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Poster 501

CAPSID ASSEMBLY MODULATOR ALG-001075 PREVENTS CCCDNA FORMATION AND HBV DNA INTEGRATION IN VITRO

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ALIGOS THERAPEUTICS

BACKGROUND

The hepatitis B virus (HBV) capsid assembly process has emerged as a key target for the treatment of chronic HBV infection.[1] Capsid assembly modulators (CAMs) are small molecules that bind the hepatitis B core protein (HBc) and prevent the encapsidation of pregenomic RNA (pgRNA), blocking HBV DNA and RNA production (primary mechanism of action). At higher concentrations, they also interfere with the disassembly of viral particles, preventing establishment of covalently closed circular DNA (cccDNA, secondary mechanism of action, Figure 1). CAMs may also prevent HBV DNA integration into the host genome by blocking the production of double-stranded linear DNA (dslDNA), the main precursor for integration (primary mechanism), and by preventing dslDNA-containing viral particles from delivering their payload into the host nucleus (secondary mechanism). Pevifoscorvir sodium, also known as ALG-000184, is a prodrug of ALG-001075, a novel capsid assembly modulator leading to the formation of empty capsids (CAM-E).[2] In clinical trials to date, ALG-000184 has demonstrated substantial reductions of HBV DNA, RNA, HBsAg, HBeAg and HBcrAg in subjects with chronic HBV infection.[3] Here, we investigated the effects of ALG-001075 on HBV cccDNA formation, dslDNA production and HBV DNA integration in vitro.

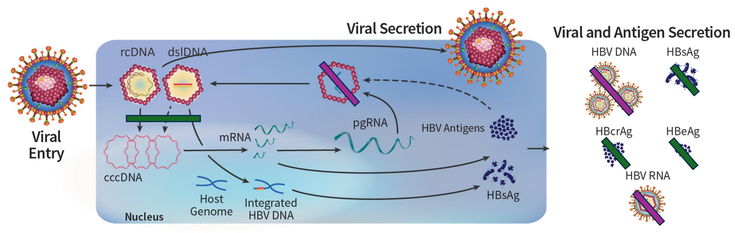


Figure 1 – HBV replication cycle with primary and secondary CAM mechanisms highlighted. HBV replication starts with viral entry of the hepatocyte through NTCP, followed by endosomal release, transfer to the nuclear pore complex and delivery of the relaxed circular DNA (rcDNA) or dslDNA to the nucleus to form cccDNA or to be integrated into the host genome, respectively. Subsequent transcription from cccDNA yields viral antigens and pgRNA, which is packaged into new viral particles that are released from the cell. CAMs interfere with HBV replication by preventing pgRNA encapsidation, reducing HBV DNA and RNA (primary mechanism, purple boxes), and by blocking capsid disassembly and cccDNA establishment, reducing viral antigens (secondary mechanism, green boxes).

METHODS

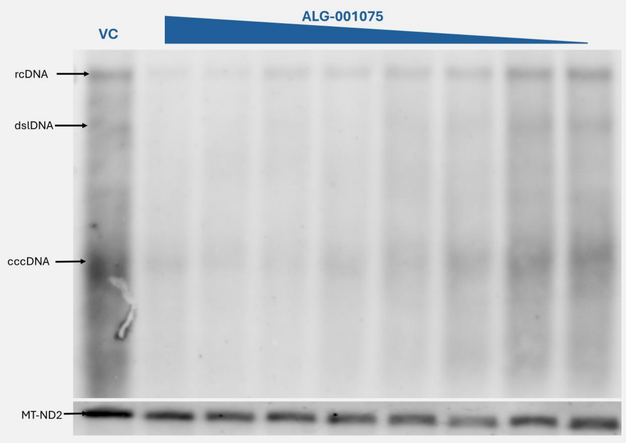
HBV cccDNA was quantified in HBV-infected PHH cells using Southern blot and digital PCR (dPCR). Production of double-stranded linear DNA (dslDNA), which can lead to HBV DNA integration, from HBV-expressing HepG2.117 cells[4] was investigated using peptide nucleic acid (PNA) clamp-based qPCR.[5] The levels of relaxed circular DNA- (rcDNA) and dslDNA-containing viral particles were assessed using an in-house dPCR assay. The level of HBV DNA integration after HBV infection of HepG2-NTCP cells was quantified using genomic DNA isolation, HBV sequence enrichment, next-generation sequencing and subsequent identification of HBV-host junctions.[6]

RESULTS

ALG-001075 PREVENTS CCCDNA ESTABLISHMENT IN PRIMARY HUMAN HEPATOCYTES

When ALG-001075 was added together with the HBV inoculum to primary human hepatocytes (PHH), it resulted in dose-dependent declines of HBV rcDNA, dslDNA and cccDNA, as assessed through Southern blot (Figure 2). Similar results were obtained through quantification of total HBV DNA and cccDNA by dPCR, with the EC_{50} value for cccDNA prevention at approximately 60 nM, in line with values reported earlier for the secondary mechanism of ALG-001075.[2] When ALG-001075 was added 6 days after infection, the amount of cccDNA was not diminished, while total HBV DNA was reduced with an EC_{50} of approximately 1 nM. The amount of secreted HBsAg and HBeAg was measured as well using commercially available chemiluminescent immunoassay (CLIA) kits. These results were comparable to the cccDNA quantification.

2A



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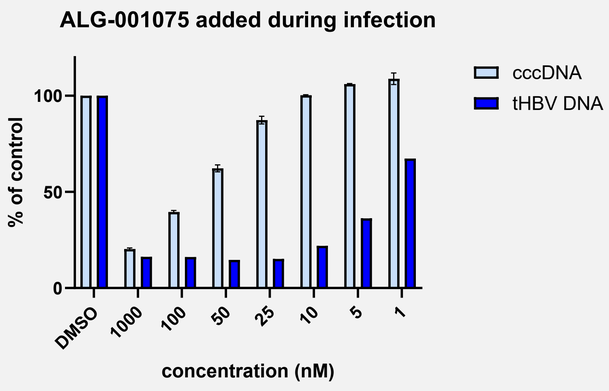

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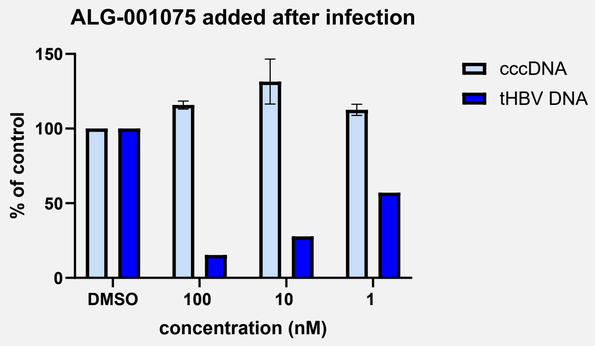

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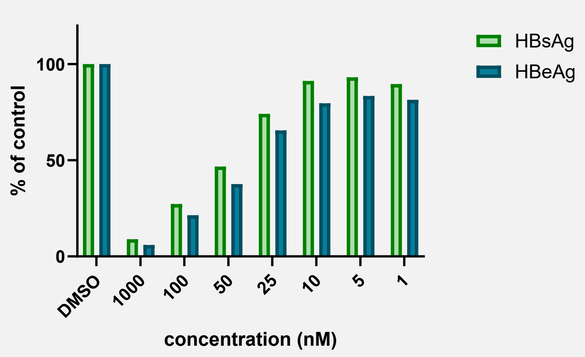


Figure 2 – ALG-001075 dose-dependently prevents cccDNA establishment when added during infection. Figure 2A (Top left): Southern blot of HBV-infected HepG2-NTCP cells, treated with ALG-001075 at 1000, 100, 50, 25, 10, 5, 1 and 0.5 nM (left to right) at the time of infection. MT-ND2 was used as loading control. **Graph 2B (Midlle left):** dPCR quantification of total HBV DNA and cccDNA on the same samples. T5 exonuclease digestion was performed before cccDNA quantification. **Graph 2C (Bottom left):** dPCR quantification of total HBV DNA and cccDNA on samples treated with ALG-001075 six days after infection. T5 exonuclease digestion was performed before cccDNA quantification. **Graph 2D (Top):** amount of HBs/eAg in culture medium of the same samples measured by CLIA. cccDNA, covalently closed circular DNA; dPCR, digital PCR; dslDNA, double-stranded linear DNA; MT-ND2, Mitochondrially Encoded NADH Dehydrogenase 2; rcDNA, relaxed circular DNA; ssDNA, single-stranded DNA; tHBV DNA, total HBV DNA; VC, virus control.

A CLOSE ANALOG OF ALG-001075 PREVENTS HBV DNA INTEGRATION IN VITRO

To assess the impact of nucleoside analogs such as entecavir (ETV) and CAM-Es on HBV DNA integration, HepG2-NTCP cells were HBV-infected and subsequently cultured for 14 days in the presence or absence of ETV or ALG-000111. Total genomic DNA (> 5 kb) was extracted, enriched for HBV-containing sequences and subjected to next-generation sequencing. HBV-host DNA junctions were identified in each sample through a dedicated bio-informatics pipeline.[6] Treatment with both ETV (100 nM) and ALG-000111 at a low concentration (10 nM, engaging the primary but not the secondary mechanism of action of CAM-Es) resulted in a roughly 3-fold reduction in the number of detected junctions (Figure 4). Increasing the concentration of ALG-000111 to 10,000 nM (within the physiologically relevant range for ALG-0001075 when dosed as prodrug pevifoscorvir sodium), resulted in an 85% reduction in the number of detected integrants. This suggests, to be tested in clinical trials, that the engagement of both primary and secondary mechanisms of CAMs could provide a better protection against HBV DNA integration compared to nucleos(t)ide analogs for which such protection has already been demonstrated clinically.[8,9]

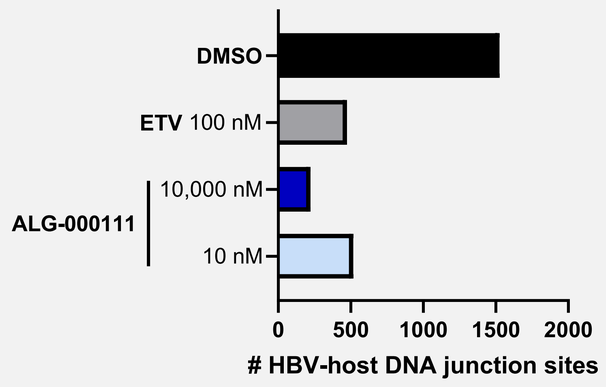
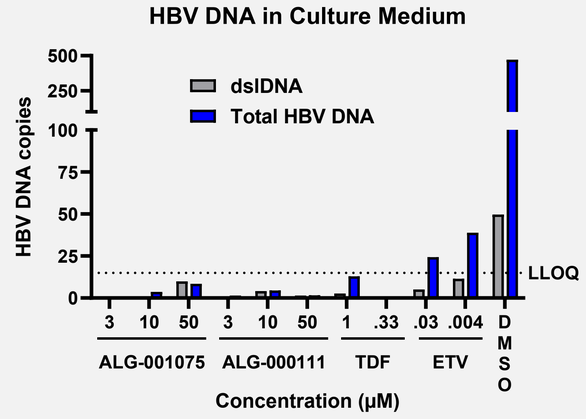


Figure 3 – CAM-E ALG-000111 prevents HBV DNA integration to a greater extent than ETV when both are added at physiologically relevant concentrations.

ALG-001075 EFFICIENTLY BLOCKS PRODUCTION OF HBV DSLDNA, PRECURSOR TO HBV DNA INTEGRATION

Production of total HBV DNA, dslDNA and rcDNA from HepG2.117 cells was assessed through a previously published PNA clamp-based qPCR method[5] and a specific dslDNA/rcDNA dPCR assay developed in-house. As expected, based on their primary mechanism, CAM-Es ALG-001075 and ALG-000111 (a close structural analog of ALG-001075)[7] resulted in pronounced reductions at clinically relevant micromolar concentrations (Figure 3), with all values falling below the lower limit of quantification of the assay. Tenofovir disoproxil fumarate (TDF) and entecavir (ETV) also reduced dslDNA levels. Testing of a broad concentration range of ALG-000111 confirmed potent inhibition of rcDNA- and dslDNA-containing viral capsids with EC_{50} values below 1 nM. In contrast, ETV showed a surprising difference in potency on rcDNA- and dslDNA-containing viral capsids.

PNA clamp



In-house dPCR

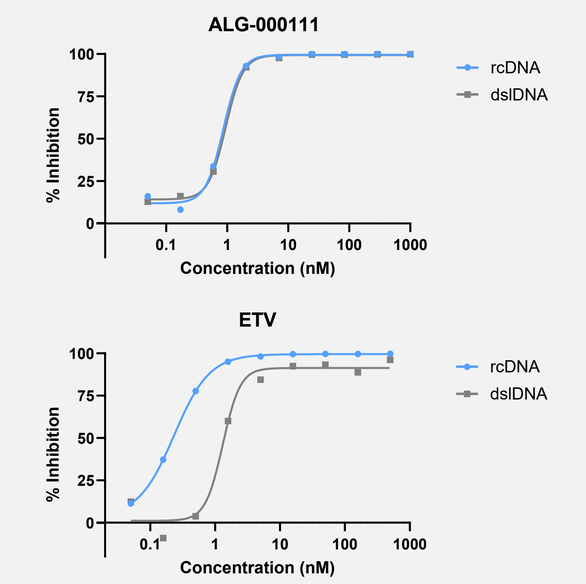


Figure 4 – CAM-Es and nucleos(t)ide analogs reduce total HBV DNA and dslDNA production in HepG2.117 cells. Top graph: dslDNA and total HBV DNA copies per condition measured by PNA clamp assay. **Bottom graphs:** Percentage inhibition of rcDNA and dslDNA in function of ALG-000111 and ETV concentrations measured by an in-house dPCR assay. dslDNA, double-stranded linear DNA; ETV, entecavir; LLOQ, lower limit of quantification; TDF, tenofovir disoproxil fumarate.

CONCLUSION

- **ALG-001075 demonstrated potent prevention of HBV cccDNA formation in cell culture experiments, confirming earlier in vitro findings of inhibition of cccDNA markers, such as HBsAg, HBcrAg and intracellular HBV RNA.[2] This provides a potential explanation for the antigen declines observed in HBeAg-positive patients with chronic HBV infection treated with Pevyfoscorvir sodium, as seen in our phase 1 clinical trial.[3]**
- **Furthermore, ALG-001075 inhibited the production of dslDNA, the main precursor for HBV DNA integration, and ALG-000111, a close structural analog of ALG-001075, was shown to directly prevent HBV DNA integration through both the primary and secondary mechanism of action.**
- **In addition, ALG-000111 showed a similar potency in inhibiting dslDNA and rcDNA formation, as opposed to ETV, which was less potent in inhibiting dslDNA compared to rcDNA.**
- **These results confirm the promising and diverse antiviral properties of ALG-001075 in vitro and suggest it may have certain advantages over commonly used nucleos(t)ide analogs, which is currently being tested in the Phase 2 B-SUPREME study of pevifoscorvir sodium in participants with chronic HBV infection.**

DISCLOSURES: All authors are directly or indirectly employed by Aligos and may own stock

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