Preclinical Characterization of a Novel Class of Highly Potent Small Molecule Hepatitis B and D Virus Entry Inhibitors

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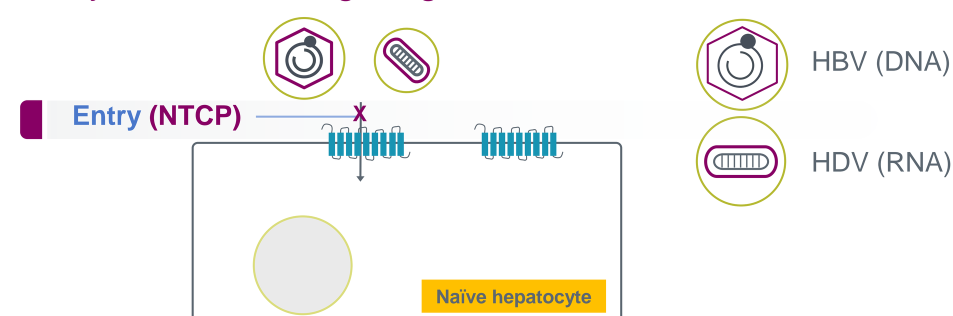
BACKGROUND

- Chronic hepatitis B virus (HBV) infection and hepatitis D virus (HDV) infection affect approximately 296 million and 12 million patients worldwide, respectively[1,2]
- HDV is a satellite virus that requires the hepatitis B surface antigen to infect hepatocytes[3,4]
- HBV patients with chronic HDV infection have faster disease progression and a greater risk of developing liver-related outcomes compared to patients without HDV[5–7]
- Entry inhibitors prevent the establishment of a productive infection (**Figure 1**)
- The daily injectable HBV/HDV entry inhibitor bulevirtide (Myrcludex B) targets the host receptor sodium taurocholate cotransporting polypeptide (NTCP), preventing HBV and HDV from entering hepatocytes[8,9]
- Bulevirtide has been shown to lower HDV serum RNA levels and normalize alanine aminotransferase levels in coinfected individuals when combined with tenofovir as well as lower hepatitis B surface antigen levels when combined with pegylated interferon-alpha.[10,11] Bulevirtide received conditional marketing authorization from the European Medicines Agency for the treatment of chronic HDV in patients with compensated liver disease[12]
- There is a need for potent orally-administered entry inhibitors to improve the treatment of HDV patients

Figure 1. Entry Inhibitors Targeting NTCP Block the Infection of Hepatocytes by HBV and HDV



HBV, hepatitis B virus; HDV, hepatitis D virus; NTCP, sodium taurocholate cotransporting polypeptide.

OBJECTIVE

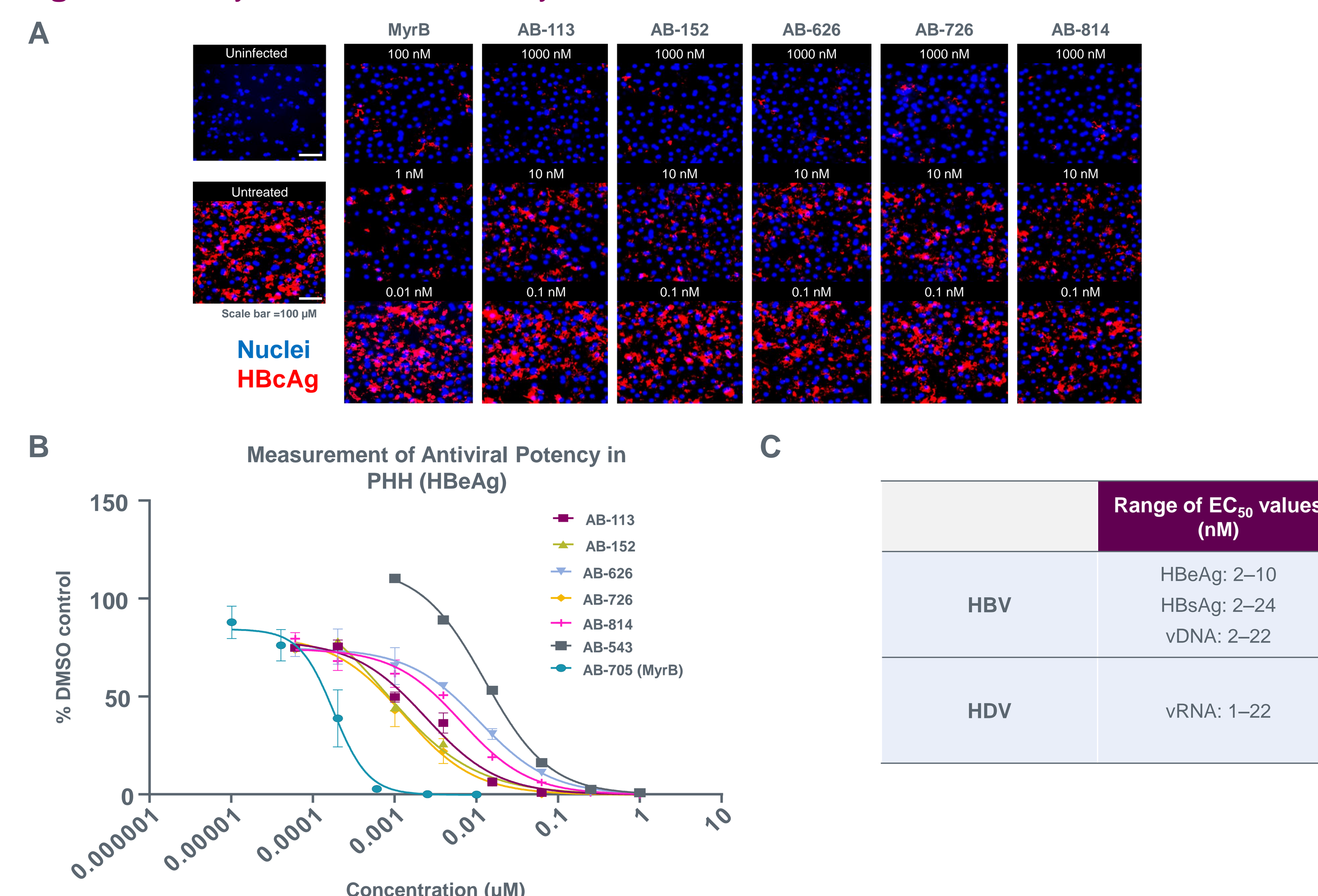
- To characterize the potency and properties of 6 structurally-differentiated orally-bioavailable small molecule HBV/HDV entry inhibitors

METHODS

- **HBV and HDV infection:**
  - **Hepatitis B e antigen (HBeAg):**
    - Primary human hepatocytes (PHHs) were infected with HBV at a multiplicity of infection (MOI) of 300 viral genome equivalents (vge)/cell and concomitantly treated with compounds. The next day, cells were washed and fresh media without inhibitors was added. Cell culture media was harvested at 8 days post-infection (dpi) and secreted HBeAg was measured via an enzyme-linked immunosorbent assay (ELISA)
    - For time-of-addition experiments, HepG2-NTCP cells were treated with compounds for 16 hours during or after infection at an MOI of 50 vge/cell. Cell culture media was harvested at 5 dpi and an HBeAg ELISA was performed
    - HepG2-NTCP cells were infected with HBV (50 vge/cell) and cotreated with inhibitors. At 5 dpi, viral supernatants were harvested and an HBeAg ELISA was performed
  - **HBV DNA:**
    - PHHs were infected as described above. At 8 dpi, cells were lysed, and total HBV DNA was quantified using a branched DNA assay probing against minus-strand HBV DNA
  - **Hepatitis B core antigen (HBcAg) immunofluorescence analysis (IFA):**
    - PHHs were pretreated with compounds 4 hours prior to infection with HBV at MOI of 10,000 vge/cell. The next day, cells were washed and fresh media without inhibitors was added. At 7 dpi, cells were processed for immunofluorescence staining of HBcAg using a custom rabbit anti-HBc polyclonal antibody
  - **HDV RNA:**
    - PHHs were pretreated with inhibitors for 1 hour followed by HDV inoculation (5 vge/cell). At 8 dpi, total cellular RNA was harvested, followed by HDV-specific reverse transcriptase-quantitative polymerase chain reaction. Cytotoxicity was assessed by a commercial cell counting kit
  - **Serum shift assay:**
    - HepG2-NTCP cells were infected with HBV in the presence of 2% fetal bovine serum (FBS) and physiologically relevant levels of human serum albumin (HSA; 45 mg/mL) and alpha1-acid glycoprotein (AAG; 0.7 mg/mL) and then were compared to a standard infection carried out in media with 2% FBS alone. To compensate for viral particles that became bound by HSA and AAG, the MOI was shifted to 500 vge/cell in comparison to the standard infection at 50 vge/cell. Half-maximal effective concentration (EC_{50}) values were generated by quantifying the secretion of HBeAg into culture supernatants by ELISA at 5 dpi
- **Bile acid transporter:**
  - HEK-293T cells expressing human NTCP were preincubated for 30 minutes with entry inhibitors, then a 2-minute incubation with a fluorescent bile acid salt derivative. Fluorescent bile acid uptake was measured by flow cytometry
- **PreS1 binding competition:**
  - HEK-293T cells stably expressing human NTCP were coincubated with myristoylated preS1-Alexa-594 peptide and inhibitors for 10 minutes. Binding of fluorescent peptide was measured by flow cytometry
- **Metabolic stability:**
  - Metabolic stability was determined at 1 μM of testing concentration with cynomolgus monkeys and human liver microsomes (LMs) using ketanserin and liquid chromatography–tandem mass spectrometry (LC-MS) detection
- **CYP and hERG inhibition:**
  - CYP inhibition was determined at 10 μM of testing concentration with human LMs using diclofenac (2C9), bufuralol (2D6), testosterone (3A4) and midazolam (3A4) as probe substrates and detected with LC-MS. hERG inhibition was determined at 10 μM of testing concentration with Chinese hamster ovary cell lines expressing hERG channels of P29
- **Pharmacokinetic (PK) studies:**
  - Selected compounds (1 mg/kg intravenous and 5 mg/kg oral) were analyzed in cynomolgus monkeys

RESULTS

Figure 2. Entry Inhibitors Efficiently Inhibit HBV and HDV Infection in PHHs

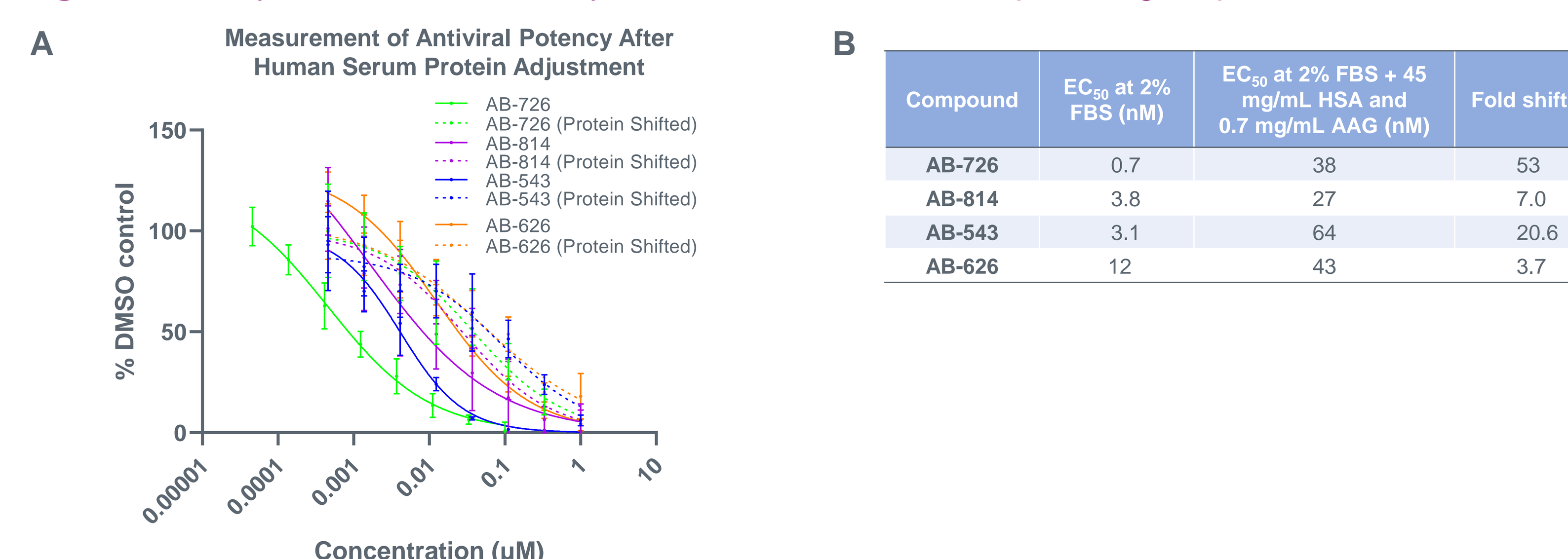


	Range of EC_{50} values (nM)
HBV	HBeAg: 2–10 HBsAg: 2–24 vDNA: 2–22
HDV	vRNA: 1–22

DMSO, dimethyl sulfoxide; EC_{50}, half-maximal effective concentration; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HDV, hepatitis D virus; MyrB, myrcludex B; PHH, primary human hepatocyte; vDNA, intracellular HBV DNA; vRNA, HDV RNA.

- Novel entry inhibitors efficiently inhibited HBV entry in PHHs (**Figure 2**)
- Entry inhibitors efficiently inhibited HDV entry in PHHs (**Figure 2C**)

Figure 3. Entry Inhibitors Efficiently Inhibit HBV in NTCP-Expressing Hepatoma Cells

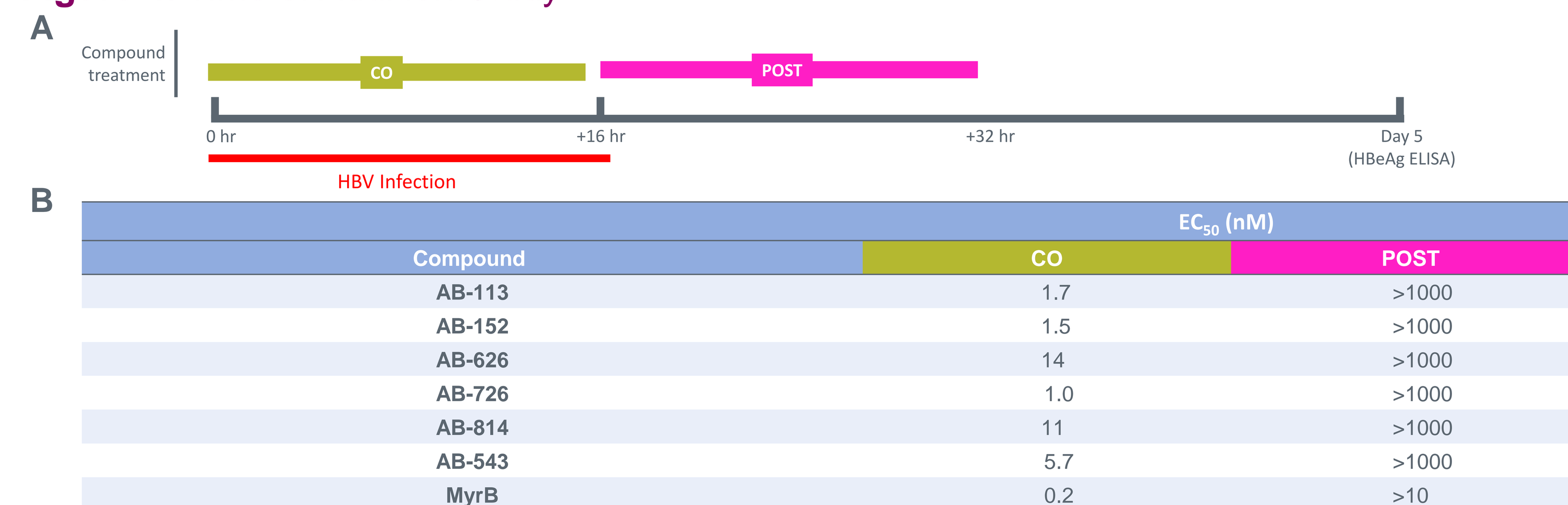


Compound	EC_{50} at 2% FBS (nM)	EC_{50} at 2% FBS + 45 mg/mL HSA and 0.7 mg/mL AAG (nM)	Fold shift
AB-726	0.7	38	53
AB-814	3.8	27	7.0
AB-543	3.1	64	20.6
AB-626	12	43	3.7

AAG, alpha1-acid glycoprotein; DMSO, dimethyl sulfoxide; EC_{50}, half-maximal effective concentration; FBS, fetal bovine serum; HBV, hepatitis B virus; HSA, human serum albumin; NTCP, sodium taurocholate cotransporting polypeptide.

- Novel entry inhibitors efficiently inhibited HBV entry in HepG2-NTCP cells (**Figure 3**)
- Human serum factors affected potency of entry inhibitors (3.7- to 53-fold changes in EC_{50} values)

Figure 4. Time of Addition Study

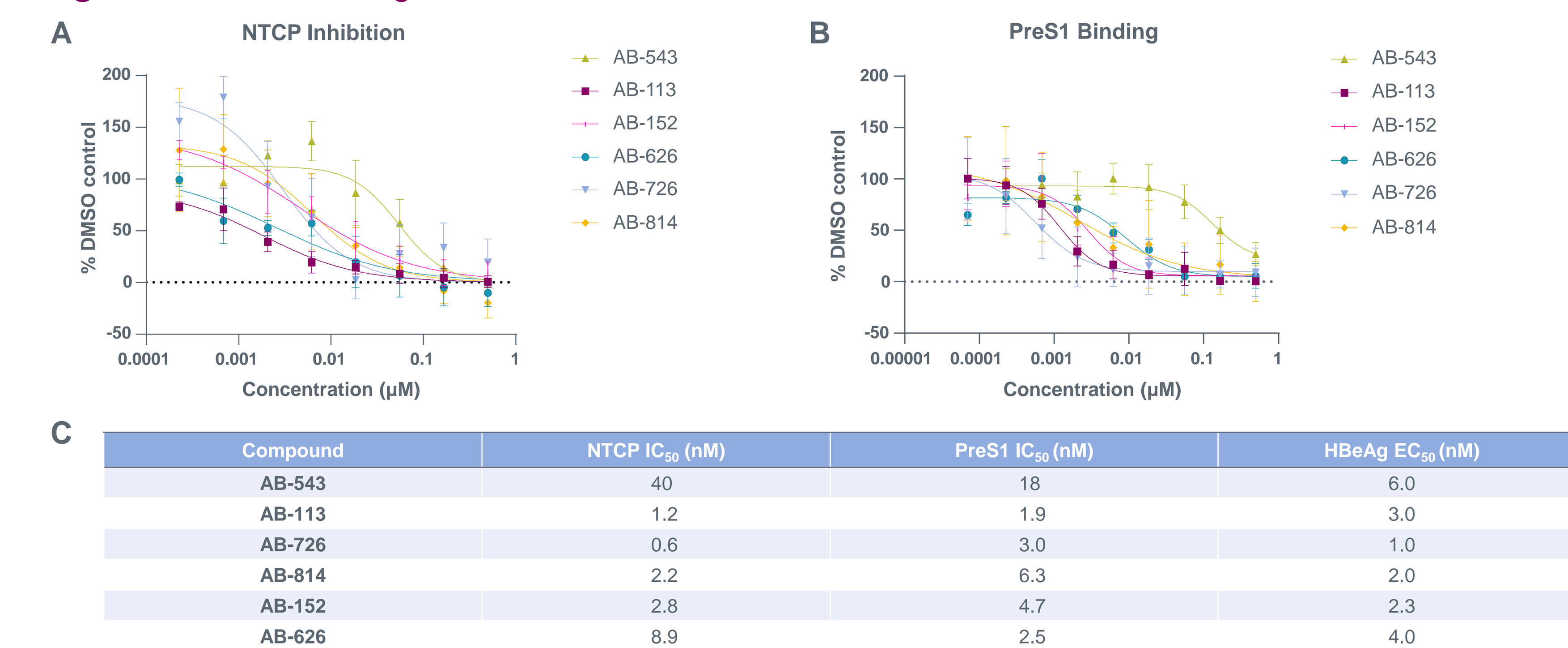


Compound	EC_{50} (nM) CO	EC_{50} (nM) POST
AB-113	1.7	>1000
AB-152	1.5	>1000
AB-626	14	>1000
AB-726	1.0	>1000
AB-814	11	>1000
AB-543	5.7	>1000
MyrB	0.2	>10

CO, cotreatment; EC_{50}, half-maximal effective concentration; ELISA, enzyme-linked immunosorbent assay; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; hr, hour; MyrB, myrcludex B; POST, posttreatment.

- Novel entry inhibitors efficiently inhibited HBV upon cotreatment during infection (**Figure 4**)
- HBV entry is not inhibited upon treatment after virus internalization (posttreatment)

Figure 5. PreS1 Binding and NTCP Inhibition

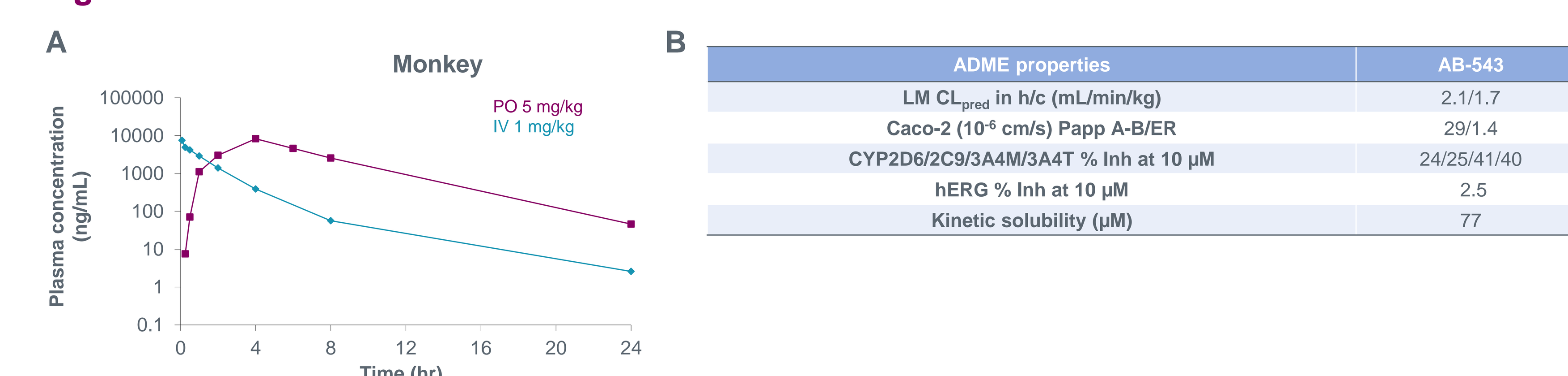


Compound	NTCP IC_{50} (nM)	PreS1 IC_{50} (nM)	HBeAg EC_{50} (nM)
AB-543	40	18	6.0
AB-113	1.2	1.9	3.0
AB-726	0.6	3.0	1.0
AB-814	2.2	6.3	2.0
AB-152	2.8	4.7	2.3
AB-626	8.9	2.5	4.0

DMSO, dimethyl sulfoxide; EC_{50}, half-maximal effective concentration; HBeAg, hepatitis B e antigen; IC_{50}, half-maximal inhibitory concentration; NTCP, sodium taurocholate cotransporting polypeptide.

- The compounds inhibited NTCP-dependent bile acid uptake (half-maximal inhibitory concentration [IC_{50}] = 0.6–40 nM; **Figure 5A and 5C**)
- The compounds inhibited preS1 binding to NTCP (IC_{50} = 1.9–18 nM; **Figure 5B and 5C**)

Figure 6. ADME Profile of AB-543



ADME properties	AB-543
LM CL_{pred} in h/c (mL/min/kg)	2.1/1.7
Caco-2 (10^{-6} cm/s) Papp A-B/ER	29/1.4
CYP2D6/2C9/3A4M/3A4T % Inh at 10 μM	24/25/41/40
hERG % Inh at 10 μM	2.5
Kinetic solubility (μM)	77

N=2.

ADME, absorption, distribution, metabolism, and excretion; CL_{pred}, predicted clearance; ER, efflux ratio; h/c, human/monkey; hr, hour; Inh, inhibition; IV, intravenous; LM, liver microsome; min, minute; Papp A-B, apical to basolateral apparent permeability; PK, pharmacokinetics; PO, by mouth, QD, once daily.

- Cynomolgus monkey is predicted to be the most relevant species for in vivo evaluation and human PK prediction
- The novel potent entry inhibitor AB-543 possesses a good PK profile (**Figure 6A**), showing 100% bioavailability in monkey and a high oral exposure with a terminal half-life of 2.6 hours
- Compound AB-543 demonstrated good absorption, distribution, metabolism, and excretion properties (**Figure 6B**) with:
  - Good in vitro-in vivo correlation and low metabolic clearance in monkey and human LMs
    - LM predicted clearance (CL_{pred}) of 1.7 mL/min/kg and 2.1 mL/min/kg in monkey and human, respectively
  - Good apparent permeability in Caco-2 cells with no efflux
  - No hERG liability
  - Moderate kinetic solubility
- AB-543 has the potential to achieve a desired minimum concentration coverage with once daily 300 mg dosing based on human PK projection using allometric scaling

CONCLUSIONS

- This study identified a novel class of highly-potent, orally-bioavailable HBV and HDV entry inhibitors with good drug-like properties
- The identified inhibitors interfere with preS1 binding and NTCP-mediated bile acid uptake, indicating that the putative molecular target is NTCP
- Lead optimization of this series of entry inhibitors is in progress, with nomination of a development candidate anticipated in the first half of 2023

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