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HAP Class I Capsid Assembly Modulators Clear Hepatitis B Virus-Infected Hepatocytes Through Core-dependent Hepatocyte Death and Subsequent Proliferation

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ALIGOS THERAPEUTICS

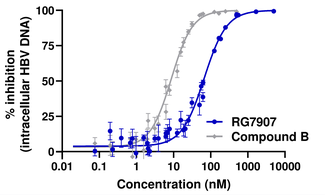
Abstract #1202

Background

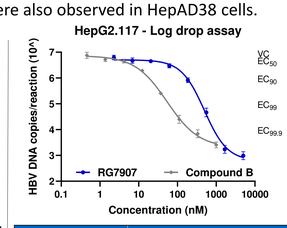
Hepatitis B virus (HBV) capsid assembly is an attractive target for the treatment of chronic hepatitis B (CHB). Class I capsid assembly modulators (CAM1s, also called CAM-A)[1] induce HBV core protein (HBc) aggregation and sustained HBsAg reduction in CHB mouse models.[1 - 4] The underlying mechanism governing this effect has not yet been elucidated. Here, we present an exploration of the mechanism of action of a reference CAM1 (CAM-A), RG7907.

RG7907 is a potent inhibitor of HBV DNA production

The antiviral activity of RG7907 was evaluated in vitro in HepG2.117 cells that carry a Tet-Off promoter driving the expression of HBV pgRNA and HBc. [5] RG7907 inhibited intracellular HBV DNA production with an EC_{50} of 63.3 ± 18 nM by blocking pgRNA encapsidation and subsequent reverse transcription. Compound B, a reference CAM2 (WO2019/175657), exhibited an EC_{50} of 8.3 ± 3 nM as previously reported. Including additional DNase digestion steps to remove non-encapsidated HBV DNA increased the dynamic detection window and allowed the demonstration of DNA inhibition over several orders of magnitude with EC_{90}, EC_{99} and $EC_{99.9}$ values of 204, 458, and 989 nM, respectively, for RG7907. Both compounds showed no toxicity up to at least 10 µM in HepG2.117 cells, and similar antiviral activity and lack of toxicity were also observed in HepAD38 cells.



	Compound B (CAM 2)	RG7907
EC_{50} (nM)	8 ± 3	63.3 ± 16
EC_{90} (nM)	36 ± 2	298.6 ± 4
CC_{50} (nM)	> 10,000	>10,000
n	≥30	

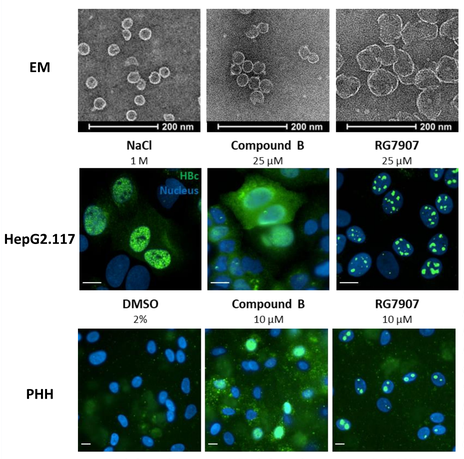


	Compound B (CAM 2)	RG7907
EC_{50} (nM)	4.1	59.6
EC_{90} (nM)	19.8	204
EC_{99} (nM)	62.6	458
$EC_{99.9}$ (nM)	227.3	989
n	4	

Figure 1 – Left: *Dose-response curves for inhibition of HBV DNA replication in HepG2.117 cells. Curves and values represent mean ± SEM from ≥30 independent experiments.* ***Right:*** *Dose-response curves for inhibition of HBV DNA replication in HepG2.117 cells in the log drop assay. Curves and values represent mean ± SEM from 4 independent experiments. VC = virus control.*

RG7907 induces HBc aggregation in vitro and in cellulo

CAM1s have been shown to induce the aggregation of HBc into aberrant structures in vitro. [1 ,7, 8] Consistently, RG7907 misdirected the assembly of HBc into large aberrant capsid-like particles, as demonstrated by electron microscopy and immunofluorescent staining for HBc in HepG2.117 cells and in a more physiologically relevant and metabolizing cells, primary human hepatocytes (PHH). In addition, western blot of HBc in HepG2.117 cells shows a profound reduction of (soluble) HBc after RG7907 treatment, suggesting a compound-induced degradation of HBc.



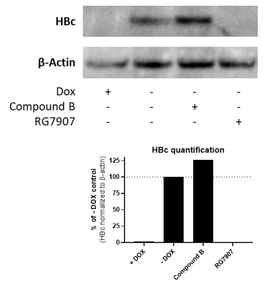


Figure 2 - Top left: *Electron microscopy images of HBc incubated with NaCl (1 M), compound B or RG7907 (25 µM). Empty capsids with normal morphology (NaCl and compound B) and aberrant particles (RG7907) can clearly be distinguished.* ***Middle and bottom left:*** *Immunofluorescent staining of HepG2.117 cells (**Middle, left**) and primary human hepatocytes (PHH, **bottom, left**) for HBc; Class I CAMs induce loss of cytoplasmic HBc and formation of nuclear HBc aggregates with different phenotypes for RG7907 and compound B.* ***Right:*** *Western blot for HBc in HepG2.117 lysates; Compound B and RG7907 both at 2 µM.*

Methods

AAV-HBV-transduced mice[9] were treated with RG7907 to evaluate viral kinetics and liver biomarkers, including serum HBV DNA, HBsAg, HBeAg, and ALT. In addition, intrahepatic pgRNA, total HBV DNA and AAV-HBV episome levels were quantified. RNAseq and immunohistochemical staining for HBc, HBsAg, hepatocyte apoptosis (TUNEL) and proliferation (Ki-67) were performed on liver samples collected at different time points during treatment. In vitro mechanistic experiments were performed in HepG2.117 and HBc- or HBsAg- overexpressing cell lines, including HBc immunostaining, cell viability and apoptosis assessments.

RG7907 strongly reduces viral markers in AAV-HBV mice

AAV-HBV mice were treated with RG7907 for 70 days and sampled at different time points to assess viral markers. RG7907 rapidly reduced serum HBV DNA levels by ~3 log_{10} IU/mL by day 7 with titers further declining afterward to reach a plateau of >5 log_{10} IU/mL reduction by the end of the treatment. ALT levels peaked at day 28 (4.5-fold increase) before returning to baseline by day 56. ALT elevation preceded reductions in serum HBsAg and HBeAg by 1.5 and 1.2 log_{10} IU/mL, respectively, at day 70.

Immunohistochemical staining (IHC) showed a marked reduction in the number of HBc- and HBsAg-positive cells, concomitant with clearance of AAV-HBV episome from hepatocytes on day 35, further decreasing by day 70. Interestingly, day 35 staining showed that the reduction in HBc and HBsAg staining occurred in a piecemeal fashion, with several high antigen-producing cells still observable at this time. Even at day 70, a few positive cells could still be found, explaining the remaining (low but stable) HBsAg serum levels at the end of treatment. Subsequent analysis of liver samples by terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) to confirm and quantify apoptosis showed a 39% and 72% increase in apoptotic cells at day 14 and 35, respectively, in RG7907-treated AAV-HBV mice over untreated animals. These kinetics align with the mild ALT elevation observed at the corresponding timepoints. RG7907 treatment also triggered hepatocyte proliferation, evident by a significant increase (1.7 – 1.8-fold over untreated control) in the number of cells expressing protein Ki-67, returning to baseline by day 70. The transient increase in cell proliferation may be a compensatory mechanism for hepatocytes lost through apoptosis.

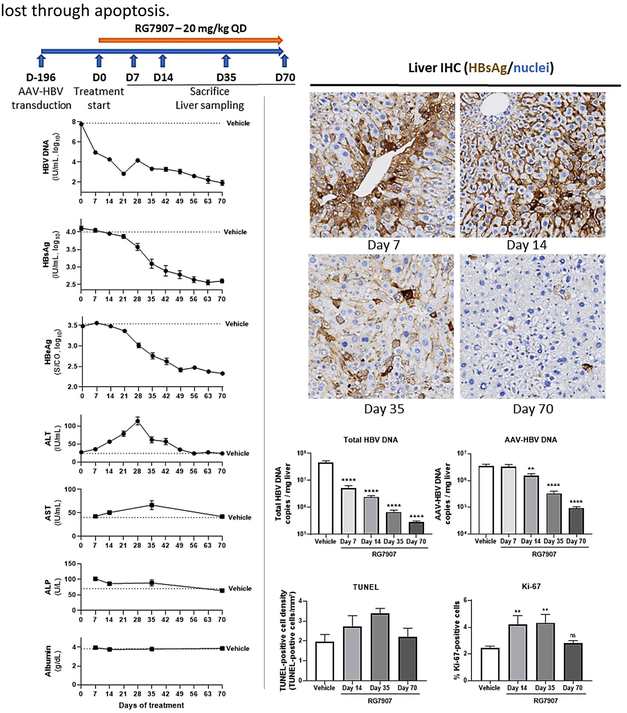


Figure 3 – Left*: RG7907 significantly reduced circulating HBV markers, HBV-positive hepatocytes and AAV-HBV episome levels in AAV-HBV mice. Study design and evolution of HBV DNA, HBsAg, HBeAg, ALT, AST, ALP, and albumin over the course of a 70-day treatment with RG7907 at 20 mg/kg QD per os.* ***Right - upper:*** *Liver IHC staining showing a dramatic loss of HBV-positive cells from day 35 on, with few positive cells remaining at end of the treatment.* ***Right – Middle:*** *Specific quantification of intrahepatic AAV-HBV DNA at different timepoints, showing a gradual reduction of both total and AAV-HBV DNA.* ***Right – lower:*** *Quantitative analysis of DNA fragmentation and double-strand DNA breaks during RG7907 treatment.* ***Right – lower:*** *Quantitative analysis of Ki-67 staining, a cell proliferation marker. Data are represented as mean ± SEM with at least n = 3 mice for each timepoint. Statistical analysis was performed by ordinary one-way ANOVA test followed by Dunnett's multiple comparisons test: **** p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05.*

RG7907 induces targeted apoptosis of infected hepatocytes and subsequent hepatocyte proliferation, accompanied by IFN signaling

RNA expression profiles of liver samples obtained at different time points during treatment were assessed. Analysis of differentially expressed genes clustered into hallmark pathways revealed considerable upregulation in apoptosis, mitosis (E2F targets, G2M checkpoint, mitotic spindle), and IFN response (alpha and gamma) pathways in addition to several others.

To further investigate the *in vivo* observation of RG7907-induced apoptosis, we assessed prolonged exposure of HBV-expressing HepG2.117 cells to RG7907 or compound B for up to 70 days, either in the presence of doxycycline (no HBc expression) or in its absence (inducing HBc expression). Interestingly, unlike compound B, RG7907 induced a time- and dose-dependent CAM1-induced cell death (CCD), starting on day 21. This effect was observed only in the absence of doxycycline, confirming the dependence on both HBc and RG7907. Annexin V and caspase 3/7 assays showed that RG7907-mediated CCD is likely mediated through apoptosis, in line with in vivo IHC and RNAseq data. Finally, we demonstrate that HBc is necessary and sufficient for CCD by using HepG2-NTCP cells expressing HA-tagged HBc and L-HBsAg. Here, treatment of cells with RG7907 for only 7 days significantly induced apoptosis in HBc-expressing cells with no effect in HBsAg-expressing cells.

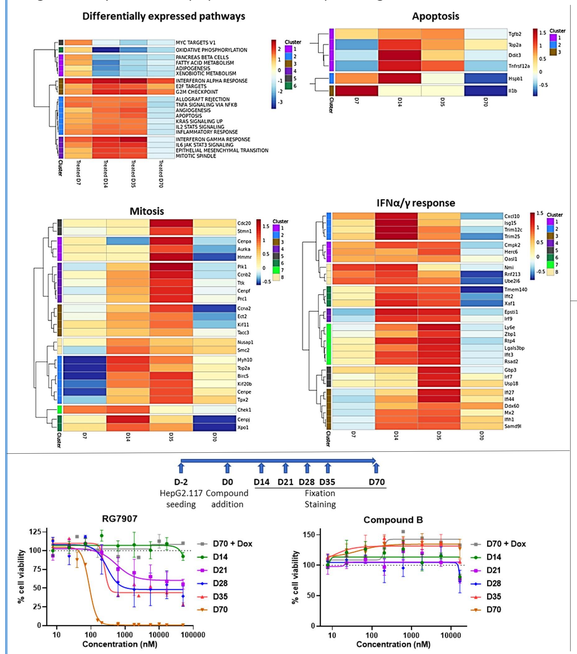


Figure 4 – Top Left: *RNAseq of livers from RG7907-treated mice showed temporal upregulation of genes involved in apoptosis, mitosis, and IFNα/γ response. Heatmap of pathways differentially expressed during RG7907 treatment compared to untreated controls. Heatmaps of individual genes involved in apoptosis, mitosis (E2F targets, G2M checkpoint, and mitotic spindle pooled together) and interferon α/γ response .* ***Bottom left****: CAM1-induced cell death (CCD) assay in HepG2.117 cells. RG7907 treatment led to HBc-dependent cell death in contrast to compound B.* ***Top Right****: Apoptosis, caspase activity and cell viability assessment after extended incubation with RG7907 showed HBc-dependent cell death in HepG2.117 cells. Annexin V and caspase 3/7 assays indicated the observed cell death was linked to apoptosis.* ***Bottom Right:*** *Apoptosis and cell viability was confirmed in HepG2-NTCP expressing HA-tagged HBc and L-HBsAg. Cell death and viability is primarily mediated by HBc. Data are represented as mean ± SEM.*

Conclusions

CAM1 (CAM-A) RG7907 induces HBc aggregation both in vitro and in vivo, which triggers a controlled level of hepatocyte apoptosis, limited to HBc-expressing cells. Compensatory hepatocyte proliferation leads to an additional loss of AAV-HBV episomes in mouse livers. The combination of both mechanisms, possibly complemented by an immune response, results in a sustained loss of HBV-positive cells in mice hepatocytes. This represents an attractive mechanism for CHB functional cure regimens.

References

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