A NOVEL HBV CAPSID INHIBITOR COMPOUND SERIES DEMONSTRATES IMPROVED INHIBITION OF HBV WT, T33N AND I105T CORE PROTEIN VARIANTS AND SHOWS A UNIQUE BINDING MODE TO CORE PROTEIN

Nagraj Mani[1], Andrew G. Cole[1], Steven G. Kultgen[1], Andrzej Ardzinski[1], Junjun Cheng[2], Andrea Cuconati[1], Bruce D. Dorsey[1], Kristi Fan[1], Fang Guo[1], Ju-Tao Guo[2], Zhanying Hu[2], Roseann Kowalski[1], Eugen Mesaros[1], Rene Rijnbrand[1], Holly M. Micolochick Steuer[1], Kim Stever[1], and Michael J. Sofia[1]

[1]Arbutus Biopharma Inc., Warminster, PA, USA and [2]Baruch S. Blumberg Institute, Doylestown, PA, USA

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Arbutus BIOPHARMA *Curing Chronic Hepatitis B*

BACKGROUND

- The process of core protein assembly into pregenomic RNA (pgRNA) containing nucleocapsids is an essential step during replication of the hepatitis B virus.
- The successful encapsidation of pgRNA by 240 core protein molecules signals the synthesis of the viral genome (relaxed circular DNA; rcDNA) and downstream events of infectious virion production and replenishment of the nuclear cccDNA pool.
- A number of small molecule inhibitors of HBV capsid assembly have moved into clinical development and have demonstrated potent antiviral activity.
- Capsid inhibitors thus represent a novel mechanistic class of anti-HBV agents with potential to be part of future curative regimens (*Cole, 2016*).
- Capsid inhibitors have been classified into two mechanistically differentiating categories: Class I capsid inhibitors form non-capsid polymers while Class II capsid inhibitors form capsids that look normal but are devoid of viral nucleic acids (pgRNA/rcDNA).

OBJECTIVES

- Optimize the antiviral profile of novel HBV capsid inhibitor compound series for improved potencies against WT and core variants T33N and I105T that confer resistance to AB-506 and other capsid inhibitors as well as for improved inhibition of nucleocapsid uncoating.

MATERIALS AND METHODS

- Antiviral activity was determined in different cell culture models of HBV using branched DNA and ELISA to measure effects on rcDNA or secreted e-antigens as described previously (*Mani et al 2018*).
- HepG2-NTCP/HBV infection systems was used to study effects on capsid uncoating (sAg) and cccDNA establishment.
- Activity against HBV core protein variants was determined using a HepG2 transient transfection assay.
- Cytotoxicity of compounds was evaluated in various cell lines using CellTiter-Glo® (CTG) assay (Promega).
- X-ray crystallography studies were conducted to determine the binding mode of representative compounds to core protein Cp-Y132A assembly deficient mutant as described previously (*Klumpp et al 2015*).
- Mode of action studies were conducted using HepDE19 or AML12-HBV10 cells to evaluate compound effects on HBV pgRNA encapsidation, capsid particle density, size and core protein phosphorylation status using particle gel assays, sucrose and CsCl density gradient centrifugations, electron microscopy and western blot analysis as described previously (*Campagna et al 2013, Mani et al 2018*).
- Immunofluorescence microscopy studies were conducted to visualize the effects of compounds on core protein localization in HBV infected PHH. AML12HBVpolY63F was used for studying effect of capsid inhibitors on core protein phosphorylation.
- Sucrose and CsCl gradient analysis of HBV capsids: HepDES19 cells were were mock-treated (DMSO) or treated with the indicated compound for 6 days in the absence of tetracycline. The cell lysates were cleared by centrifugation and loaded onto a 25% sucrose cushion and centrifuged at 46,000 rpm for 3 h (Beckman, Rotor SW55). Pellets were dissolved in 1 mL of TNE buffer containing proteinase and phosphatase inhibitors and loaded onto a linear sucrose gradient in TNE buffer and spun at 27,000 rpm for 4 h (Beckman, Rotor SW28). Fractions were collected from the bottom of the centrifugation tubes using a blood collection kit and analyzed by dot blot assays to detect viral capsids with anti-HBcAg antibody and core DNA by hybridization. HBV capsids from sucrose gradient centrifugation were pelleted and dissolved in 300 µL TNE buffer, and mixed with CsCl solution to achieve a final concentration of 1.25 g/mL CsCl and centrifugated at 28,000 rpm for 48 h (Beckman, Rotor SW28). Fractions were collected from the bottom of the centrifugation tube and analyzed by dot blot assays as before. Sucrose and CsCl concentrations of each fraction was measured using a Refractometer (Mettler Toledo).
- Electron microscopy of HBV capsids: Capsid enriched fractions from sucrose gradient centrifugation were pooled, diluted in TNE buffer and centrifuged at 27,000 rpm for 4 h (Beckman, Rotor SW28). Pellets were dissolved in TNE buffer and detected by EM following negative staining with Uranyless and imaged on an FEI Tecnai 12 Spirit/Biotwin (LaB6 filament), operating at 100 kV, with an AMT 2k x 2k CCD camera.

RESULTS

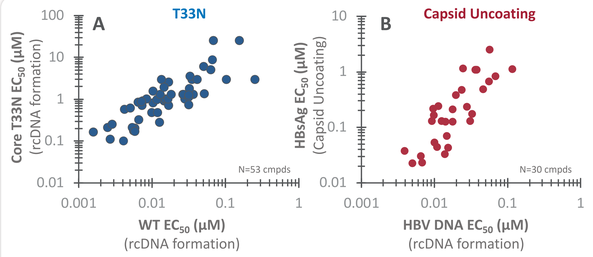
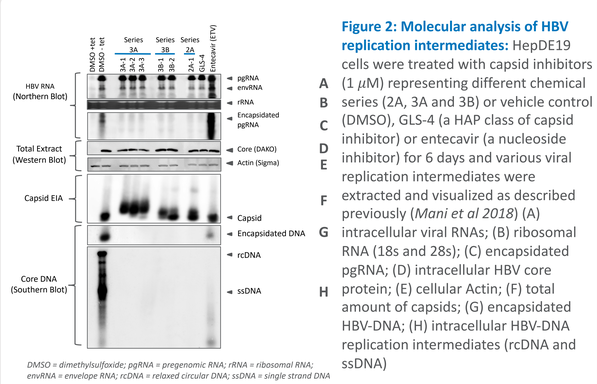


Figure 1: A) GT-D WT vs T33N variant potencies (EC_{50} values for rcDNA inhibition); B) HBV infected HepG2-NTCP potencies (EC_{50} values for HBV DNA vs HBsAg inhibition).

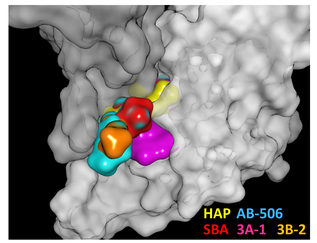
Table 1: Antiviral profile of representative compounds from chemical series 2A, 3A and 3B

Compound	HepDE19		HepG2-NTCP/HBV		HepG2/GT-D transient transfection		
	EC_{50} µM (rcDNA)	CC_{50} µM (CTG)	EC_{50} µM (DNA)	EC_{50} µM (sAg)	WT EC_{50} µM (rcDNA)	T33N EC_{50} µM (rcDNA)	I105T EC_{50} µM (rcDNA)
AB-506	0.077	>25	0.101	1.430	0.065	23.230	1.255
Cmpd 2A-1	0.048	>25	0.098	0.987	0.050	15.453	0.702
Cmpd 3A-1	0.007	>25	0.010	0.053	0.010	0.820	ND
Cmpd 3A-2	0.007	>25	0.007	0.063	0.007	0.716	ND
Cmpd 3A-4	0.006	>25	0.007	0.125	0.008	0.867	0.098
Cmpd 3A-5	0.002	24	0.005	0.023	0.003	0.098	0.016
Cmpd 3A-6	0.013	>25	0.012	0.221	0.017	1.346	0.139
Cmpd 3A-7	0.011	>25	0.014	0.193	0.011	0.718	0.152
Cmpd 3B-1	0.013	>25	0.018	0.125	0.014	>3	ND
Cmpd 3B-2	0.052	>25	0.074	1.436	0.056	>25	0.979



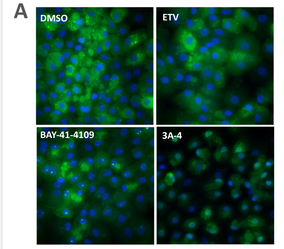
DMSO = dimethylsulfoxide; pgRNA = pregenomic RNA; rRNA = ribosomal RNA; envRNA = envelope RNA; rcDNA = relaxed circular DNA; ssDNA = single strand DNA

Figure 2: Molecular analysis of HBV replication intermediates: HepDE19 cells were treated with capsid inhibitors (1 μM) representing different chemical series (2A, 3A and 3B) or vehicle control (DMSO), GLS-4 (a HAP class of capsid inhibitor) or entecavir (a nucleoside inhibitor) for 6 days and various viral replication intermediates were extracted and visualized as described previously (*Mani et al 2018*) (A) intracellular viral RNAs; (B) ribosomal RNA (18s and 28s); (C) encapsidated pgRNA; (D) intracellular HBV core protein; (E) cellular Actin; (F) total amount of capsids; (G) encapsidated HBV-DNA; (H) intracellular HBV-DNA replication intermediates (rcDNA and ssDNA)



HAP = heteroarydihydropyrimidine; SBA = sulfamoylbenzamide

Figure 3: A space-filling model of cmpds bound to core protein dimer:dimer interface vs HAP, SBA, & AB-506. Capsid inhibitors are a structurally diverse set of small molecule compounds that share a common binding site. An overlay of X-ray structures of 5 compounds representing different capsid inhibitor chemotypes is shown at the dimer:dimer interface of core protein Cp-Y132A mutant. Binding of capsid inhibitors to the dimer:dimer interface facilitates faster assembly kinetics leading to defects in viral replication such as formation of empty capsids or misassembled non-capsid polymers. Cmpd 3A-1 shows a differential binding mode to core protein vs AB-506 and 3B-2 and others.



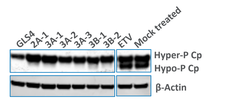


Figure 4: Immunofluoresence microscopy and core protein phosphorylation studies A) Core protein localization in HBV infected primary human hepatocytes treated with inhibitors. B) Western blot analyses of core protein phosphorylation status in AML12HBVpolY63F treated with either capsid inhibitors, entecavir or mock.

GLS-4 and BAY 41-4109 are HAPs (heteroaryldihydropyrimidines); Cmpds 2A, 3A and 3B represent different chemical series of Class II mechanism of action capsid inhibitors; ETV = entecavir; DMSO = mock treated cells.

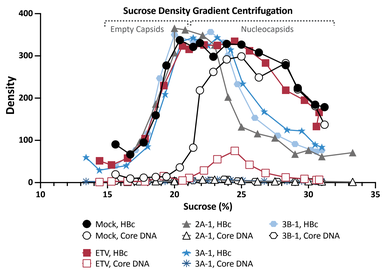


Figure 5: Sucrose gradient centrifugation analysis: HBV capsids isolated from HEPDES19 cells treated with compounds representing series 2A, 3A and 3B. Empty capsids produced in capsid inhibitor-treated cells showed similar sedimentation velocity with the empty capsids formed in the mock- or ETV-treated cells. As expected, empty capsids showed reduced sedimentation velocity than the nucleocapsids.

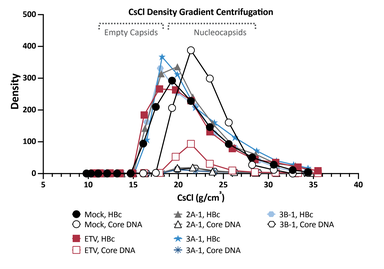


Figure 6: CsCl density gradient centrifugation analysis of HBV capsids. HBV capsids isolated from HEPDES19 cells treated with compounds representing series 2A, 3A and 3B showed similar densities.

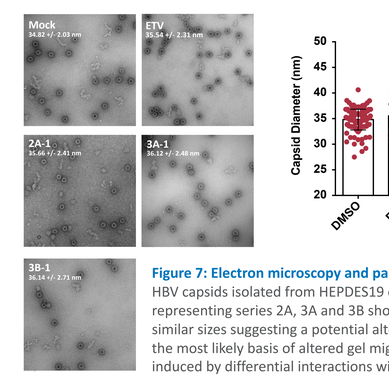


Figure 7: Electron microscopy and particle size measurements. HBV capsids isolated from HEPDES19 cells treated with compounds representing series 2A, 3A and 3B show capsid particles with similar sizes suggesting a potential altered capsid conformation as the most likely basis of altered gel migration pattern observed induced by differential interactions with capsid inhibitors

CONCLUSIONS

- Optimization of the *in vitro* and *in vivo* potencies and drug-like properties of two novel chemical series (3A and 3B) of HBV capsid inhibitors led to compounds with EC_{50} values ≤10 nM in cell culture models.
- In HepAD38 cells, compounds from series 3A and 3B blocked pgRNA encapsidation and produced HBV capsids devoid of viral nucleic acids without reducing intracellular pgRNA and core protein levels (class II capsid inhibitor) similar to compound series 2A (2A-1 and AB-506).
- X-ray crystallography studies showed a compound from series 3A bound to the dimer:dimer interface and displayed a differential binding mode compared to AB-506, 2A, 3B and reported structures, and displayed a slower gel migration pattern of capsids formed in HepAD38 cells upon compound treatment
- Sucrose and CsCl density gradient centrifugation analyses and electron microscopy studies suggested that compound treatment did not alter the particle density, size or phosphorylation patterns of core protein during pgRNA encapsidation attributing the differences to potential alterations in capsid shape/conformation induced upon capsid inhibitor binding.
- Multiple examples of compounds from series 3A showed improved *in vitro* potencies against a T33N and a I105T variant of core protein as well as improved inhibition of capsid uncoating and cccDNA establishment in comparison to AB-506.
- Compounds from series 3A are being evaluated for advancement into IND-enabling studies

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CONTACT INFORMATION AND DISCLOSURES

NAGRAJ MANI, Ph.D., Sr. Principal Scientist,

- Arbutus Biopharma Inc., 701 Veterans Circle, Warminster, PA 18974.
- Email: nmani@arbutusbio.com
- Tel: 1-267-420-2712

- Authors affiliated with Arbutus Biopharma are employees and may own company stock

WEBSITE:

www.arbutusbio.com