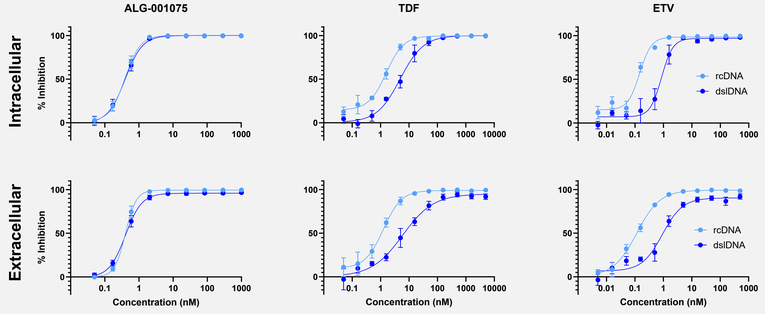


Figure 3. Percentage inhibition of rcDNA and dslDNA as a function of ALG-001075, TDF and ETV concentrations in HepG2.117 cultures, measured by dPCR. Data represent mean ± SEM from at least three independent biological replicates.

Fraction	Compound	EC_{50} rcDNA (nM)	EC_{50} dslDNA (nM)	p-value
Intracellular	ALG-001075	0.53 ± 0.24	0.55 ± 0.27	0.7695
	TDF	0.91 ± 0.48	4.19 ± 0.60	<0.0001
	ETV	0.099 ± 0.041	0.56 ± 0.25	<0.0001
Secreted	ALG-001075	0.46 ± 0.08	0.50 ± 0.10	0.3308
	TDF	0.85 ± 0.32	4.50 ± 3.18	0.0072
	ETV	0.105 ± 0.016	0.72 ± 0.37	<0.0001

Table 1. EC_{50} values for rcDNA and dslDNA across intracellular and secreted HBV DNA fractions. Values are shown as mean ± SD across HepG2.117, HepAD38 and HepG2-NTCP cell models. p-values represent the comparison between rcDNA and dslDNA EC_{50} values.

ANTIVIRAL EFFECTS ON HBV rcDNA AND dslDNA IN PRIMARY HUMAN HEPATOCYTES

To further evaluate the effects of NAs and CAMs in a more physiologically relevant infection model, intracellular HBV DNA was quantified in HBV-infected PHH. Both rcDNA and dslDNA were inhibited in a dose-dependent manner. Consistent with the results obtained in HBV-producing cell lines, TDF and ETV showed lower EC_{50} values for rcDNA than for dslDNA, whereas ALG-001075 inhibited both forms with similar potency (Figure 4).

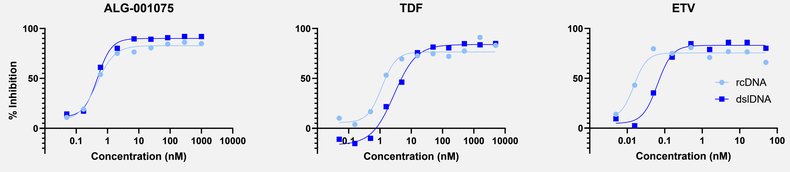


Figure 4. Percentage inhibition of rcDNA and dslDNA as a function of ALG-001075, TDF and ETV concentrations in HBV-infected primary human hepatocytes, measured by dPCR.

HBV rcDNA AND dslDNA ARE DETECTABLE IN PRECLINICAL AND CLINICAL SERUM SAMPLES

In HBV-infected chimeric human liver mice, both rcDNA and dslDNA were detected (Figure 5A), with 72% of circulating DNA identified as rcDNA (Figure 5B). In samples from treatment-naive participants in a Phase 1 study of pevy, both DNA forms were present at higher levels in $HBeAg^+$ (n=7) than in $HBeAg^-$ (n=11) samples (Figure 5C), while rcDNA was the predominant circulating DNA form in both groups (Figure 5D).

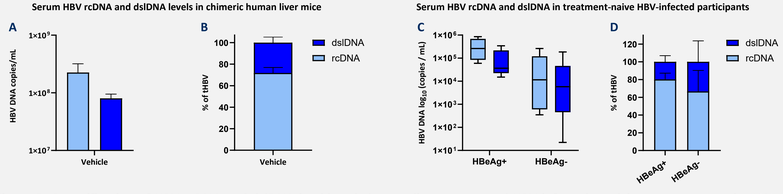


Figure 5. Quantification of HBV rcDNA and dslDNA in chimeric human liver mouse and HBV-infected participant serum samples. (A) Serum rcDNA and dslDNA levels in chimeric human liver mice treated with vehicle or the CAM-A RG7907. **(B)** Relative proportions of rcDNA and dslDNA in the same samples. **(C)** Serum rcDNA and dslDNA levels in treatment-naive HBV-infected participant samples, grouped by HBeAg status. **(D)** Relative proportions of rcDNA and dslDNA according to HBeAg status. Absolute HBV DNA levels are expressed as copies/mL serum. Bars show mean ± SEM; box-and-whisker plots show the median and interquartile range, with whiskers indicating the minimum and maximum values.

CONCLUSION

- **The duplex dPCR assay enabled differential quantification of HBV rcDNA and dslDNA in cellular and serum samples**
- **TDF and ETV showed consistently higher EC_{50} values for dslDNA formation than for rcDNA across the tested cellular systems**
- **CAM-E ALG-001075 inhibited both DNA forms equipotently in vitro, consistent with its mechanism of action upstream of reverse transcription**
- **CAMs may have greater potential than NAs to prevent HBV DNA integration by more effectively reducing HBV DNA levels and suppressing dslDNA production**
- **Differential quantification of HBV DNA forms may provide additional insight into antiviral activity and support the evaluation of treatments targeting HBV DNA integration**

Abbreviations: CAM, capsid assembly modulator; cccDNA, covalently closed circular DNA; dPCR, digital PCR; dslDNA, double-stranded linear DNA; ETV, entecavir; MBN, Mung Bean Nuclease; NAs, nucleos(t)ide analogs; pevy, pevifoscorvir sodium; pgRNA, pregenomic RNA; PHH, primary human hepatocytes; rcDNA, relaxed circular DNA; TDF, tenofovir disoproxil fumarate.

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