Host poly(A) polymerases PAPD5 and PAPD7 provide two layers of protection that ensure the integrity and stability of hepatitis B virus RNA.


1 Arbutus Biopharma, Warminster, Pennsylvania, USA

# Corresponding authors: Fei Liu, fliu@arbutusbio.com; Min Gao, mgao@arbutusbio.com

* Present address: Fang Guo, Novartis Institutes for Biomedical Research, Shanghai, China; Lauren Bailey, Merck Research Labs (MRL), West Point, Pennsylvania, USA; Rene Rijnbrand, Dicerna, Lexington, Massachusetts, USA.

Key Words: RNA integrity, RNA stability, PAPD5, PAPD7, HBV PRE and AB-452
Abstract

Noncanonical poly(A) polymerases PAPD5 and PAPD7 (PAPD5/7) stabilize HBV RNA via the interaction with the viral post-transcriptional regulatory element (PRE), representing new antiviral targets to control HBV RNA metabolism, HBsAg production and viral replication. Inhibitors targeting these proteins are being developed as antiviral therapies, therefore it is important to understand how PAPD5/7 coordinate to stabilize HBV RNA. Here, we utilized a potent small-molecule AB-452 as a chemical probe, along with genetic analyses to dissect the individual roles of PAPD5/7 in HBV RNA stability. AB-452 inhibits PAPD5/7 enzymatic activities and reduces HBsAg both \textit{in vitro} (EC$_{50}$ ranged from 1.4 to 6.8 nM) and \textit{in vivo} by 0.93 log$_{10}$. Our genetic studies demonstrate that the stem-loop alpha sequence within PRE is essential for both maintaining HBV poly(A) tail integrity and determining sensitivity towards the inhibitory effect of AB-452. Although neither single knock-out (KO) of \textit{PAPD5} nor \textit{PAPD7} reduces HBsAg RNA and protein production, \textit{PAPD5} KO does impair poly(A) tail integrity and confers partial resistance to AB-452. In contrast, \textit{PAPD7} KO did not result in any measurable changes within the HBV poly(A) tails, but cells with both \textit{PAPD5} and \textit{PAPD7} KO show reduced HBsAg production and conferred complete resistance to AB-452 treatment. Our results indicate that PAPD5 plays a dominant role in stabilizing viral RNA by protecting the integrity of its poly(A) tail, while PAPD7 serves as a second line of protection. These findings inform PAPD5 targeted therapeutic strategies and open avenues for further investigating PAPD5/7 in HBV replication.
Importance

Chronic hepatitis B affects more than 250 million patients and is a major public health concern worldwide. HBsAg plays a central role in maintaining HBV persistence and as such, therapies that aim at reducing HBsAg through destabilizing or degrading HBV RNA have been extensively investigated. Besides directly degrading HBV transcripts through antisense oligonucleotides or RNA silencing technologies, small-molecule compounds targeting host factors such as the noncanonical poly(A) polymerase PAPD5 and PAPD7 have been reported to interfere with HBV RNA metabolism. Herein, our antiviral and genetic studies using relevant HBV infection and replication models further characterize the interplays between the cis-element within the viral sequence and the trans-elements from the host factors. PAPD5/7 targeting inhibitors, with oral bioavailability, thus represent an opportunity to reduce HBsAg through destabilizing HBV RNA.
**Introduction**

Globally, more than 250 million patients are chronically infected with hepatitis B virus (HBV) (World Health Organization), but a functional cure of chronic hepatitis B (CHB) is rarely achieved even after years of treatment with nucleos(t)ide analogues (NAs) such as entecavir (ETV) and tenofovir disoproxil fumarate (TDF) (1). Pegylated IFN-α enhances antiviral immune response, but the cure rate remains low and side effects are often difficult to tolerate (2, 3). The major obstacles to curing CHB include the persistence of the episomal covalently closed circular DNA (cccDNA), and an immune system that is tolerized to HBV, likely due to the excess amount of circulating Hepatitis B surface antigen (HBsAg) levels (4-6).

The HBV envelope proteins preS1, preS2 and HBsAg are synthesized in the endoplasmic reticulum and are secreted as both viral and subviral particles (7, 8). HBV virions are double-shelled particles with an outer lipoprotein bilayer containing the envelope proteins, and an inner nucleocapsid that encloses the HBV DNA and viral polymerase. The subviral particles devoid of nucleocapsids and HBV DNA (9, 10) are up to 100,000-fold in excess relative to the virions in the blood of infected patients (11). Such high levels of subviral particles are believed to play a key role in immune tolerance and maintenance of persistent HBV infection (5, 6). In patients with chronic hepatitis B, HBV-specific T cells are depleted or functionally impaired (12-15), and circulating and intrahepatic antiviral B cells are defective in the production of antibodies against HBsAg, with an expansion of atypical memory B cells (16, 17). HBsAg has also been linked to the inhibition of innate immunity and functionality of other immune cell types (18). Therefore, antiviral strategies that aim to target the HBV RNA transcripts could suppress HBsAg production and may break the immune tolerance state to potentially increase the functional cure rate.
Regulation of HBV RNA metabolism involves the post-transcriptional regulatory element (PRE), which is a stretch of ribonucleotides spanning positions 1151-1582 on the viral transcripts that is essential to HBV subgenomic RNA (sRNA) nuclear export and regulation of pregenomic RNA (pgRNA) splicing (19-22). The PRE contains three sub-elements, PREα, PREβ1 and PREβ2. Each sub-element is sufficient to support sRNA nuclear export and HBsAg production, but all three together exhibit much greater activity (23, 24). RNA secondary structure prediction and phylogenetic covariations analysis suggest that two stem-loop structures (SLα and SLβ1) localized either in PREα or PREβ1 sub-elements may exist in vivo and serve as protein binding sites (23, 25). These two stem-loop structures are highly conserved not only in HBV variants but also throughout the different mammalian hepadnaviruses, and mutations in the stem regions reduced HBsAg production (23). The PRE is complexed with several RNA binding proteins, including T-cell intracellular antigen 1, La protein, polypyrimidine tract binding protein, ZC3H18 and ZCCHC14 (26-32). These PRE binding proteins may serve to regulate the export and stability of HBV RNAs. In particular, the CAGGC pentaloop sequence/structure of stem-loop alpha (SLα) within the PREα sub-element has been predicted to bind sterile-alpha-motif domain containing proteins (24). Recently, ZCCHC14 (a sterile-alpha-motif containing protein), together with PAPD5 and PAPD7 (the non-canonical poly(A) RNA polymerase associated domain containing proteins 5 and 7), were identified as the cellular binding proteins that interacted with the HBV SLα sequence (33).

The small-molecule compound, RG7834, targets PAPD5/7 and destabilizes HBV RNAs (34-37). Using a genome-wide CRISPR screen, it was subsequently observed that ZCCHC14 and PAPD5 were associated with the antiviral activity of RG7834 (32). Interestingly, individual knockdown of PAPD5 or PAPD7 had minimal effect against HBsAg production, while knockdown of
or double knockdown of PAPD5/7 had a profound anti-HBsAg activity similar to that observed when cells were treated with RG7843 (32, 37). It was further demonstrated that double knockout of PAPD5/7 reduced guanosine incorporation frequency within HBV RNA poly(A) tails, leading to a proposed model in which HBV RNA recruits the PAPD5/7-ZCCHC14 complex via the CNGGN pentaloop of PRE SLα to enable the extension of mixed tailing on HBV poly(A) tails, which subsequently protects the viral RNAs from cellular poly(A) ribonucleases (33).

To gain further insights into how small-molecule inhibitors destabilize HBV RNAs, mechanistic studies were performed using AB-452, an analogue of RG7834, to evaluate its effect in HBV replicating cells and in cells transfected with constructs containing mutations within the PRE sequence. To better understand how PAPD5 and PAPD7 coordinate in the protection of HBV RNAs, both HBV RNA transcripts and their poly(A) tails were analyzed in cells with PAPD5 and/or PAPD7 knockout. Our results reveal that HBV utilizes two layers of protection mechanism provided by PAPD5 and PAPD7 to protect their poly(A) tail integrity and RNA stability.
Results

AB-452 inhibits HBV *in vitro* and *in vivo*.

AB-452 and RG7834 both belong to the dihydroquinolizinones chemical class. The antiviral activities of AB-452 and its diastereomer ARB-169451 were evaluated using multiple *in vitro* HBV replication models including HepG2.2.15 cells (constitutively express HBV through the integrated viral genome), PLC/PRF/5 cells (a patient-derived hepatocellular carcinoma cell line only expressing HBsAg), and HBV infected HepG2-NTCP cells or primary human hepatocytes (PHH) in which viral replication was dependent on cccDNA transcription (Table 1). AB-452 reduced HBsAg, HBeAg, and HBV DNA production with EC$_{50}$ values ranging from 0.28 to 6.8 nM, while its diastereomer ARB-169451 was more than 1,000-fold weaker towards HBsAg inhibition when compared to AB-452 (Table 1). AB-452 antiviral activity was specific for HBV as the compound was inactive against a panel of ten different RNA and DNA viruses with EC$_{50}$ values of >30 μM (Table 2). In addition, the cytotoxicity of AB-452 was evaluated in several cell lines from different tissue origins showing CC$_{50}$ values of >30 μM (the highest concentration tested) (Table 3), demonstrating the selectivity of AB-452.

To evaluate the effects of AB-452 against the different stages of the viral life cycle, HBV replication intermediates and viral proteins were analyzed from HepG2.2.15 cells treated with AB-452 at a concentration of 50-fold above its EC$_{50}$ value (Fig. 1A). The nucleoside analog ETV and two classes of HBV capsid inhibitors GLS-4 (class I) and compound A (cmpdA, class II) were included as controls (structures not shown) targeting the polymerase and core/capsid proteins, respectively. ETV strongly inhibited HBV DNA replication, but did not reduce the production of intracellular HBV RNA, core protein levels, or capsid assembly. Consistent with
their mechanism of action, capsid inhibitors inhibited pgRNA encapsidation and HBV DNA replication, but had no effect against total pgRNA and sRNA transcripts. On the contrary, AB-452 displayed a unique antiviral phenotype reducing intracellular pgRNA, sRNA, core protein, native capsids, encapsidated pgRNA, and replicating HBV DNA (Fig. 1A). Furthermore, the effect of AB-452 against intracellular pgRNA and sRNA was dose dependent and appeared to reach a plateau at >100 nM, with approximately 25% pgRNA and 18% sRNA remaining detectable at the highest concentration tested (1 μM) (Fig. 1B). Results from the time course studies showed that AB-452 induced reduction of pgRNA and sRNA starting at 8 h post treatment and the levels continued to decline through the 48 h treatment period (Fig. 1C).

An AAV-HBV-transduced mouse model was used to assess the anti-HBV effect of AB-452 in vivo (Fig. 2). Compared to the vehicle control, oral administration of AB-452 for 7 days at 0.1, 0.3, and 1 mg/kg twice daily resulted in mean 0.68, 0.72 and 0.93 log_{10} reduction of serum HBsAg (Fig. 2A) and mean 0.79, 1.16 and 0.94 log_{10} reduction of serum HBV DNA (Fig. 2B), respectively. Inhibition of circulating HBV markers at 0.1, 0.3 and 1 mg/kg doses was found to be correlated with dose-dependent reductions of viral products in the liver: intrahepatic HBsAg levels were reduced by 64, 69 and 83% (Fig. 2C), intrahepatic total HBV RNA levels were reduced by 35, 55, and 66%, and intrahepatic pgRNA levels were reduced by 43, 55, and 63% (Figs. 2D and 2E), respectively. AB-452 treatments were well-tolerated, with no significant change or reduction in body weight in mice throughout the course of the compound treatment compared to those receiving the vehicle control (Fig. 2F). The in vitro observation that AB-452 suppressed intracellular HBV RNA was therefore translatable to the in vivo AAV-HBV transduced mouse model when treated with AB-452.
AB-452 promotes HBV RNA degradation through inhibiting PAPD5/7 and blocking guanosine incorporation within HBV poly(A) tails.

To investigate the molecular mechanism of how AB-452 inhibits HBV RNA, studies were performed using HepAD38 cells in which HBV transcription is under tetracycline (Tet) regulation. Tet was first removed to induce transcription and accumulation of viral RNAs, and the capsid inhibitor GLS-4 was added to prevent pgRNA encapsidation. Six days later, Tet was added back to shut down further transcription and cells were treated with both GLS-4 and AB-452 for an additional 16 h. The effect of AB-452 on HBV transcripts was evaluated by collecting cells at 0, 2, 4, 8 and 16 h post treatment, and decay of the transcribed HBV RNA was monitored by Northern blot analysis. In the absence of AB-452, HBV RNA levels reduced over time due to natural decay (Fig. 3A). In the presence of AB-452, both pgRNA and sRNA exhibited faster migration starting at 2 h post-treatment and their levels were significantly reduced at 8 and 16 h post-treatment (Fig. 3A). Determination of the pgRNA half-lives (T½) showed that AB-452 treatment reduced the T½ values from 4.5 h to 2.4 h compared to those from untreated cells (Fig. 3B).

Destabilization of HBV RNA by RG7834 was reported to be mediated through inhibiting the PAPD5 and PAPD7 proteins(32, 37). We determined the effect of AB-452 on the enzymatic activity of recombinant human PAPD5 and PAPD7 using an ATP depletion biochemical assay (Fig. 3C). Results showed that AB-452 efficiently inhibited PAPD5 with an IC₅₀ of 94 nM (Fig. 3C). RG7834 also inhibited PAPD5, although the potency (IC₅₀ = 167 nM) was lower than previously reported (IC₅₀ = 1.3 nM) (32). We speculate that the reduction in the inhibitory effect against the recombinant enzymes could be due to the truncated PAPD5 form that was used in the current study. Both AB-452 and RG7834 inhibited PAPD7 with IC₅₀ values of 498 nM and 1093
nM, respectively. In contrast, the enantiomer ARB-169451 was unable to effectively inhibit PAPD5 (IC$_{50}$ = 27,000 nM) or PAPD7 (IC$_{50}$ > 50,000 nM) (Fig. 3C).

RNA metabolism in most eukaryotic mRNAs employs the 3’ deadenylation pathway, in which poly(A) tail shortening is often observed prior to mRNA degradation (38-40). We therefore determined the HBV poly(A) tail length and composition from HepAD38 cells in the presence or absence of AB-452. To amplify the HBV poly(A) tail, G/I (guanosine and inosine nucleotides) tailing was added to the 3′-ends of mRNA transcripts and the newly added G/I tails were used as the priming sites to synthesize the cDNA that would be used for amplification of HBV poly(A) tails. The lengths and compositions of the amplicons containing the HBV RNA poly(A) tails were analyzed by next generation sequencing (PacBio Sequel Sequencing platform). Results showed that majority of the HBV poly(A) tails from untreated samples ranged between 50 to 200 nucleotides in length, with an average length of around 100 nucleotides. In contrast, AB-452 treatment reduced the HBV RNA poly(A) tail length by almost 50%, to an average of 58 nucleotides (Fig. 3D and 3E). The poly(A) tails amplified from β-actin cDNAs served as the negative control, which was not affected by the treatment (Fig. 3D).

It was recently reported that PAPD5/7 extended HBV mRNA poly(A) tails with intermittent guanosine (G), and the incorporation of G could shield them from rapid de-adenylation by cellular deadenylases (33). Since AB-452 inhibited PAPD5/7 enzymatic activities and shortened poly(A) tail lengths, we therefore hypothesized that the G content within the HBV poly(A) tails would be affected by AB-452 treatment. Quantification of the non-adenosine nucleosides within the HBV poly(A) tails indeed revealed that the frequency of G was significantly reduced in the presence of AB-452 (Fig. 3F). The fraction of poly(A) tails containing internal G was reduced from 64% to 25% in the presence of AB-452 compared to those from untreated HepAD38 cells.
Taken together, the data indicate that inhibition of PAPD5/7 by AB-452 led to blockage of G incorporation and shortening of the poly(A) tail.

**SLα within the PRE sequence is required for HBV RNA integrity and AB-452 susceptibility.**

We and others have determined that reduction of HBV RNA by RG7834 is dependent on the HBV PRE(32, 36), which partially overlapped with the HBx coding region. To further define the involvement of the sub-elements within PRE on HBV RNA stability and AB-452 susceptibility, several reporter plasmids were constructed (Fig. 4A): 1) H133 is the wild type construct supporting the expression of 2.1 kb HBV sRNA expression, 2) H133_Gluc is derived from H133 but with the HBsAg coding sequence replaced with *Gaussia* luciferase, 3) Gluc_dHBx is derived from H133_Gluc but with most of the HBx coding sequence deleted (nucleotide 1389 to 1991) and the HBV poly(A) replaced with the SV40 poly(A) signal, and 4) Gluc_rcSLα is derived from Gluc_dHBx with an inverted SLα sequence.

AB-452, but not its enantiomer ARB-1694151, inhibited both HBsAg and Gluc expression in cells transfected with H133 (EC\textsubscript{50} = 2.5 nM), H133_Gluc (EC\textsubscript{50} = 10.0 nM), or the Gluc_dHBx construct (EC\textsubscript{50} = 4.2 nM) (Fig. 4A). These data indicate that AB-452 antiviral activity was not dependent on the HBsAg sequence, HBx sequence or the HBV poly(A) signal sequence. On the other hand, inverting the SLα sequence (Gluc_rcSLα) abolished sensitivity to AB-452 (EC\textsubscript{50} >100 nM) (Figs. 4A and 4B). Interestingly, we observed that the transcribed RNA from the Gluc_rcSLα transfected cells showed reduction in RNA levels and appeared smaller in size when compared to the RNA from cells transfected with the Gluc_dHBx plasmid, with or without AB-452 treatment (Fig. 4B). The rates of HBV RNA decay revealed that AB-452 treatment reduced
Gluc_dHBx RNA half-lives (T½) from 10 h to 5.1 h when compared to DMSO treated cells (Fig. 4C). In contrast, the Gluc_rcSLα RNA was unstable (T½ = 5.6 h) and its T½ was only slightly reduced by AB-452 (T½ = 4.2 h) (Fig. 4C).

In addition to SLα, HBV PREα contains another cis-acting element known as La protein binding element, these two cis-acting elements were included in a 109 nucleotides sequence that was critical for RG7834 sensitivity (36). The requirement of these two elements was studied by generating two additional H133 derived constructs, H133_dSLα and H133_dLa, in which the SLα sequence and the La element was deleted, respectively. AB-452 inhibited HBsAg production in H133_dLa transfected cells with similar efficiencies as the wildtype H133 construct (EC50 = 4.3 and 2.5 nM, respectively), indicating that the La protein binding element was not essential for susceptibility to AB-452. Alike to the results observed in the Gluc_rcSLα transfection, AB-452 was inactive against the H133_dSLα (EC50 >100 nM) (Fig. 5A). Deleting the SLα sequence also led to the shortening and reduction of sRNA level (Fig. 5B), as well as reduced transcripts T½ (Fig. 5D). Cells transfected with H133_dSLα showed reduced sensitivity to AB-452 compared to the wild type H133 transfected cells (Fig. 5B and 5C). The transcript half-life from the H133_dSLα transfected cells treated with AB-452 was only slightly reduced from 6.2 h to 5.5 h when compared to those treated with DMSO (Fig. 5D).

NGS analysis of the sRNA poly(A) tails showed that AB-452 reduced the average poly(A) tail length of H133 transcripts from 124 to 64 nucleotides (Fig. 5E). The poly(A) tails from the H133_dSLα transcripts were 62 and 71 nucleotides with and without AB-452 treatment, respectively (Fig. 5E). In terms of the poly(A) tail composition, the guanylation frequency was highest in cells transfected with the wildtype PRE (H133), and the overall guanylation frequency was reduced from about 60% to 24% in the presence of AB-452 (Figs. 5F and 5G). In contrast,
the guanylation frequency in the H133_dSLα transcripts already appeared low (22% to 27%) with and without AB-452 treatment (Figs. 5F and 5G). Taken together, these data provide first line evidence demonstrating that the SLα sequence serves to stabilize the viral transcripts through maintaining the poly(A) tail lengths and mixed-nucleotides composition.

PAPD5 and PAPD7 determine HBV RNA integrity and stability.

To understand the individual role of PAPD5 and PAPD7 in regulating HBV RNA stability and poly(A) tail integrity, knockout (KO) cell lines with deletion of PAPD5 (P5_KO), PAPD7 (P7_KO), or both PAPD5/7 (double knockout, P5/7_DKO) were isolated using CRISPR-Cas9 gene editing and HepG2-NTCP cells. It was reported that ZCCHC14 (Z14), which interacts with PAPD5/7 and the HBV PRE, plays an important role in maintaining HBV RNA integrity and stability (33). Z14 KO cell lines (Z14_KO) were generated to assess the involvement of Z14 on regulating HBV RNA. In addition to the parental wildtype (WT) HepG2-NTCP cell line, two additional WT cell clones (T3-4 and T2-14) were included as clonal controls. The full-allelic KO genotype for all the individual cell clones was confirmed by DNA sequencing (Fig. 6A), and PAPD5 and Z14 knockout were also confirmed at the protein level (Fig. 6B). PAPD7 protein expression could not be evaluated by Western blot due to the lack of an efficient PAPD7 specific antibody, but the PAPD7 KO genotype was confirmed by DNA sequencing (Fig. 6A).

Overall, cell proliferation analysis suggests that PAPD5, PAPD7, PAPD5/7 and Z14 were not critical for cell survival (Fig. 7A). The effect of knocking out PAPD5/7 and Z14 on viral protein production and HBV replication was examined by using two independent systems: adenovirus-encoded HBsAg transduction and HBV infection (Figs. 7B-7C). In the adenovirus transduction studies, single KO of PAPD5 or PAPD7 did not reduce HBsAg production compared to the WT cell clones. HBsAg expression in the P5/7_DKO and Z14_KO clones was about 50% lower than
that of the WT or PAPD5/7 single KO clones in the 5 days culture (Fig. 7B). In the HBV
infection studies, the levels of viral proteins and HBV DNA were much lower in the P5/7_DKO
and Z14_KO clones compared to the WT or PAPD5/7 single KO clones in the 9 days culture
(Fig. 7C).

We next examined the impact of deleting PAPD5, PAPD7, Z14, or both PAPD5/7 on compound
sensitivity. In adenovirus transduced cells, AB-452 inhibited HBsAg production from WT (EC_{50}
= 9.0 nM) and P7_KO cell clones (EC_{50} = 7.2 and 10.0 nM) with similar EC_{50} values. However,
AB-452 was about 6- to 8-fold less active against the P5_KO cell clones (EC_{50} = 56.0 and 71.6
nM) (Table 4). Susceptibility to AB-452 was also evaluated using HBV infected HepG2-NTCP
cells: results showed that AB-452 inhibited WT (EC_{50} = 2.7 nM) and P7_KO (EC_{50} = 3.2 and 3.8
nM) cells with similar efficiencies but was again about 10-fold less active against the P5_KO
cells (EC_{50} = 37.7 and 39.3 nM) (Table 4). A similar trend was also observed with RG7834,
suggesting this differentiated antiviral activity was not AB-452 specific. The antiviral data are
consistent with the finding that AB-452 and RG7834 were more efficient against PAPD5 than
PAPD7 in the enzymatic assays (Fig. 3C). Among the P5/7_DKO and Z14_KO cell lines, AB-
452 treatment did not show further inhibition compared to the untreated controls (Figs. 7D-7G).

Interestingly, while there was no appreciative reduction of HBV protein and DNA production
observed from the P5_KO cells (Figs. 7B-7C), we observed that the sRNA migrated faster than
those from the WT and P7_KO cells (Fig. 7H-7I). Intrigued by this observation, the sRNA from
WT and the various KO cells were further characterized by NGS analysis. Results revealed that
knocking out PAPD5 alone, but not PAPD7, reduced both the poly(A) tail lengths (from >136 bp
to 94 bp) and guanylation frequency of sRNA (from ~50% to 30%) when compared to the WT
cells (Figs. 7J-7K). AB-452 treatment led to reduction of poly(A) tail length (from >136 bp to
~60 bp) and guanylation (from ~50% to ~10%) in both WT and P7_KO cells. The P5_KO cells appeared less sensitive to AB-452 in its shortening of poly(A) tail lengths and guanosine incorporation. Cells with Z14_KO and PAPD5/7_DKO already showed drastically reduced levels of sRNA, poly(A) tail lengths (51-58 bp) and guanosine incorporation (~10%), with and without AB-452 treatment. Taken together, these results suggest that of the two noncanonical poly(A) polymerases, PAPD5 appeared to play a major role in determining viral poly(A) tail integrity, guanosine incorporation and AB-452 sensitivity.
Current therapies for chronic hepatitis B patients rarely achieve functional cure, which is characterized as sustained loss of HBsAg with or without HBsAg antibody seroconversion (41). The discovery of RG7834 has raised significant interest as this class of small-molecule inhibitors has the potential to reduce both HBV RNA and viral proteins, which are distinct from direct-acting antivirals targeting the HBV polymerase and capsid proteins (34, 36, 37). AB-452 is an analog of RG7834 with a similarly broad antiviral effect against multiple HBV replication intermediates. It has been appreciated that integrated HBV DNA is a major source of HBsAg expression in HBeAg negative patients (42). Our data indicate that AB-452 can reduce HBsAg produced from cccDNA in HBV-infected cells as well as from integrated HBV DNA in patient-derived hepatocellular carcinoma cells (Table 1). Furthermore, oral administration of AB-452 substantially reduced HBV DNA, HBsAg, HBeAg, and intrahepatic HBV RNA from AAV-HBV-infected mice (Fig. 2). Our studies here provide insights into the mode of action for AB-452 and further characterize the RNA stabilization mechanisms utilized by the virus. Our results demonstrate that the cis-acting SLα viral sequence and the trans-acting host factors PAPD5 and PAPD7 coordinate to protect viral RNA. Interference of such viral-host interactions through small-molecule compounds treatment or genetic mutations led to destabilization of viral transcripts and reduction of HBsAg.

The requirement of PAPD5/7 and ZCCHC14 to form a complex with HBV RNA through the PRE element for stabilizing HBV RNA has been described (32, 33). Since the ZCCHC14/PAPD5/7 complex is recruited onto the SLα sequence, it is conceivable that mutating the SLα sequence may disrupt the binding of the ZCCHC14/PAPD5/7 complex and
consequently affect HBV RNA stability. Here, our studies provided the genetic evidence that an intact SLα sequence is indeed critical for maintaining HBV poly(A) tail integrity and stability, as inverting or deleting this sequence both destabilize HBV RNA. Notably, the phenotype of the SLα deletion and inversion mutants resembled the antiviral effect of AB-452: cells treated with AB-452 display the phenotypes of HBV poly(A) tail shortening, reduced guanosine incorporation, and HBV RNA degradation.

Initial studies suggest that PAPD5 and PAPD7 may provide redundant if not identical role(s) in protecting HBV RNA stability (32, 33, 37, 43). However, our results from the \textit{P5\_KO} and \textit{P7\_KO} cell lines would argue that PAPD5 and PAPD7 may serve two lines of protection in maintaining the stability of HBV RNA. \textit{P5\_KO}, but not \textit{P7\_KO}, impaired poly(A) tail integrity. Moreover, the phenotypic measurements we monitored so far indicate that the \textit{P7\_KO} cells were similar to WT cells, further supporting that PAPD5 expression alone could support viral RNA integrity and stabilization (Fig. 7). These data suggest that PAPD7 did not actively contribute to HBV RNA protection in the presence of PAPD5, but instead served as a second line of protection by moderately extending HBV poly(A) tail when PAPD5 was depleted (Figs. 7I-7K).

Results from the enzymatic assays show that PAPD5 was more robust than PAPD7 in the extension of poly(A) tails (data not shown), supporting our argument that PAPD5 would be the major host factor in protecting HBV RNA. Immune precipitation experiments conducted by two independent research groups indicated that both PAPD5 and PAPD7 were bound to HBV mRNA, with PAPD7 at a lower level compared to PAPD5 (33, 43). Further studies would be required to clarify the role of PAPD7 in HBV RNA metabolism in WT cells.

Another noteworthy observation from this study is that the two HBV RNA destabilizers, AB-452 and RG7834, displayed different inhibitory efficiencies against PAPD5 and PAPD7. Both
compounds were 5- to 7-fold less efficient against the enzymatic activities of PAPD7 compared
to PAPD5, which was in turn consistent with the results from cell-based studies in which AB-452 and RG7834 displayed a 6- to 10-fold reduction in activities against HBsAg production in
the P5_KO cells (in which PAPD7 is present) when compared to those from the WT and P7_KO
cells (in which PAPD5 is present). These data suggest that it may not be critical to completely
inhibit PAPD7 to achieve HBV RNA destabilization. These data, together with the genetic
studies, support the hypothesis that PAPD5 could be more essential than PAPD7 in stabilizing
HBV RNA. Our results further suggest that developing PAPD5-selective inhibitors of HBV
replication could be pharmacologically feasible.

Here, we propose a working model of the interplay between HBV transcripts and the cellular
ZCCHC14/PAPD5/7 RNA metabolism machineries (Fig. 8). Maintenance of HBV RNA stability
is a dynamic process regulated by canonical and non-canonical poly(A) polymerases and
deadenylases. PAPD5 could form a complex with ZCCHC14, which directs the non-canonical
polymerase onto the viral transcripts through the SLα within the HBV PRE sequence. Assembly
of the ZCCHC14/PAPD5 onto SLα within the HBV PRE sequence facilitates the addition of G
while extending the poly(A) tail. This guanylation process may stall the cellular poly(A)
exonuclease and terminate further deadenylation, thus protecting the RNA from degradation.
When PAPD5 is depleted, ZCCHC14/PAPD7 complex may bind to HBV RNA and protects its
degradation, however PAPD7 is less effective for poly(A) extension and guanylation
incorporation. When HBV is challenged by HBV RNA destabilizers such as AB-452 or PRE
mutations, viral RNA integrity and stability are disrupted due to either the inhibition of PAPD5/7
enzymatic activities or disarraying of the ZCCHC14/PAPD5/7 complex from interacting with the
SLα sequence, respectively.
Funding

This study was sponsored by Arbutus Biopharma.
Methods and materials

Cell lines and culture
HepG2.2.15, HepAD38, HepDE19 cells and PLC/PRF/5 cells were cultured in DMEM/F12 medium (Corning, NY, USA), supplemented with 10% fetal bovine serum (Gemini, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. Huh-7 cells (Creative Bioarray, NY, USA) were cultured in RPMI 1640 medium (Basel, Switzerland) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. HepG2-hNTCP-C4 cells were cultured in DMEM medium (Gibco™, MA, USA) containing 10% fetal bovine serum and 10 mM HEPES.

Antiviral studies
HepG2.2.15 (30,000 cells/well) were plated in 96-well collagen treated plates and incubated at 37°C. Compounds were half-log serially diluted and added in duplicate to 96 well microtiter plates. The plates were incubated for a total duration of 6 days, after which the culture media was removed for the HBsAg and HBeAg CLIA assays (AutoBio Diagnostics Co, China) performed according to manufacturer’s instructions. Secreted HBV DNA was extracted (Realtime Ready Cell Lysis Kit, Roche, Mannheim, Germany) and quantified in a qPCR assay (LightCycler® 480 SYBR Green I Master, Roche) with the 5’-GGC TTT CGG AAA ATT CCT ATG-3’ (sense) and 5’-AGC CCT ACG AAC CAC TGA AC-3’ (antisense) primers using the PCR conditions of denaturing at 95 °C for 5 min, followed by 40 cycles of amplification at 95 °C for 15 s and 60 °C for 30 s. Antiviral selectivity studies against a panel of different DNA and RNA viruses were performed at ImQuest BioSicences (Frederick, MD, USA). Briefly, test compounds were
evaluated using 6 doses including the highest concentration of 30 µM and five serial half-logarithmic dilutions in triplicate for the antiviral assays.

**Infection of HepG2-hNTCP-C4 cells and primary human hepatocytes (PHHs)**

HepG2-hNTCP-C4 cells were seeded into collagen-coated 10-cm dishes at a density of $8.6 \times 10^6$ cells per dish and cultured in 10 mL complete DMEM medium containing 2% DMSO. One day later, the cells were infected with HBV at 100-250 genome equivalent (GE)/cell in DMEM containing 4% PEG-8000. The inoculums were removed 24 h later and the infected cells were washed 4 times with PBS and seeded in 96-well plates at a density of $5 \times 10^4$/well following trypsinization. Serial dilutions of compounds were added to the plates and refreshed at day 4, 8 and 11 post infection. The supernatants were harvested for HBsAg and HBeAg analysis (ELISAs, International Immuno-Diagnostics, CA, USA). HBV DNA was extracted from cell lysates per manufacturer’s instructions (Qiagen DNeasy 96 Blood and Tissue Kit, Qiagen, Hilden, Germany). HBV DNA was detected by qPCR using primers and probe as follows: 5’-GTC CTC AAY TTG TCC TGG-3’ (sense), TGA GGC ATA GCA GCA GGA-3’ (antisense), and Probe /56-FAM/CTG GAT GTG TCT GCG GCG TTT TAT CAT/36-TAMSp/. For the infection of PHHs, cells were placed in collagen coated 96-well plates (65,000 cells/well) overnight, then infected with HBV at 250 GE per cell in media containing 4% PEG-8000. The inoculums were removed 24 h later and compounds were added or replenished on days 4, 7, 9, 11, and 14 post infection. On day 16, medium was removed, and HBV antigens, DNA and RNA quantification were monitored as described above.

**Detection of intracellular HBV RNA by Northern blot analysis**

Total cellular RNA was extracted from HepG2.2.15 or HepAD38 cells treated with or without antiviral compounds using TRIzol reagent according to the manufacturer’s direction.
Northern blot was performed as described previously (1). Briefly, total RNA was separated in a 1.5% agarose gel and transferred onto an Amersham Hybond-XL membrane (GERPN303S, GE Healthcare, IL, USA). Membranes were probed with an \( \alpha^{32} \)-P-UTP (Perkin Elmer, CT, USA) labeled minus-strand specific full-length HBV riboprobe (3.2 kb) transcribed from plasmid pSP65_HBV (+) DNA. Membranes were exposed to a phosphoimager screen and the hybridization signals were quantified using Image Studio software.

**Detection of intracellular HBV DNA by Southern blot analysis**

Total intracellular viral core-associated DNA was extracted as described previously (1) and analyzed by Southern blot hybridization with an \( \alpha^{32} \)-P-UTP labeled full-length HBV riboprobe (3.2 kb) that specifically hybridized to minus-strand of viral DNA. Membranes were exposed to a phosphoimager screen and the hybridization signals were quantified using Image Studio software.

**Detection of intracellular HBCAg, PAPD5, and \( \beta \)-actin by Western blot analysis**

HepG2.2.15 or HepAD38 cells cultured in a 12-well plate were lysed with 200 \( \mu \)L Laemmli sample buffer (Bio-Rad, PA, USA) supplemented with 2.5% 2-mercaptoethanol (Sigma-Aldrich, MO, USA). Cell lysates were subjected to denaturing gel electrophoresis with 12% Criterion™ TGX Stain-Free™ Precast Gels and Tris/Glycine/SDS running buffer (Bio-Rad). Proteins were transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane Trans-Blot® Turbo™ Transfer System (Bio-Rad). Membranes were blocked with 5% nonfat milk in TBS-0.1% tween for 1 h and incubated with the primary antibody overnight at 4°C. After washing with TBST (TBS containing 0.1% Tween 20), the membrane was incubated with the secondary antibody. Membranes were again washed 3 times with TBST and soaked with 200 \( \mu \)L Clarity™.
Western ECL Substrate (Bio-Rad) and imaged with the iBright Imaging Systems (ThermoFisher Scientific). The primary antibodies used in the present study include anti-HBc antibody (Dako cat. no. B0586, United Kingdom), anti-PAPD5 antibody (Atlas Antibodies cat. no. HPA042968, Bromma, Sweden), and anti-β-actin antibody (Abcam cat. no. ab8227, Cambridge, United Kingdom).

**Particle gel for viral nucleocapsid and encapsidated HBV DNA analysis**

For intracellular viral nucleocapsid analysis, HepG2.2.15 cells were lysed in buffer containing 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, and 0.1% NP-40. Cell debris was removed by centrifugation and the viral particles were fractionated through nondenaturing 1% agarose gels electrophoresis and transferred to a nitrocellulose filter by blotting with TNE buffer (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, and 1 mM EDTA). To detect HBV core antigens, membranes were probed with polyclonal antibody against HBV core protein (Dako cat. no. B0586, United Kingdom). Bound antibodies were revealed by horseradish peroxidase (HRP)-labeled secondary antibodies (ThermoFisher Scientific) and visualized with the iBright Imaging Systems according to the protocol of the manufacturer. For the detection of encapsidated HBV DNA, the DNA-containing particles on the membrane were denatured with a solution containing 0.5 N NaOH and 1.5 M NaCl, and this step was followed by neutralization with a solution containing 1 M Tris-HCl (pH 7.6) and 1.5 M NaCl. HBV DNA was detected by hybridization with an α-32P-UTP labeled full-length HBV riboprobe (3.2 kb) that specifically hybridized to minus-strand of viral DNA.

**Detection of encapsidated pgRNA**

To detect intracellular encapsidated pgRNA, HepG2.2.15 or HepAD38 cells were lysed with 300 μL of lysis buffer (10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA and 0.1% NP-40) per
Cell debris and nuclei were removed by centrifugation and the supernatants were digested with 20 U/mL of micrococcal nuclease (MNase) at 37 °C for 30 min. The core particles were precipitated with 35% PEG 8000 dissolved in 1.5 M NaCl on ice for 60 min, isolated by centrifugation and dissolved in TNE buffer (10 mM Tris-HCl, pH 7.6; 150 mM NaCl and 1 mM EDTA). Encapsidated pgRNA in core particles was extracted with TRIzol reagent, and pgRNA was quantified in a qPCR assay (SuperScript™ III Platinum™ SYBR™ Green One-Step qPCR Kit w/ROX, Invitrogen™) with the 5' - GGT CCC CTA GAA GAA CCT CTC-3' (sense) and 5' - CAT TGA GAT TCC CGA GAT TGA GAT-3' (antisense) primers using the PCR conditions of 50°C for 30 minutes hold (cDNA synthesis), denaturing at 95 °C for 5 min, followed by 40 cycles of amplification at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s.

**In vivo antiviral activity in a mouse model of HBV**

HBV mouse experiments were conducted at Arbutus Biopharma (Burnaby, Canada) in accordance with Canadian Council on Animal Care (CCAC) Guidelines on Good Animal Practices and using protocols approved through the CCAC-certified Institutional Animal Care and Use Program. Male C57BL/6J mice, 6 weeks old, were each inoculated with 1E11 genomes of adeno-associated virus (AAV) vector AAV-HBV1.2 containing a 1.2× overlength sequence of HBV genome (genotype D, Genbank accession no. V01460) (2). Mice were administered the AAV2/8-type vector via intravenous tail vein injection. 28 days after AAV infection, animals were randomized into groups (n = 5) based on serum HBsAg concentration. Animals were administered vehicle only or AB-452 at 0.1, 0.3 or 1 mg/kg by oral gavage twice daily for 7 days and terminated 12 h after the last dose. Serum and liver HBsAg concentrations were determined using the BIO-RAD EIA GS HBsAg 3.0 kit according to manufacturer's instructions. Serum HBV DNA concentrations were measured from total extracted DNA using primer/probe.
sequences described previously (3). Total HBV RNA concentrations in the liver were quantified using a branched DNA assay (QuantiGene 2.0, ThermoFisher Scientific) with probes targeting the shared 3’ region of HBV transcripts whereas 3.5 kb HBV RNA concentrations were quantified with probes targeting the unique 5’ region of the pgRNA; in both cases signal was normalized against mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

**Purification of recombinant PAPD5 and PAPD7 proteins**

Molecular cloning, expression, and purification of the recombinant human PAPD5 and PAPD7 proteins were performed at Xtal BioStructures (MA, USA). PAPD5 (amino acids 186-518, NCBI Reference Sequence: XM_011523275) and PAPD7 (amino acids 226-558, NCBI Reference Sequence: NM_006999.6) genes comprising of nucleotidyltransferase and PAP-associated domains were codon-optimized, synthesized and inserted in the expression vector pET-24a(+) for E. coli expression with 10XHis-Flag-TEV-tags at the N-terminal. The pET-24a(+) constructs were transformed into E. coli competent cells BL21 (DE3). Cells were grown in TB-Kan medium (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 72 mM K2HPO4, 17 mM KH2PO4 + 50 μg Kanamycin/mL + 100 mM sodium phosphate pH 7.0 + 2 mM MgSO4) at 37 °C until OD600 ~0.7. Expression of protein was induced for 16 h with 0.2 mM IPTG at 18 °C. Cells were lysed in Lysis buffer (25 mM HEPES pH 7.6, 300 mM KCl, 5 % glycerol) containing lysozyme (1 mg/ml), EDTA-free Protease inhibitor tablets (cOmplete™ Protease Inhibitor, Roche). Cell suspension was sonicated 8 × 20 s at 27-30 W (power level 3.5-4.0) with 40 s breaks between each pulse. Lysate was cleared by centrifugation in a 50 mL conical tube at 20,000 × g for 30 min (Fiberlite F13-14x50cy rotor in a Sorvall RC6 centrifuge). Supernatant was incubated with Ni-charged MagBeads (GenScript, L00295) equilibrated with the binding Buffer (25 mM HEPES pH 7.5, 300 mM KCl, 5% glycerol, 1 mM TCEP). Bound proteins were washed with 50
mL the binding Buffer and eluted in elution buffer containing a gradient of imidazole ranging from 5 to 500 mM. Peak fractions were pooled and stored in 50% glycerol at -80 °C.

**PAPD5 and PAPD7 ATP depletion assay**

Reactions were carried out in duplicate in 96-well low profile, skirted, white plates (Catalog number: AB0800WL) where each well contained 10 μl of the reaction mixture consists of purified recombinant PAPD5 or PAPD7 proteins (12.5 nM) in 10 mM Tris-HCl (pH 8.0), 100 mM KCl, 5 mM MgCl₂, 250 nM RNA substrate (CALM1) 5′-GCC UUU CAU CUC UAA CUG CGA AAA AAA AAA-3′, 750 nM ATP, 0.1 mM EDTA, 1 mM TCEP and 0.002% NP-40. The reactions were incubated at room temperature for 3 h and ATP depletion was monitored by using Kinase-Glo® Luminescent Kinase kit following manufacturer’s instructions (Promega cat. no. V6712, WI, USA).

**Poly(A) tail-length analysis of HBV transcripts**

The poly(A) tail of HBV transcripts was measured with the Poly(A) Tail-Length Assay Kit (cat. no. 76455, Thermofisher) per manufacturer’s instructions. Total RNA was added with poly G/I tail at the 3’ end and reverse transcribed by the primer specific to the poly G/I tail. HBV mRNA poly(A) tail was amplified by HBV specific primer (5′-CAC CAG CAC CAT GCA ACT TT-3′, nt 1806-1825 on HBV genome) and the universal primer that anneals to the G/I tail. HBV gene specific PCR (GSP) was conducted using primers targeting nt 1633-1702 of HBV RNA with 5′CCG AAT GTT GCC CAA GGT CT-3′ (sense) and 5′-CTC AAG GTC GGT CGT TGA CA-3′ (antisense). ACTB mRNA poly(A) tail was amplified by (sense) 5′-TTG CCA TCC TAA AAG CCA CC-3′ and the universal primer. ACTB GSP primers include 5′-CCC AGC ACA ATG AAG ATC AAG-3′ (sense) and 5′- GAC TCG TCA TAC TCC TGC TTG-3′(antisense). GSP-amplified products were used as loading control. The obtained amplicon products were
resolved on a 2 % agarose gel. HBV poly(A) PCR products were sequenced on PacBio Sequel 530 Sequencing platform. CCS (circular consensus sequencing) reads were generated from raw subreads using PacBio SMRTLINK software (v5.1). Accuracy cutoff was default 0.9. PCR primer sequences (allowing two mismatches) were used to identify segments potentially containing poly(A) tails in CCS reads. A CCS reads could contain multiple such segments because of PCR conditions. These segments were processed separately in downstream analysis. Poly(A) tails were identified in each segment. Notes: it was unsuccessful to sequence the sample “DMSO-treated WT (parent) cells” initially (Fig. 6J-6K). In the second sequencing, two samples including “DMSO-treated WT (parent) cells” and “DMSO-treated P7_KO (T2-22) cells” were sequenced and the sample “DMSO-treated P7_KO (T2-22) cells” served as a bridge to normalize two batches of sequencing data.

**HBV PRE cis-elements analysis**

Constructs containing either HBsAg or the *Gaussia* luciferase (Gluc) reporter genes were synthesized (GenScript). The H133 encodes the full HBsAg transcript sequence (spanning nt 2 - 1991, U95551) under the regulation of tetracycline controlled CMV promoter. The H133_dSLα and H133_dLa are derived from H133 with either the SLα sequence (nt 1294 - 1322) or the La protein binding site (nt 1271 - 1294) deleted, respectively. In the luciferase-based constructs the HBsAg encoding region was replaced with Gluc (the Gluc constructs). Variants were introduced into the Gluc constructs in which the HBx coding sequence was deleted with either wild type SLα (Gluc_dHBx) or an inverted SLα sequence (Gluc_rcSLα). Huh-7 cells were transfected with the HBsAg or luciferase reporter derived plasmids per manufacturer’s instructions (Lipofectamine 3000, Invitrogen, MA, USA). Cells were treated with the indicated compounds for 5 days. Culture supernatants were used for HBsAg or luciferase measurement (Pierce Gaussia
Luciferase Glow Assay Kit, ThermoFisher Scientific, Waltham, MA, USA). Cells were collected for HBV RNA transcript and cellular ribosomal RNA analysis by Northern blots.

**CRISPR/Cas9 knockout generation**

PAPD5 (Gene ID: 64282), PAPD7 (Gene ID: 11044) and ZCCHC14 (Gene ID: 23174) were knocked out with the CRISPR/Cas9 gene editing in the HepG2-hNTCP-C4 cells at GenScript Biotech Corporation (NJ, USA). Briefly, gRNAs were designed and expressed in the plasmid pSpCas9 BB-2A-GFP PX458. Three gRNAs were evaluated for each gene, the gRNA with the highest cleavage efficiency was selected to knock out the target gene: PAPD5 gRNA (5’-GAC ATC GAC CTA GTG GTG TTT GG-3’), PAPD7 gRNA (5’-ATA TTT GGC AGC TTT AGT ACA GG-3’), and ZCCHC14 gRNA (5’-GCG TGA GAC CCG CAC CCC CG-3’). HepG2-hNTCP-C4 cells were transfected with validated gRNA-Cas9 plasmids. Transfected cells were sorted by FACS through EGFP. The obtained cell pool was expanded for single cell cloning. Genomic DNA from the single cell clones was extracted for PCR amplification with primers flaking the target site followed by Sanger sequencing.

**Acknowledgments**

We thank Ingrid Graves, Agnes Jarosz, Chris Pasetka and Alice Li for the analysis of in vivo data. Thank Jorge Quintero for scaling up the production of cmpdA. Thank Tianlun Zhou for providing HBsAg-encoded adenoviruses.
All the authors listed in the manuscript are either current or previous employees of Arbutus Biopharma. Amy C.H. Lee, Fang Guo, Andrew S. Kondratowicz, Angela Miller, Lauren D. Bailey, and Rene Rijnbrand were Arbutus employees at the time of data generation.

FL and MG conceived and designed the research. FL, ACHL, FG, ASK, HMS, AM, LB, XW, SC, SGK and AGC performed the research. All authors analyzed the data. FL, ACHL and MG wrote the paper, and AGC, DG, BDD, RR (designed plasmids), AL and MJS revised the paper.
References


**Figure legends**

**Fig. 1.** AB-452 interferes with multiple steps of HBV life cycle by reducing HBV RNA. (A) HepG2.2.15 cells were treated with DMSO, ETV (1 μM), GLS-4 (1 μM), cmpdA (1 μM), or AB-452 (70 nM) for 6 days. Intracellular HBV pgRNA (3.5 kb) and sRNA (2.4 kb and 2.1 kb) were analyzed by Northern blot. Intracellular core protein was detected by Western blot. Intracellular HBV capsids were detected by the capsid particle gel assay based on agarose gel electrophoresis. Intracellular HBV DNA was detected by Southern blot. Encapsidated pgRNA (capsid pgRNA) was quantitated by qRT-PCR and expressed as percentage of untreated controls (DMSO). Ribosomal RNA (18S and 28S) and β-actin serve as loading controls for analysis of HBV RNA and core protein, respectively. (B) Levels of intracellular pgRNA and sRNA in HepG2.2.15 cells treated with increasing concentrations of AB-452 (0.14 to 1000 nM) for 48 h. Percentages of pgRNA and sRNA were determined by normalizing to untreated controls. (C) Time course analysis of HBV RNAs from cells treated with and without 70 nM AB-452. Total intracellular RNA was extracted from cells harvested at 4, 8, 12, 24 and 48 h time points post-treatment. HBV pgRNA and sRNA were analyzed by Northern blotting. Percentages of pgRNA and sRNA were determined by normalizing to untreated controls at each time point.

**Fig. 2.** Antiviral activity of AB-452 in an AAV-HBV-transduced mouse model. AAV-HBV transduced animals received AB-452 at 0.1, 0.3, 1 mg/kg or vehicle orally twice daily for 7 days. Effect of AB-452 on the production of (A) serum HBsAg, (B) serum HBV DNA, (C) intrahepatic HBsAg, (D) intrahepatic total HBV RNA and (E) intrahepatic 3.5 kb HBV pgRNA on day 7 post-treatment. (F) Effect of AB-452 on body weight through the 7-day treatment. Data represent group mean (n = 5) ± SD. Statistically significant difference (p<0.05) from vehicle
control was determined using one-way ANOVA (Dunn’s multiple comparisons test) and is denoted by an asterisk (*).

**Fig. 3. AB-452 promotes HBV RNA degradation through inhibiting PAPD5 and PAPD7 enzymatic activities and blockage of guanosine incorporation into viral RNA poly(A) tails.**

HepAD38 cells were cultured in the absence of Tet to promote HBV transcription, and in the presence of the capsid inhibitor GLS4 to prevent pgRNA encapsidation for 6 days. On day 7, Tet was added back with media containing either DMSO or AB-452 (70 nM), and cells were harvested either before treatment (time 0 h) or at 2, 4, 8, and 16 h post-treatment. (A) HBV mRNA was analyzed by Northern blot, with ribosomal RNAs as loading control. The positions of HBV pgRNA (3.5 kb) and subgenomic RNAs (2.4 kb and 2.1 kb) are indicated. (B) Decay rate of HBV pgRNA in the presence or absence of AB-452, with calculated T½ labeled for each treatment (n = 2). (C) Effect of AB-452, RG7834, and AB-169451 on the enzymatic activity of PAPD5 and PAPD7. Half-maximal inhibition (IC₅₀) for the three compounds were determined based on the dose response curves and reported in the table below each figure. Mean values (± standard derivations) are presented from duplicate experiments. (D to G) Analysis of HBV poly(A) tails from HepAD38 cells treated with or without AB-452 (70 nM). (D) Cells were incubated with AB-452 for 4 h prior to isolation of intracellular RNA. Total RNA was tagged with a poly-G/I tail at the 3’ end and reverse transcribed by poly-G/I specific primer. Both HBV and β-actin mRNA poly(A) tails were specifically amplified using one gene specific primer and the universal primer that anneals to the G/I tail. Gene specific PCR (GSP) was used as loading control. The poly(A) tail length of β-actin mRNA served as the negative control. (E) HBV RNA poly(A) tails were subjected to next generation sequencing for tail lengths analysis. (F) Frequency of non-A modifications (C, cytidylation, G/GG, guanylation; U, uridylation) was
analyzed within the HBV poly(A) tails. Guanosines were often clustered; tandem GG analysis was made to reflect this observation. (G) Determination of G nucleotide frequency located within the HBV poly(A) tails. The numbers of guanylated tail reads and total tail reads obtained from the NSG sequencing were indicated under each sample. The frequency of guanylated tails of viral mRNAs was calculated for poly(A) tail length of ≥ 10 nt.

Fig. 4. SLα sequence within the HBV PREα sub-element is essential for RNA stability and AB-452 activity. Evaluation of AB-452 against HBsAg and Gaussia luciferase (Gluc) encoded plasmids containing either wildtype HBV PREα (H133, H133_Gluc, or Gluc_dHBx) or SLα inversion-derived mutant (Gluc_rcSLα). (A) Schematic representation of H133 (wild type) and the Gluc-derived constructs. Huh-7 cells were transfected with each of these plasmids and susceptibility to AB-452 was evaluated by monitoring HBsAg or Gluc activity. The inactive enantiomer ARB-169451 was included as a negative control. Mean values (± standard derivations) are presented from triplicate experiments. (B) The HBx deletion variants containing either the WT SLα (Gluc_dHBx) or the inverted SLα (Gluc_rcSLα) sequence were transfected into Huh-7 cells, which were treated with DMSO, AB-452 (100 nM) or ARB-169451 (100 nM) for 5 days. Effect of AB-452 against HBV RNA was analyzed by Northern blot with ribosomal RNAs as loading control. (C) Kinetics of HBV RNA degradation in cells transfected with Gluc_dHBx or Gluc_rcSLα. Transcription proceeded for 2 days prior to the addition of tetracycline with or without AB-452 (100 nM). Cells were harvested before treatment (time 0 h) and at 4, 8, and 16 h post treatment. HBV RNA decays were monitored by qRT-PCR assay, with calculated decay T_{1/2} labeled under each treatment (n = 3). Data and error bars represent mean % HBV RNA and standard deviations relative to time 0 of each condition from at least three independent experiments.
Fig. 5. SLα determines AB-452 sensitivity and HBV RNA poly(A) tail integrity. Huh-7 cells were transfected with H133 (wild type), H133_dLa or H133_dSLα plasmids and treated with AB-452 for 5 days. (A) Schematic representation of H133 (wild type), H133_dSLα (SLα deleted, nt 1294-1322), and H133_dLa mutants (La binding site deleted, nt 1271-1294). The activities of AB-452 and ARB-169451 against HBsAg production were determined and EC$_{50}$ values summarized. Mean values (± standard derivations) are determined from triplicate experiments. (B-C) H133 and H133_dSLα were transfected into Huh-7 cells and treated with ETV (1 μM), AB-452 (0.1 μM), or ARB-169451 (0.1 μM) for 5 days. Intracellular HBV sRNA and secreted HBsAg were analyzed by Northern blot or ELISA assay, respectively. Levels of HBV RNA and HBsAg were normalized to those from cells treated with DMSO. Data represent average values ± standard deviations from at least three independent experiments. (D) Kinetics of HBV RNA degradation in Huh-7 cells transfected with H133 and H133_dSLα plasmids. Transcription proceeded for 2 days prior to the addition of tetracycline with or without AB-452 (100 nM). Cells were harvested before treatment (time 0 h) and at 4, 8, and 16 h post treatment. HBV RNA decays were monitored by qRT-PCR assay, with calculated decay T$_{1/2}$ labeled (n = 3). (E) HBV RNA poly(A) tails were sequenced and analyzed for frequency of tail lengths from cells transfected with H133 or H133_dSLα treated with or without AB-452 (100 nM). (F) Frequency of non-A modifications (G, guanylation; U, uridylation; C, cytidylation) within the poly(A) tail of HBV mRNAs were analyzed. Tandem GG analysis was performed to analyze clustered guanosines. (G) The frequency of guanylated tails of viral mRNAs was calculated with a poly(A) tail length of ≥ 10 nt. The numbers of guanylated tail reads and total tail reads obtained from the NSG sequencing were indicated under each sample.
Fig. 6. Confirmation of the PAPD5, PAPD7 and ZCCHC14 CRISPR-Cas9 mediated knockouts in the representative clones. (A) Insertion-deletion mutations (INDELs) are annotated with the bps of insertion (+) or deletion (-) on alleles, in which “/” is used to separate INDELs among different alleles. The regions targeted by gRNAs are highlighted in black. INDELs are detected by sequencing trace analysis with CAT tool (CRISPR analysis tool). (B) PAPD5 and ZCCHC14 were detected with the indicated antibodies in the Western blots. The * asterisk indicates a cross-reacting band.

Fig. 7. Knockout of PAPD5/7 and ZCCHC14 destabilizes and desensitizes HBV RNA to AB-452. PAPD5, PAPD7, ZCCHC14, or both PAPD5 and PAPD7 were knocked out in HepG2-NTCP cells by CRISPR-Cas9 gene editing. (A) Cell proliferation of PAPD5, PAPD7 and Z14 KO or WT clones was analyzed. Percentage cell growth relative to the WT parent HepG2-NTCP cells was determined for each tested clone. (B) Adenoviruses carrying HBsAg coding sequence were used to transduce either WT, PAPD5, PAPD7 or ZCCHC14 KO cell clones, extracellular HBsAg was measured on day 5 post transduction. Percentage of HBsAg relative to the WT parent HepG2-NTCP cells was determined. (C) HepG2-NTCP cells were infected with HBV inoculum. HBsAg, HBeAg and HBV DNA were measured on day 9 post infection in the KO clones and normalized to the WT parent cells. (D) AB-452 activity of HBsAg inhibition was evaluated in the PAPD5/7 single or double KO and ZCCHC14 KO clones infected with adenoviruses. (E-G) AB-452 antiviral activity was evaluated in HBV infected HepG2-NTCP clones. (H) HBV sRNA was analyzed by Northern Blot in the PAPD5/7 single or double KO and ZCCHC14 KO cell clones treated with and without AB-452 for 5 days. (I) HBV mRNA poly(A) tails were amplified, and the obtained amplicons were resolved in a 2 % agarose gel. Gene specific PCR (GSP) of HBV RNA was used as loading control. (J) HBV sRNA poly(A) tails
were sequenced for the analysis of tail lengths and (K) guanylation incorporation frequency. The mean values and standard derivations were plotted at least from duplicate experiments for the Figs. A-G. *P < 0.05, **P < 0.01, ***P < 0.001 compared with three WT cell lines or DMSO treated cells. P values were calculated by unpaired two-tailed t-test.

**Fig. 8.** A proposed model illustrating the interplay between HBV cis-element SLα and the host factors PAPD5 and PAPD7 in maintaining HBV RNA integrity and stability.
Table 1. *In vitro* anti-HBV effect of AB-452 and ARB-169451

<table>
<thead>
<tr>
<th>HBV replication cells</th>
<th>HBV biomarkers</th>
<th>AB-452 EC(_{50}) (nM)*</th>
<th>ARB-169451 EC(_{50}) (nM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2.2.15</td>
<td>HBsAg</td>
<td>1.4 ± 0.2</td>
<td>2,233 ± 1,185</td>
</tr>
<tr>
<td></td>
<td>HBeAg</td>
<td>2.8 ± 2.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HBV DNA</td>
<td>0.28 ± 0.01</td>
<td>-</td>
</tr>
<tr>
<td>HepG2/NTCP</td>
<td>HBsAg</td>
<td>6.8 ± 1.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HBeAg</td>
<td>4.0 ± 2.3</td>
<td>-</td>
</tr>
<tr>
<td>PHH</td>
<td>HBsAg</td>
<td>3.0 ± 2.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HBeAg</td>
<td>3.7 ± 1.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HBV DNA</td>
<td>4.2 ± 3.8</td>
<td>-</td>
</tr>
<tr>
<td>PLC/PRF/5</td>
<td>HBsAg</td>
<td>2.3 ± 0.36</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

*Mean EC\(_{50}\) ± standard deviations were determined from at least three independent experiments.
Table 2. Antiviral selectivity of AB-452 against a panel of DNA and RNA viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell Line</th>
<th>Measurement</th>
<th>AB-452 EC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV</td>
<td>HepG2-2.2.15</td>
<td>HBsAg</td>
<td>0.0012</td>
</tr>
<tr>
<td>HSV-1 / HSV-2</td>
<td>Vero</td>
<td>CPE*</td>
<td>&gt;30</td>
</tr>
<tr>
<td>HCMV</td>
<td>MRC-5</td>
<td>CPE</td>
<td>&gt;30</td>
</tr>
<tr>
<td>HIV</td>
<td>CEM-SS</td>
<td>CPE</td>
<td>&gt;30</td>
</tr>
<tr>
<td>HCV</td>
<td>Huh-7</td>
<td>Luciferase</td>
<td>&gt;30</td>
</tr>
<tr>
<td>DENV2</td>
<td>BHK21</td>
<td>CPE</td>
<td>&gt;30</td>
</tr>
<tr>
<td>WNV</td>
<td>Vero76</td>
<td>CPE</td>
<td>&gt;30</td>
</tr>
<tr>
<td>RSV</td>
<td>HEp2</td>
<td>CPE</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Human Rhinovirus</td>
<td>H1-Hela</td>
<td>CPE</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Influenza</td>
<td>MDBK</td>
<td>CPE</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

*CPE, the cytopathic effect
### Table 3. Cytotoxicity of AB-452 against cells derived from various tissues

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Tissue Origin</th>
<th>AB-452 CC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2-2.2.15</td>
<td>Liver</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Huh-luc/neo-ET</td>
<td>Liver</td>
<td>&gt;30</td>
</tr>
<tr>
<td>CEM-SS</td>
<td>T lymphoblast</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Vero</td>
<td>Kidney</td>
<td>&gt;30</td>
</tr>
<tr>
<td>MDBK</td>
<td>Kidney</td>
<td>&gt;30</td>
</tr>
<tr>
<td>MRC5</td>
<td>Lung fibroblasts</td>
<td>&gt;30</td>
</tr>
<tr>
<td>HEp2</td>
<td>Human epithelial cells</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Hela</td>
<td>Human cervical cancer cells</td>
<td>&gt;30</td>
</tr>
<tr>
<td>BHK21</td>
<td>Kidney</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>
Table 4. Anti-HBV effect of AB-452 and RG7834 in PAPD5 or PAPD7 KO cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Compound</th>
<th>Adeno infection</th>
<th>HBV infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EC$_{50}$ (nM)</td>
<td>#FC vs WT</td>
</tr>
<tr>
<td>WT (parent)</td>
<td>AB-452</td>
<td>9.0 ± 4.6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>RG7834</td>
<td>11.8 ± 9.6</td>
<td>1</td>
</tr>
<tr>
<td>P5_KO (T3-4)</td>
<td>AB-452</td>
<td>71.6 ± 31.2</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>RG7834</td>
<td>126 ± 74.7</td>
<td>10.7</td>
</tr>
<tr>
<td>P5_KO (T3-15)</td>
<td>AB-452</td>
<td>56.0 ± 34.0</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>RG7834</td>
<td>85.7 ± 59.8</td>
<td>7.3</td>
</tr>
<tr>
<td>P7_KO (T2-8)</td>
<td>AB-452</td>
<td>10.0 ± 1.0</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>RG7834</td>
<td>23.7 ± 15.2</td>
<td>2.0</td>
</tr>
<tr>
<td>P7_KO (T2-22)</td>
<td>AB-452</td>
<td>7.1 ± 1.3</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>RG7834</td>
<td>10.8 ± 2.1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Mean EC$_{50}$ ± standard deviations were determined from three independent experiments. #FC: fold change.
Fig. 1.

A

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>ETV</th>
<th>GLS-4</th>
<th>cmpdA</th>
<th>AB-452</th>
</tr>
</thead>
<tbody>
<tr>
<td>(nM)</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.5 kb</td>
<td>2.4 kb</td>
<td>2.1 kb</td>
<td>28S</td>
<td>18S</td>
</tr>
<tr>
<td></td>
<td>core</td>
<td>β-actin</td>
<td>capsid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>104</td>
<td>96</td>
<td>182</td>
<td>168</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Capsid DNA</td>
<td>rcDNA</td>
<td>ssDNA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>DMSO</th>
<th>AB-452</th>
</tr>
</thead>
<tbody>
<tr>
<td>(nM)</td>
<td>0</td>
<td>1,000</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.5 kb</td>
<td>2.4 kb</td>
<td>2.1 kb</td>
</tr>
<tr>
<td></td>
<td>28S</td>
<td>18S</td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th></th>
<th>4h</th>
<th>8h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>*+</td>
<td>*+</td>
<td>*+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>*+</td>
<td>*+</td>
<td>*+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>*+</td>
<td>*+</td>
<td>*+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3.5 kb</td>
<td>2.4 kb</td>
<td>2.1 kb</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>28S</td>
<td>18S</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

pgRNA (%) 
sRNA (%)
Fig. 2

A. Serum HBsAg

B. Serum HBV DNA

C. Liver HBsAg

D. Liver Total HBV RNA

E. Liver 3.5 kb HBV RNA

F. Body Weight

Vehicle, 0.1 mg/kg, 0.3 mg/kg, 1 mg/kg

* statistically significant difference compared to Vehicle
Fig. 3.

A

0 h 2 h 4 h 8 h 16 h

DMSO AB-452

2.4 kb 2.1 kb 3.5 kb

28S 18S

B

Decay rate (%)

T_{1/2} = 2.4 h  T_{1/2} = 4.5 h

DMSO AB-452

C

<table>
<thead>
<tr>
<th></th>
<th>AB-452</th>
<th>ARB-169451</th>
<th>RG7834</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC_{50} (nM)</td>
<td>94 ± 11</td>
<td>27,002 ± 928</td>
<td>164 ± 3.6</td>
</tr>
</tbody>
</table>

D

IC_{50} (nM) | 498 ± 51 | >50,000 | 1,093 ± 52

E

Frequency, %

Poly(A) tail length (bp)

F

Non-A analysis, %

Poly(A) tail length (bp)

G

Guanylated tails

Frequency, %

DMSO AB-452

G reads Total reads

38,579 42,451
Fig. 4.

<table>
<thead>
<tr>
<th>AB-452 (EC50 ± SD, nM)</th>
<th>ARB-169451 (EC50 ± SD, nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H133</td>
<td>H133_Gluc</td>
</tr>
<tr>
<td>2.5 ± 0.6</td>
<td>10 ± 0.6</td>
</tr>
<tr>
<td>Gluc_dHBx</td>
<td>Gluc_dHBx</td>
</tr>
<tr>
<td>4.2 ± 0.8</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Gluc_rcSLa</td>
<td>Gluc_rcSLa</td>
</tr>
<tr>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>HBV RNA, % 0 h</td>
<td></td>
</tr>
</tbody>
</table>

A

B

C

DMSO

AB-452

ARB-169451
**Fig. 5.**

<table>
<thead>
<tr>
<th></th>
<th>AB-452 (EC_{50} ± SD, nM)</th>
<th>ARB-169451 (EC_{50}, nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H133</td>
<td>2.5 ± 0.61</td>
<td></td>
</tr>
<tr>
<td>H133_dLa</td>
<td>4.3 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>H133_dSLα</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>H133_dLa</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>H133_dSLα</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

**C**

**D**

**E**

**G**

**HBSa, % DMSO**

**HBV RNA, % 0 h**

**HBsAg, % DMSO**

**Non-A analysis, %**

**n(A) tail length (bp)**

**Guanylated tails**

**Total reads**

**DMO RNA AB-452**

**DMO RNA AB-452**

**DMO RNA AB-452**

**DMO RNA AB-452**
Fig. 6.

A

WT (T3-1)

P5_KO (T3-4)
-5/-1/+1 bp

P7_KO (T2-22), -2 bp

P5/7_DKO (T3-25)
-2/+1 bp

Z14_KO (T1-11)
-1/+1 bp

B

WT (T3-1)
P5_KO (T3-4)
P7_KO (T2-22), -2 bp
P5/7_DKO (T3-25)
Z14_KO

PAPD5

ZCCHC14

β-actin
Fig. 7.
Fig. 8.

Working Model

HBV mRNAs

PAPD5

130-140 nt

PAPD7

Poly(A) nuclease(s)

SLɑ ZCCHC14     PAPD5 PAPD7

Working Model

PAPD5 KO

~ 90 nt

AB-452

~ 60 nt

Degradation