In Vitro and *in Vivo* Profiling of Orally Bioavailable Small Molecules Inhibiting Hepatitis B Virus by Mimicking Interferon Alpha

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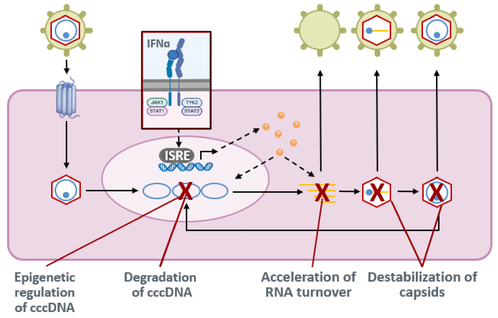
BACKGROUND

- Chronic hepatitis B virus infection (cHBV) is a significant global health problem
  - Worldwide, an estimated 296 million people have cHBV, resulting in about 820,000 deaths each year, mostly due to cirrhosis and hepatocellular carcinoma[1]
- Nucleos(t)ide reverse transcriptase inhibitors (NrtIs) reduce HBV DNA, but demonstrate a low rate of functional cure (hepatitis B surface antigen clearance), necessitating lifelong administration[2,3]
- Interferon-alpha (IFNα) interferes with multiple steps of the viral life cycle via activation of interferon-stimulated genes (ISGs; **Figure 1**)[4,5]
  - Pegylated (PEG)-IFNα has immunomodulatory and antiviral activities, leading to functional cure in some patients[6,7] and at a higher rate than for NrtIs.[8,9] However, poor tolerability of IFNα limits its use in the clinic[10]
- Orally bioavailable, liver-targeted IFNα-like small molecules with improved tolerability have the potential to increase the proportion of patients achieving functional cure through this mechanism

Figure 1. HBV Replication Cycle and IFNα



Orange circles: ISG effector proteins.
cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; IFNα, interferon-alpha; ISG, interferon-stimulated genes; ISRE, interferon-stimulated response element; JAK, Janus kinase; STAT, signal transducer and activator of transcription; TYK, tyrosine kinase.

OBJECTIVE

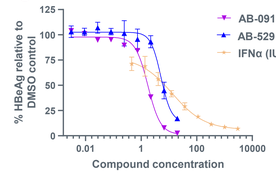
- To characterize a novel class of orally bioavailable small molecules that inhibit HBV through activation of IFN signaling

METHODS

- HBV infection of primary human hepatocytes (PHHs):
  - PHHs were infected with HBV at 300 viral genome equivalents/cell and treated with IFNα receptor (IFNAR) agonists at 3 hours post-infection. The next day, cells were washed, and fresh medium with IFNAR agonists or IFNα was added. Cell culture medium was harvested at 8 days post-infection, and secreted hepatitis B e antigen (HBeAg) was measured via an enzyme-linked immunosorbent assay (ELISA)
- Hepatitis C virus (HCV) replicon cells (NanoLuc luciferase reporter assay):
  - Huh-7 cells stably replicating HCV were treated with agonists for 2 days post-plating. Luciferase activity was measured via Nano-Glo luciferase assay
- Determination of signal transducer and activator of transcription (STAT) phosphorylation:
  - Huh-7 HCV replicon cells were treated with dimethyl sulfoxide (DMSO), IFNAR agonist, or IFNα for 30 minutes. STAT phosphorylation was assessed by Western blot or ELISA
- ISG induction and cytokine secretion:
  - *In vitro*: a) PHHs were treated with DMSO, IFNAR agonist, or IFNα. Cells were lysed, RNA isolated for hybridization to an nCounter Host Response version 1.1 Panel and analyzed using the nanoString nCounter Analysis System. b) Human peripheral blood mononuclear cells (PBMCs) were treated with medium only, DMSO, IFNAR agonist, or IFNα for 24 hours. Cytokine secretion was analyzed in cell supernatants by Luminex
  - *In vivo* (mice): RNA was extracted from liver and PBMCs of mice treated with IFNAR agonist or murine IFNα (mIFNα). Real-time quantitative polymerase chain reaction (qRT-PCR) analysis was conducted analyzing 22 selected mouse ISGs
- Pharmacokinetic (PK) and pharmacodynamic (PD) studies:
  - PK parameters in plasma were assessed at given time points
  - PD parameters in PBMCs and liver biopsies (non-human primates [NHPs]) were assessed at given time points. Total RNA was isolated and analyzed by qRT-PCR or genome-wide RNA sequencing of mouse and NHP samples, respectively

RESULTS

Figure 2. AB-091 and AB-529 Inhibit HBV Infection in PHHs

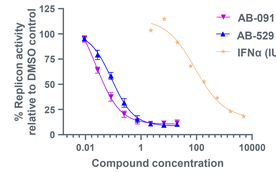


EC_{50} (μM)		EC_{50} (IU/mL)
AB-091	AB-529	IFNα
1.8	5.2	13.2

DMSO, dimethyl sulfoxide; EC_{50}, half-maximal effective concentration; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IFNα, interferon-alpha; PHHs, primary human hepatocytes.

- Novel IFNAR agonists (AB-091 and AB-529) inhibit HBV as measured by inhibition of HBeAg secretion (**Figure 2**)

Figure 3. AB-091 and AB-529 Inhibit HCV RNA Replication in Huh-7 HCV Replicon Cells

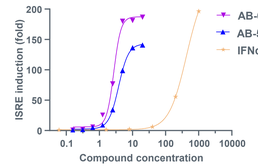


EC_{50} (μM)		EC_{50} (IU/mL)
AB-091	AB-529	IFNα
0.02	0.09	90.0

DMSO, dimethyl sulfoxide; EC_{50}, half-maximal effective concentration; HCV, hepatitis C virus; IFNα, interferon-alpha.

- IFNAR agonists efficiently inhibit HCV replication with half-maximal effective concentration (EC_{50}) values between approximately 0.02 and 0.09 μM (**Figure 3**)

Figure 4. AB-091 and AB-529 Induce ISRE Reporter Activity in HEK293 Cells



EC_{50} (μM)		EC_{50} (IU/mL)
AB-091	AB-529	IFNα
2.7	3.8	425.7

EC_{50}, half-maximal effective concentration; HEK, human embryonic kidney; ISRE, interferon-stimulated response element.

- IFNAR agonists induce interferon-sensitive response element (ISRE) reporter activity in human embryonic kidney (HEK)293-ISRE reporter cells (**Figure 4**)
- EC_{50} values of ISRE reporter activity range from approximately 3 to 4 μM (**Figure 4**)
  - The IFNAR agonists show differences in maximum stimulation

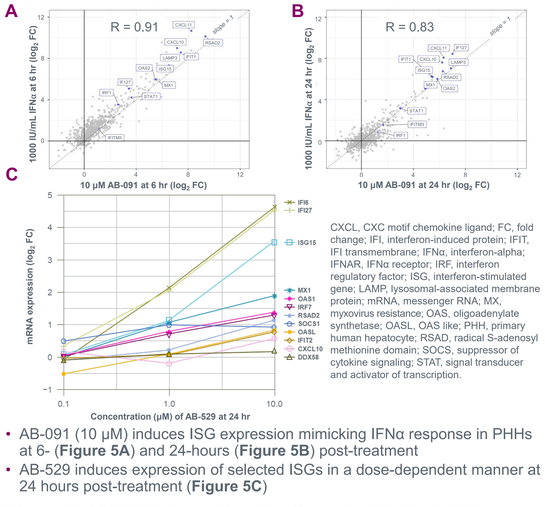
Table 1. AB-091 Mimics IFNα STAT Phosphorylation in Huh-7 HCV Replicon Cells

STAT	Treatment		
	DMSO	IFNα	AB-091
p-STAT1	–	+	+
p-STAT2	–	+	+
p-STAT3	–	+	+
p-STAT4	–	–	–
p-STAT5	–	–	–
p-STAT6[11,12]	–	–	+

DMSO, dimethyl sulfoxide; HCV, hepatitis C virus; p, phosphorylated; STAT, signal transducer and activator of transcription.

- AB-091 induces phosphorylation of STATs comparable to IFNα (**Table 1**)
  - AB-091 (20 μM) induces phosphorylation of STAT1, 2, 3, and 6, but not STAT4 and 5
  - IFNα (1000 IU/mL) induces phosphorylation of STAT1, 2, and 3, but not STAT4, 5, and 6
  - Previous studies have shown that IFNα-2a treatment can also induce phosphorylation of STAT6 in naïve Huh-7 cells[11], and IFNα-14 treatment resulted in phosphorylated STAT6 in PHHs[12]

Figure 5. IFNAR Agonists Induce ISGs Mimicking IFNα in PHHs

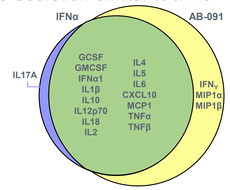


CXCL, CXC motif chemokine ligand; FC, fold change; IFI, interferon-induced protein; IFIT, IFI transmembrane; IFNα, interferon-alpha; IFNAR, IFNα receptor; IRF, interferon regulatory factor; ISG, interferon-stimulated gene; LAMP, lysosomal-associated membrane protein; mRNA, messenger RNA; MX, myxovirus resistance; OAS, oligoadenylate synthetase; OASL, OAS like; PHH, primary human hepatocyte; RSAD, radical S-adenosyl methionine domain; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription.

- AB-091 (10 μM) induces ISG expression mimicking IFNα response in PHHs at 6- (**Figure 5A**) and 24-hours (**Figure 5B**) post-treatment
- AB-529 induces expression of selected ISGs in a dose-dependent manner at 24 hours post-treatment (**Figure 5C**)

Figure 6. AB-091 Induces Cytokine Secretion Similar to IFNα Treatment in Human PBMCs

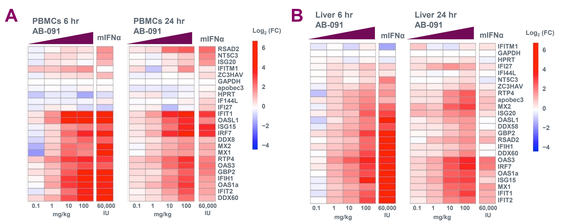
The Venn diagram shows the names of cytokines with significant increases in secretion compared to medium (for IFNα) or DMSO (for AB-091).



CXCL, CXC motif chemokine ligand; DMSO, dimethyl sulfoxide; GCSF, granulocyte colony-stimulating factor; GMCSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PMBC, peripheral blood mononuclear cells; TNF, tumor necrosis factor.

- AB-091 (20 μM) and IFNα (1000 IU/mL) induce cytokine secretion in human PBMCs, showing activation of immune responses *in vitro* (**Figure 6**)
- Cytokines exclusively secreted upon IFNα or AB-091 treatment are shown in the blue and yellow circles, respectively. Cytokines secreted upon treatment with IFNα and AB-091 are shown in the green intersection

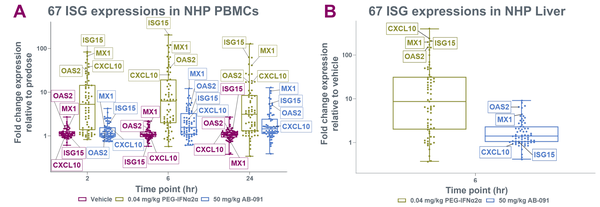
Figure 7. AB-091 Oral Dosing in Mice Induces Selected ISGs in PBMCs and Liver



apobec, apolipoprotein B editing complex; CXCL, CXC motif chemokine ligand; DDX, DExD/H-box helicase; FC, fold change; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBP, guanylate-binding protein; HPRT, hypoxanthine phosphoribosyltransferase; IFI, interferon-induced protein; IFIH, IFI helicase; IFIT, IFI transmembrane; IFNα, interferon-alpha; IFNAR, IFNα receptor; IRF, interferon regulatory factor; ISG, interferon-stimulated gene; LAMP, lysosomal-associated membrane protein; mIFNα, murine IFNα; MX, myxovirus resistance; NT5C3, cytosolic 5′-nucleotidase 3; OAS, oligoadenylate synthetase; OASL, OAS-like; PBMC, peripheral blood mononuclear cell; RSAD, radical S-adenosyl methionine domain; RTP, receptor transporter protein; STAT, signal transducer and activator of transcription; ZC3HAV, zinc finger CCCH-type antiviral protein.

- AB-091 mimics IFN by inducing ISGs comparably to mIFNα in mouse PMBCs (**Figure 7A**) and liver (**Figure 7B**)
- ISG induction is observed in a dose dependent manner (0.1 to 100 mg/kg; **Figure 7A and B**)
- PK information is shown in **Figure 9A**

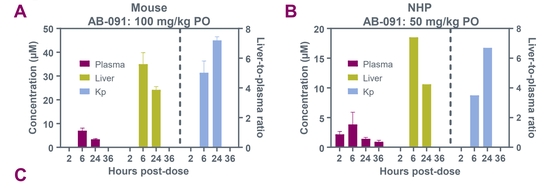
Figure 8. AB-091 Oral Dosing in NHPs Induces Selected ISGs in PBMCs and Liver



CXCL, CXC motif chemokine ligand; ISG, interferon-stimulated gene; NHP, non-human primate; OAS, oligoadenylate synthetase; PBMC, peripheral blood mononuclear cell; PEG-IFNα2a, pegylated interferon-alpha 2.

- AB-091(50 mg/kg) administered orally to NHPs induces ISG expression at 6- and 24-hours post-dose in PBMCs **(Figure 8A)** and at 6-hours post-dose in liver **(Figure 8B)**
- PK information is shown in **Figure 9B**
- The fold induction of e.g., the myxovirus resistance 1 (MX1) gene by AB-091 is comparable to MX1 induction in PBMCs and livers of patients chronically infected with HCV who are treated with IFNα[13]
- A dose of 0.04 mg/kg PEG-IFNα, which is 4 times greater than the dose used clinically, induces ISGs at all given time points to a greater extent than AB-091

Figure 9. AB-091 Pharmacokinetics in Mice and NHPs



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Mouse PK Parameters	AB-091
$t_{1/2}$ (hours)	14
Liver-to-plasma ratio (Kp)	5[a]
%F	75

[a]Measured at 6-hours post–AB-091 administration.
F, bioavailability; Kp, partition coefficient; NHP, non-human primate; PK, pharmacokinetics; PO, by mouth; $t_{1/2}$, terminal half-life.

- The PK profiles of AB-091 in mice (**Figure 9A**) and NHPs (**Figure 9B**) demonstrate that:
  - At 6- and 24-hours post-dose, AB-091 liver-to-plasma ratios are in between 5- to 7-fold in mice and 4- to 6-fold in NHPs
  - AB-091 has a terminal half-life of 14 hours, a liver-to-plasma ratio of 5 in the liver, and oral bioavailability of 75% in mice (**Figure 9C**)

CONCLUSIONS

- Novel small molecule IFNAR agonists inhibit HBV and HCV *in vitro*
- The IFNAR agonists tested in this study closely mimic IFNα by activating *in vitro* ISRE signaling, STAT phosphorylation, cytokine secretion in human PBMCs, and ISG induction in human liver cells. *In vivo*, ISG induction was also observed in the liver and PBMCs of NHPs and mice
- PK data demonstrate that the selected agonist has good liver exposure and oral absorption in mice and NHPs
- Lead optimization of multiple IFNAR agonists is in progress

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