P-140

HBV INTERNATIONAL MEETING

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Using a Toolbox of New and Established Mechanistic Assays to Determine How Core Protein Allosteric Modifiers (CpAMs) Inhibit HBV cccDNA Production

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Background

HBV Core protein (Cp) is an attractive target for new HBV antiviral therapies due to its functional involvement in multiple stages of the viral life cycle.[1] A novel class of direct-acting antivirals targeting HBV Cp, termed Core protein Allosteric Modifiers (CpAMs), has been recently discovered and advanced into clinical development.[2] Here, we develop and utilize a series of new and existing cell based assays to explore the antiviral and cccDNA inhibitory activities of a panel of chemically distinct CpAMs.[2,3,4,5,6]

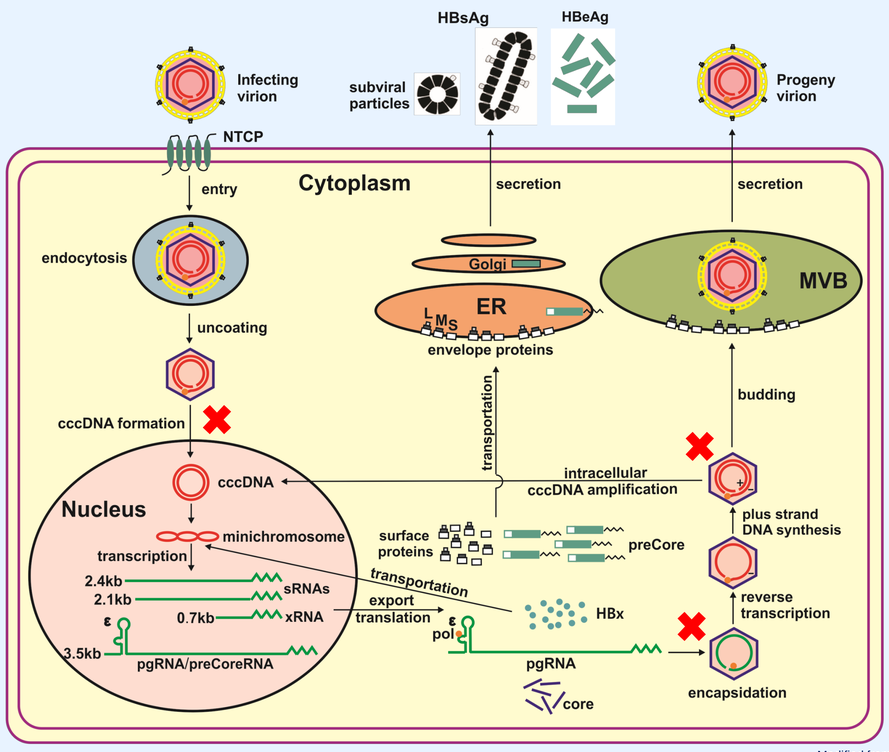
Methods

HBV Capsid Assays: Total RNA/encapsidated pgRNA, Cp, viral capsid, and capsid associated core DNA were detected by Northern blot, immunostaining, Enzyme Immunoassay (EIA), and Southern blot, qPCR and RT-qPCR respectively.[7,8]

HBV Infection Assay: HepG2-NTCP cells were infected with HBV and viral DNA/pgRNA levels were detected by Southern blot.[7] HBeAg levels were quantified by ECL ELISA, as described previously.[8]

HBV cccDNA Southern Blot: Infected cells were harvested and protein-free DNA extracted by a modified Hirt method. Non-cccDNA was removed *via* T5 exonuclease digestion. EcoRI endonuclease was then used to linearize HBV cccDNA before electrophoresis.[9]

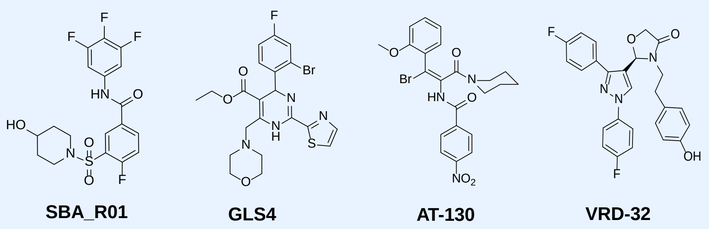
Cp is Involved in Multiple Steps of HBV Lifecycle



Modified from Block TM et al.[10]

✖ **Steps potentially inhibited by CpAMs: cccDNA formation/amplification, pgRNA encapsidation and capsid maturation**

Chemically Distinct CpAMs

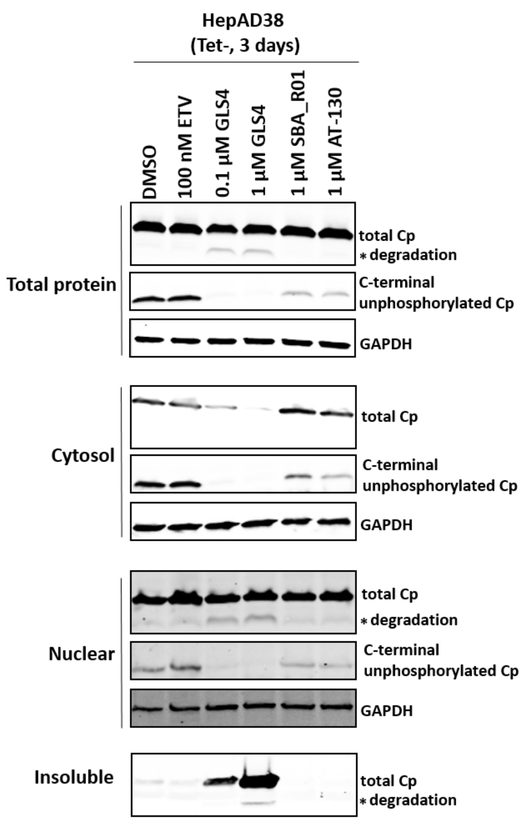


Compound	Anti HBV EC_{50} (nM) HepG2.2.15	Reference
SBA_R01	260	*4*
GLS4	15	*3*
AT-130	127 (<98-207)	*5*
VRD-32	<50	*6*

MOA	Assay	Compound EC_{50} (nM)					
		Entecavir	SBA_R01	GLS4	AT-130	VRD-32	ABI-H0808 (Prototype)
HBV Replication	HepAD38 HBV DNA	0.56	259	20	67	71	147
cccDNA Formation	NTCP Infection HBeAg	>1,000	>10,000	549	>5,000	>5,000	372

CpAMs Perturb Cp Localization and Modification

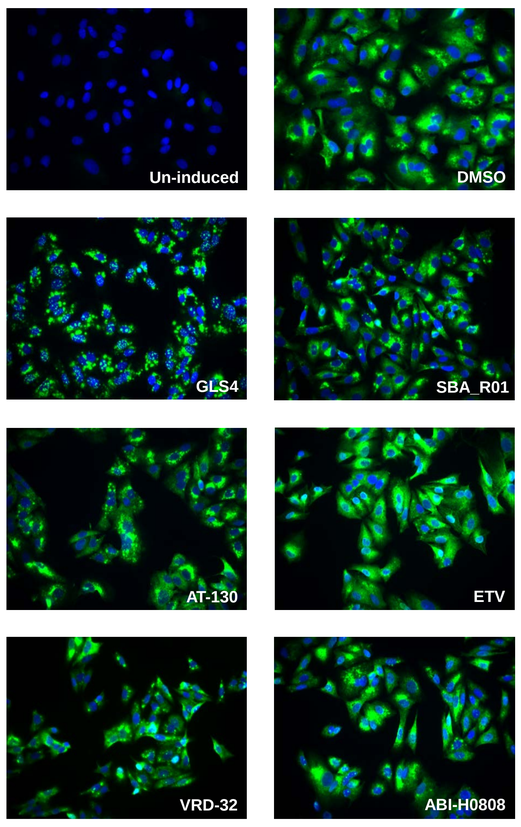
Cp Localization and Phosphorylation



Total Cp was detected by Dako core antibody, whereas unphosphorylated Cp was detected by an antibody generated against Cp peptide (aa170-183)

- Overall Cp levels not affected by CpAMs
- CpAMs appear to induce hyper-phosphorylation of Cp
- GLS4 induced Cp to form insoluble aggregates, resulting in reduced cytosolic fraction of Cp, significance is unknown

Cp Immunostaining Pattern

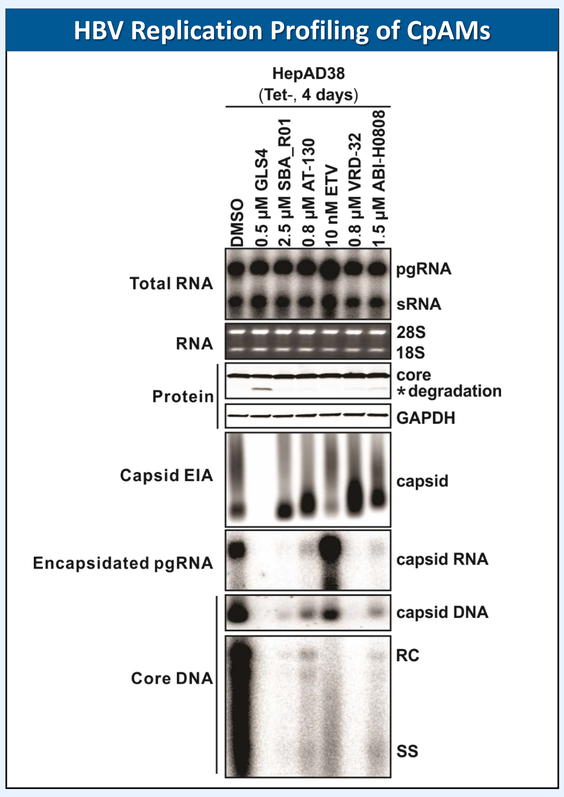


HepAD38 cells were induced and treated with compounds for 4 days at 1 μM (ETV at 0.1 μM) and displayed at a similar level of magnification

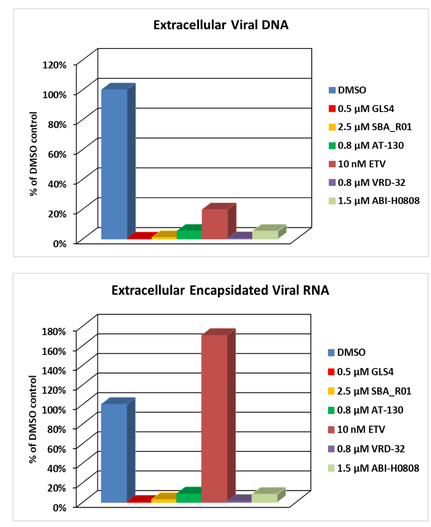
- Cp (in green) was stained using Dako core antibody
- Nuclei (in blue) were stained by DAPI
- CpAMs induced Cp cytoplasmic aggregation and formation of large nuclear foci.
- GLS4 had the most dramatic effect at 1 μM

CpAMs Inhibit HBV Replication by Blocking pgRNA Encapsidation

HBV Replication Profiling of CpAMs



Viral Productivity Profiling of CpAMs

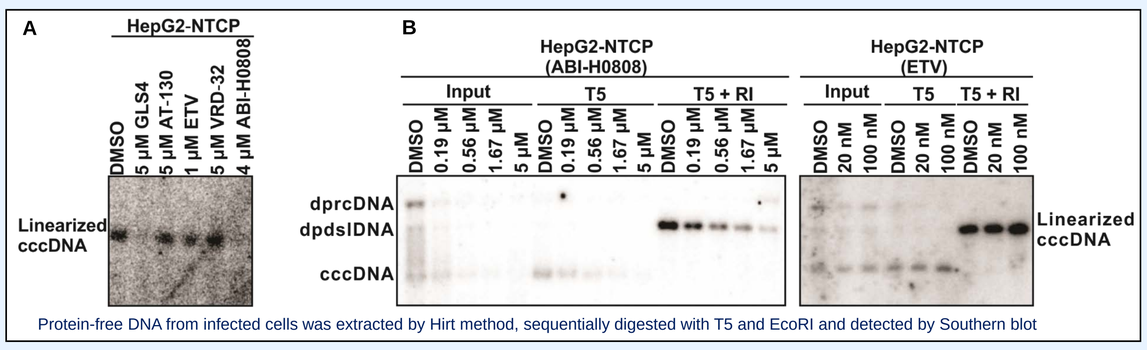


- Secreted HBV particles were devoid of both HBV DNA and pgRNA from CpAMs treated cells

- CpAMs perturbed capsid assembly (Capsid EIA)
- CpAMs inhibited, while ETV increased the amount of encapsidated pgRNA
- Blockage of pgRNA encapsidation resulted in inhibition of viral core DNA synthesis

2nd Generation CpAMs Block cccDNA Formation

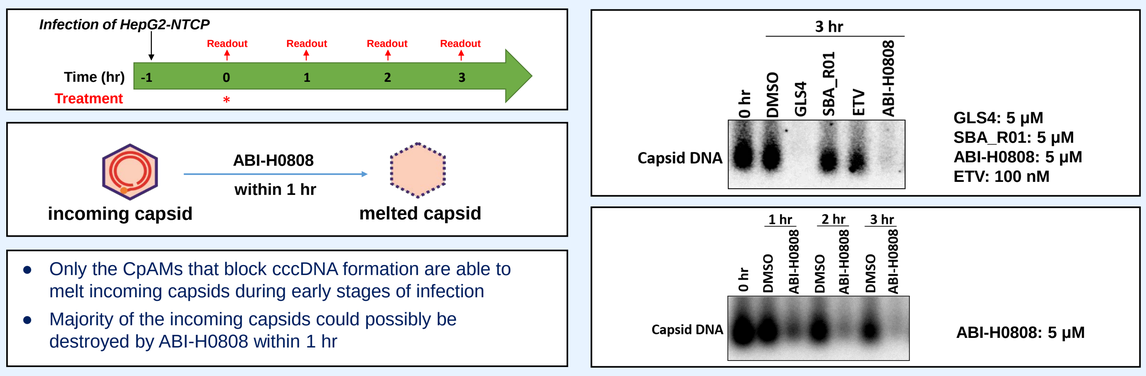
Certain CpAMs Block cccDNA Formation in HepG2-NTCP *de novo* Infection Model



Protein-free DNA from infected cells was extracted by Hirt method, sequentially digested with T5 and EcoRI and detected by Southern blot

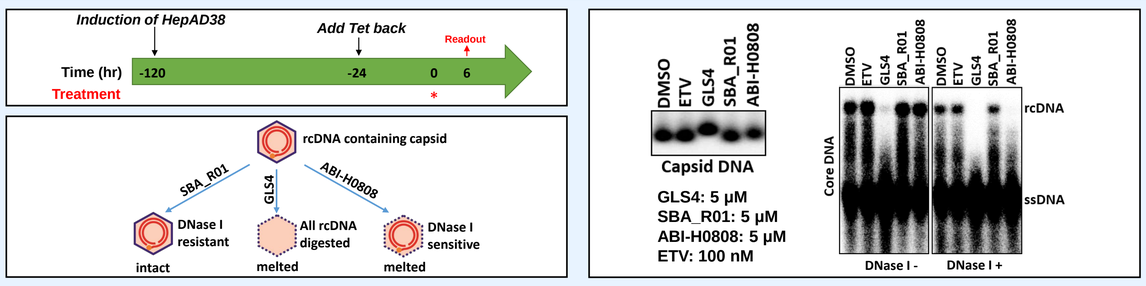
- GLS4 and ABI-H0808, but not other CpAMs block cccDNA formation (A)
- ABI-H0808 reduced HBV cccDNA formation in a dose-dependent manner (B)
- ETV (125-200x EC_{50} concentrations) had minimal effect on cccDNA levels (B)

CpAMs Block cccDNA Formation by Prematurely Melting Incoming Capsid



- Only the CpAMs that block cccDNA formation are able to melt incoming capsids during early stages of infection
- Majority of the incoming capsids could possibly be destroyed by ABI-H0808 within 1 hr

CpAMs Modify Pre-formed Mature Capsid



- Chemically distinct CpAMs had different effect on pre-formed capsids in HepAD38 cells
- GLS4 disrupted rcDNA containing (mature) capsids
- ABI-H0808 damaged mature capsids, rendering it DNase I sensitive
- SBA_R01 and ETV had no effect on pre-formed capsids
- CpAMs had minimal effect on ssDNA-containing (immature) capsids

Summary

- **A series of distinct mechanistic assays has been developed and validated to explore functional activities of CpAMs and their impact on cccDNA generation**
- **CpAMs change Cp distribution, modify Cp staining patterns, alter phosphorylation status, and accelerate Cp degradation**
- **Characterization of a diverse series of CpAMs suggested that some CpAMs are capable of inhibiting multiple steps of cccDNA production, with distinct activities/mechanisms beyond inducing empty capsids and inhibiting viral DNA replication**
- **A subset of CpAMs target both intermediate precursors of cccDNA and incoming/mature capsids to prevent cccDNA establishment and amplification, which are critical to cccDNA formation and longevity**
- **CpAMs have the potential to play a critical role in future curative therapies for HBV**

References

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2. **Pei Y. et al.** Past, Current, and Future Developments of Therapeutic Agents for Treatment of Chronic Hepatitis B Virus Infection., ***J. Med. Chem. 2017;*** *60, 6461−6479*
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