

Non-HAP class I capsid assembly modulators have distinct profiles and a differentiated mechanism of action

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Abstract 1208

Background

Hepatitis B virus (HBV) capsid assembly is an attractive target for the treatment of chronic hepatitis B. In addition to inhibiting HBV RNA encapsidation and formation of infectious HBV particles, class I capsid assembly modulators (CAM1s, also called CAM-A) induce HBV core protein (HBc) aggregation and sustained HBsAg reduction in AAV-HBV mice.¹⁻³ Most known CAM1s are heteroaryldihydropyrimidines (HAPs), such as RG7907 and GLS4,^{4,5} which have several drawbacks including suboptimal PK profiles and potential for drug-drug-interactions. We recently reported on ALG-005398, the first non-HAP CAM1.⁶ Here we present a characterization of two CAM1 profiles distinct from the typical HAP profile and explore their differentiation vs. HAP RG7907.

Methods

Antiviral activity on HBV DNA was determined in HepG2.117 cells using qPCR. Further characterization was performed using electron microscopy visualization and immunofluorescent HBc staining (spotting). HBc-dependent CAM1-induced cell death (CCD) was investigated by prolonged treatment of HepG2.117 cells. In vivo antiviral efficacy was assessed in the AAV-HBV mouse model.⁷

HAPs, profile A, and profile B CAM1s induce different HBc aggregate types

To overcome the drawbacks associated with the CAM1 HAP chemotype, we initiated a drug discovery effort to identify and optimize non-HAP CAM1s. This led to the discovery of several novel CAM1 chemotypes, with biological properties clearly distinct from reference HAPs.⁵ Interestingly, these new molecules could be classified into two differentiated biological phenotypes, designated profile A and profile B, as opposed to the HAP profile. For the following characterization of these profiles, ALG-005398 and ALG-006162 are representative profile A compounds,⁶ ALG-005863 represents profile B, and RG7907 (RO7049389, in phase II clinical development)^{3,5} is a reference HAP CAM1. First, the impact of selected compounds on purified HBc assembly into capsids was examined by electron microscopy. Thus, formation of aberrant capsids could be visualized, which is the hallmark of CAM1s. ALG-005398 induced smaller particles (40-50 nm in diameter) than the typical HAP-induced aberrant capsids observed for RG7907 (60-70 nm), but still larger than the 30-35 nm empty capsids formed in NaCl. Conversely, ALG-005863 induced the formation of larger irregular particles (70-80 nm).

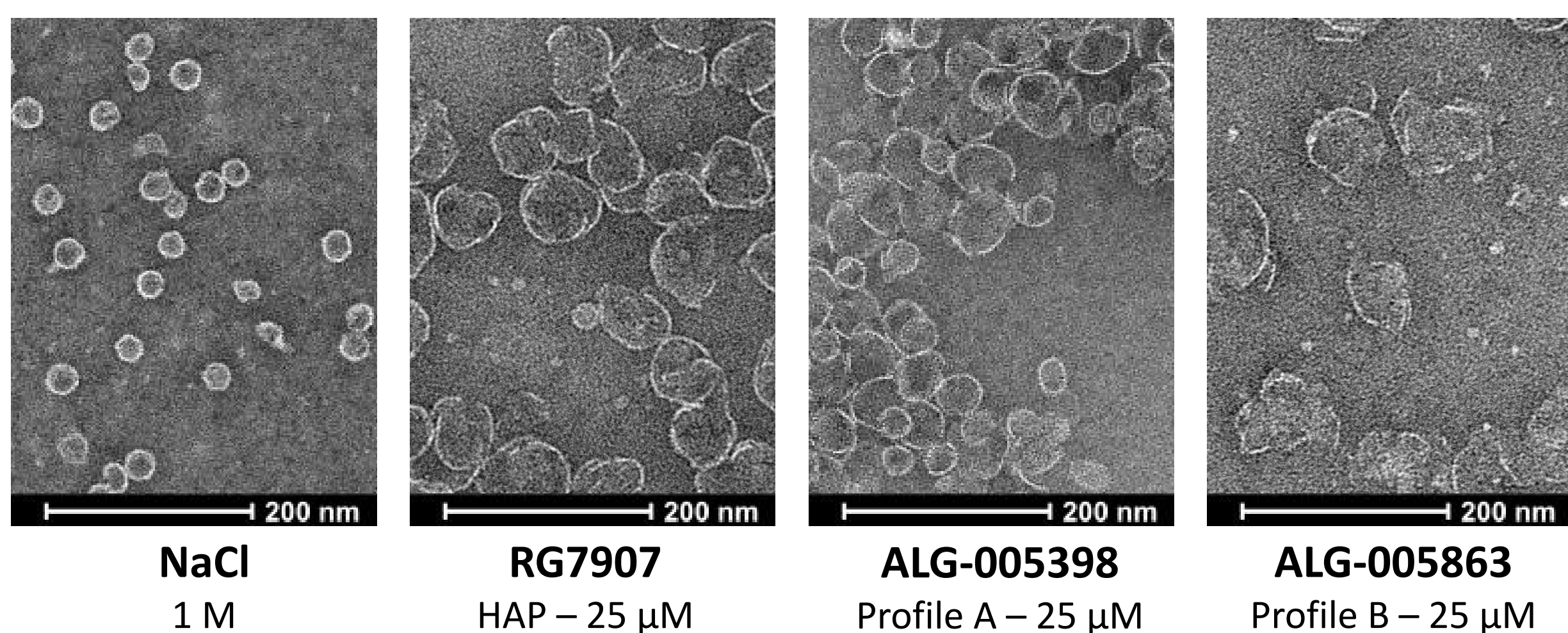


Figure 1: Electron microscopy images of HBc incubated with NaCl (1 M), RG7907, ALG-005398, or ALG-005863 (25 μM). Empty capsids with normal morphology (NaCl) and aberrant particles of different sizes and morphologies (CAM1s) can be distinguished.

CAM1s induce the formation of nuclear HBc spots, representing compound-induced HBc aggregates.⁸ Such spots were also found when HepG2.117 cells were treated with ALG-005398 and ALG-005863, although different phenotypes were observed compared to RG7907. ALG-005398-induced spots were more numerous but smaller in size, whereas ALG-005863 induced larger and more irregular aggregates. These observations are in line with electron microscopy results. Quantification of the total spot area normalized to cell number and relative to RG7907 at 10 μM allowed the determination of EC₅₀ and EC₉₀ values, showing that ALG-005398 and ALG-005863 are approximately 10x more potent than RG7907 in inducing HBc spots.

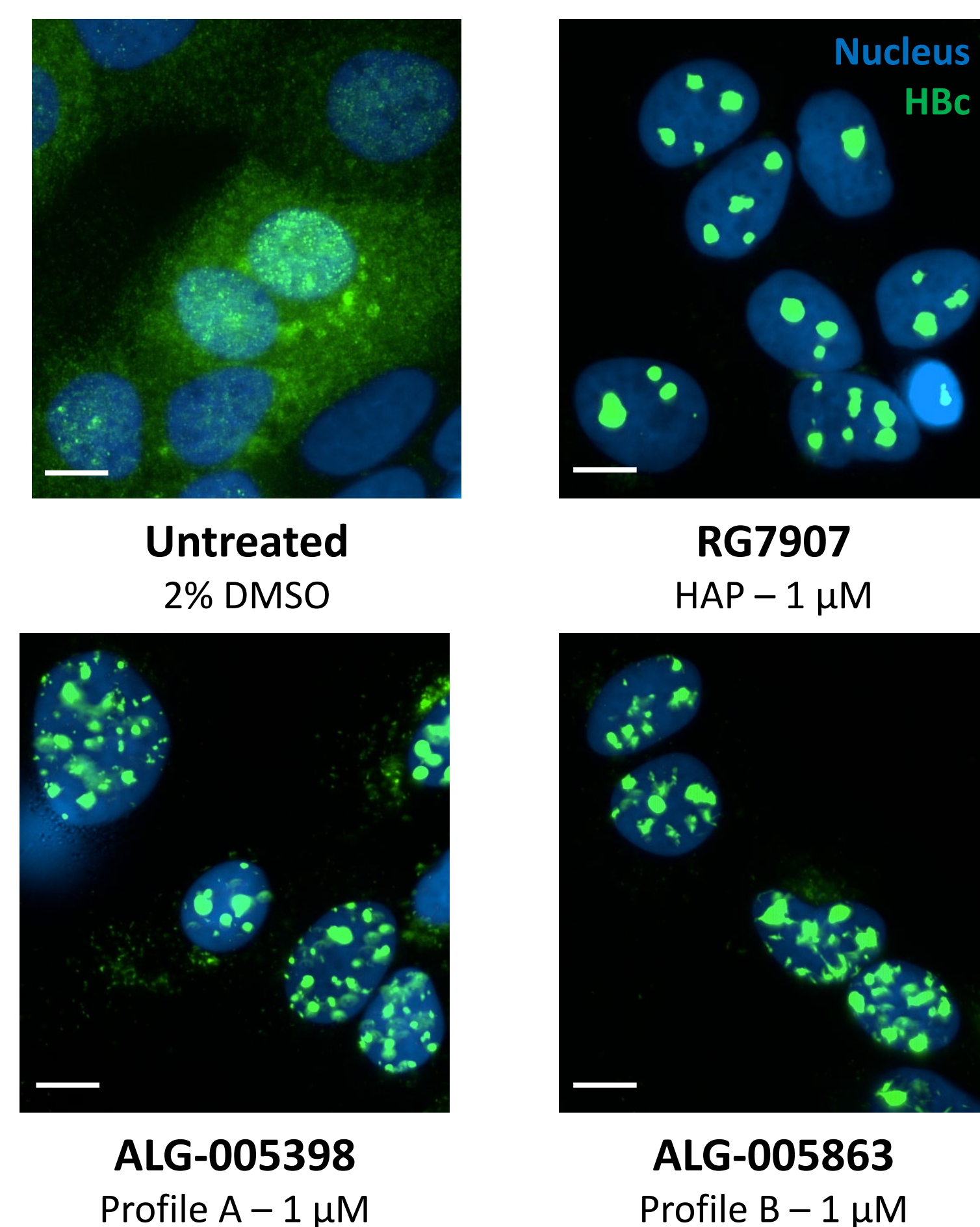
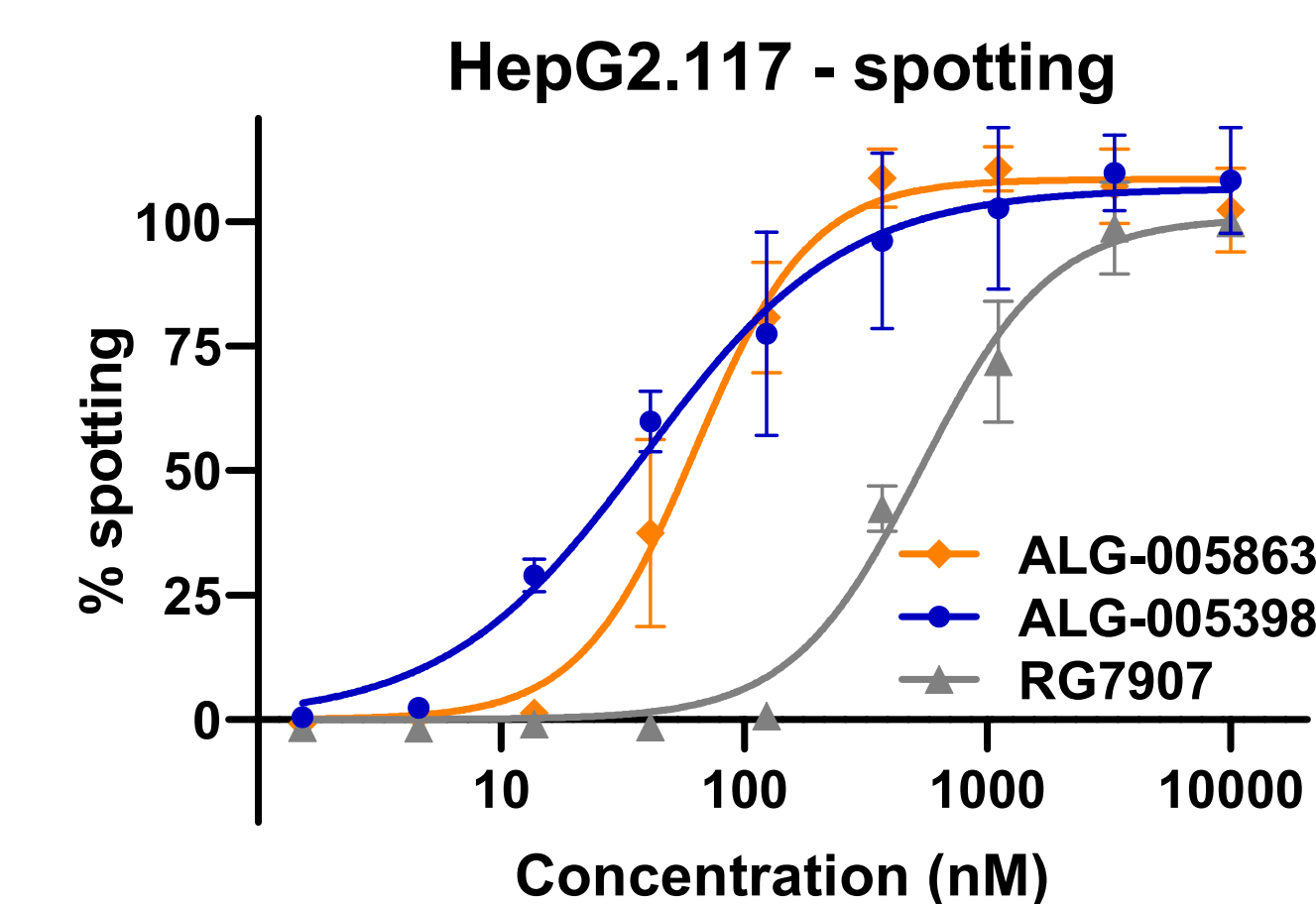


Figure 2 – Left: Immunofluorescent staining of HepG2.117 cells for HBc; CAM1s induce loss of cytoplasmic HBc and formation of nuclear HBc aggregates with different phenotypes for RG7907, ALG-005398, and ALG-005863. **Right:** Quantification of spotting by automated image analysis (total spot area normalized to cell number and relative to RG7907 at 10 μM). Curves and values represent mean ± SEM from 4 independent experiments. Bars are 10 μm.



Spotting	EC ₅₀ (nM)	EC ₉₀ (nM)
RG7907	529	1970
ALG-005398	34.7	192
ALG-005863	57.7	150

Profile B CAM1s have distinct HBV DNA inhibition characteristics

All three tested compounds effectively reduced intracellular HBV DNA in HepG2.117 cells. Interestingly, profile B reference ALG-005863 displayed a considerably steeper curve (slope of 2.5) compared to RG7907 and ALG-005398 (slopes of 1.4 and 1.5 respectively), suggesting different HBc binding kinetics. When comparing EC₅₀ values for HBV DNA and HBc spotting, ALG-005863 again behaved differently with only a 2-fold difference between the EC₅₀ values for both phenomena, compared to a 10-fold ratio for the other compounds. These trends were observed for many more molecules categorized into HAP, profile A, and profile B, based on their chemotype and spotting phenotype (data not shown).

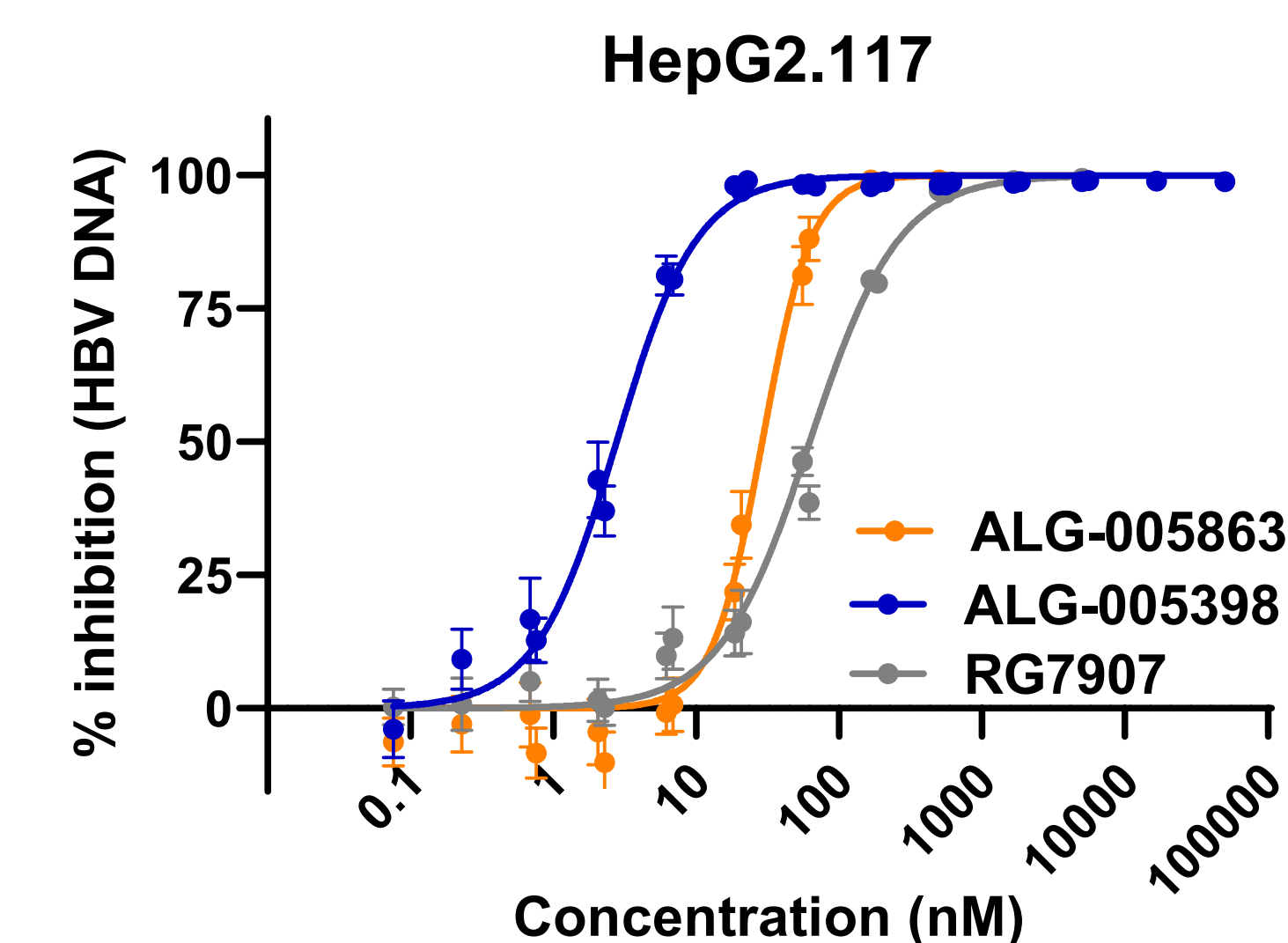


Figure 3 – Left: Dose-response curves for inhibition of HBV DNA replication in HepG2.117 cells. Curves and values represent mean ± SEM from ≥20 independent experiments. **Right:** Summary of HBV DNA EC₅₀ obtained in HepG2.117 cells and ratio of EC₅₀ spotting (HBc immunofluorescent staining) over EC₅₀ for HBV DNA inhibition, showing a clear difference between profile B reference ALG-005863 and the other 2 molecules.

	HBV DNA EC ₅₀ (nM)	EC ₅₀ ratio (spotting / DNA)	Slope
RG7907	62.2	8.50	1.4
ALG-005398	2.76	12.6	1.5
ALG-005863	28.9	2.00	2.5

HBc-dependent CAM1-induced cell death kinetics differ between profiles

As described in the accompanying poster by Kum et al (abstract 1202), CAM1 RG7907 induces a HBc-dependent cytotoxicity (CCD) upon prolonged treatment that may help explain the observed loss of HBV-infected hepatocytes in the AAV-HBV mouse model. To investigate whether the different CAM1 profiles translate to different CCD phenotypes, HepG2.117 cells were incubated with different compound concentrations for prolonged periods of time, in the absence or presence of doxycycline (with or without HBc expression, respectively). RG7907 showed a gradual appearance of CCD from day 21, reaching its maximum effect after 70 days of incubation. Conversely, profile A ALG-005398 did not show any CCD at all, with virtually identical curves in the presence or absence of doxycycline. Profile B ALG-005863 did show CCD, but displayed delayed kinetics compared to RG7907 with the effect only becoming apparent from day 42 on.

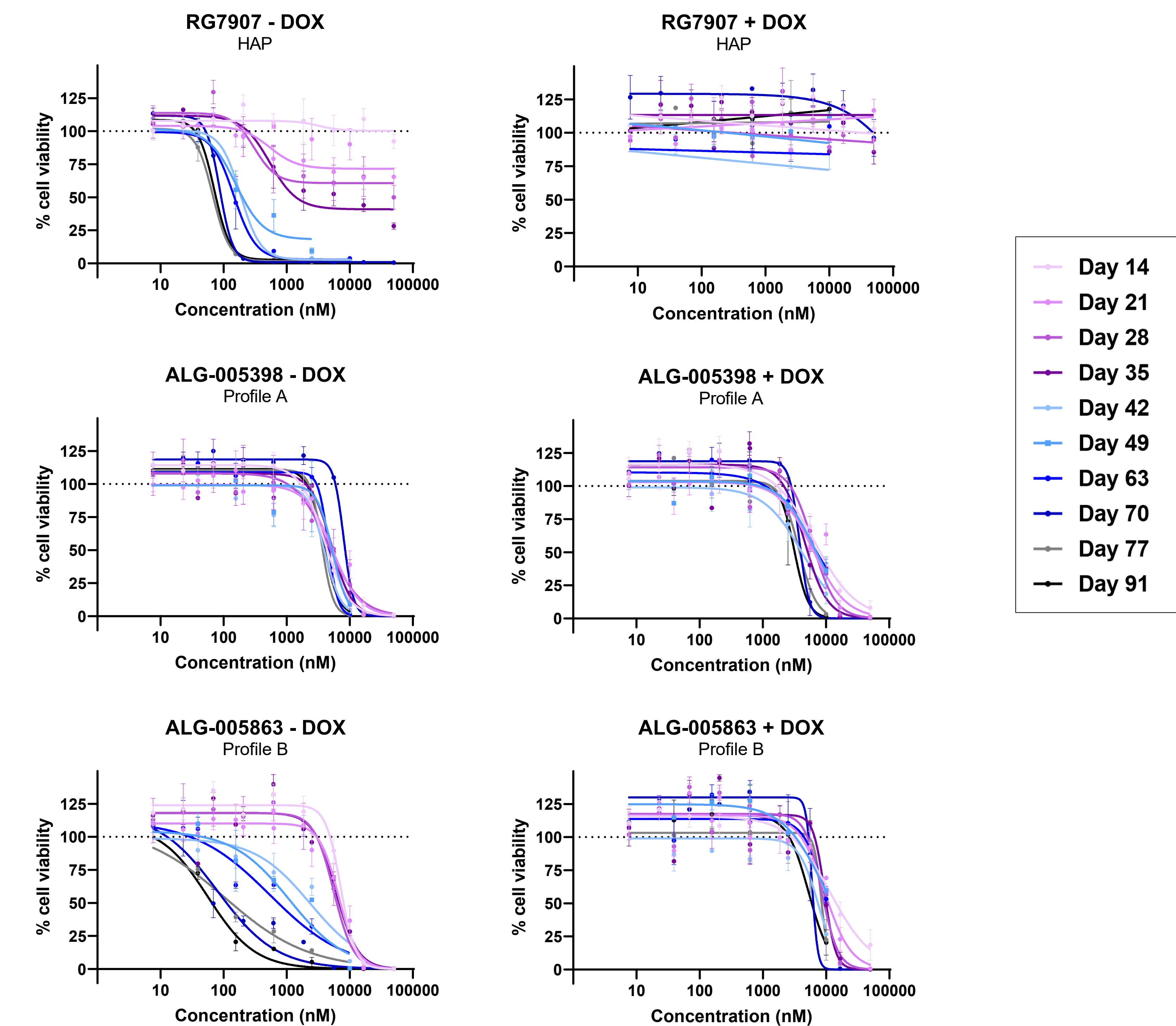


Figure 4: Cell viability assessment of HepG2.117 cells treated for extended periods with the indicated compounds in the presence of HBc expression (- DOX) or in its absence (+ DOX). Curves and values represent mean ± SEM from ≥3 independent experiments. DOX, doxycycline.

HAPs – but not profile A or B – CAM1s induce HBc-PML co-localization

Nuclear co-localization of HBc with promyelocytic leukemia (PML) bodies has been described upon HAP CAM1 treatment of HBc-expressing cells.⁹ This observation was confirmed in HepG2.117 cells. Interestingly, both profile A and profile B CAM1s did not display a similar co-localization, with the bulk of the observed PML bodies not showing appreciable HBc aggregates.

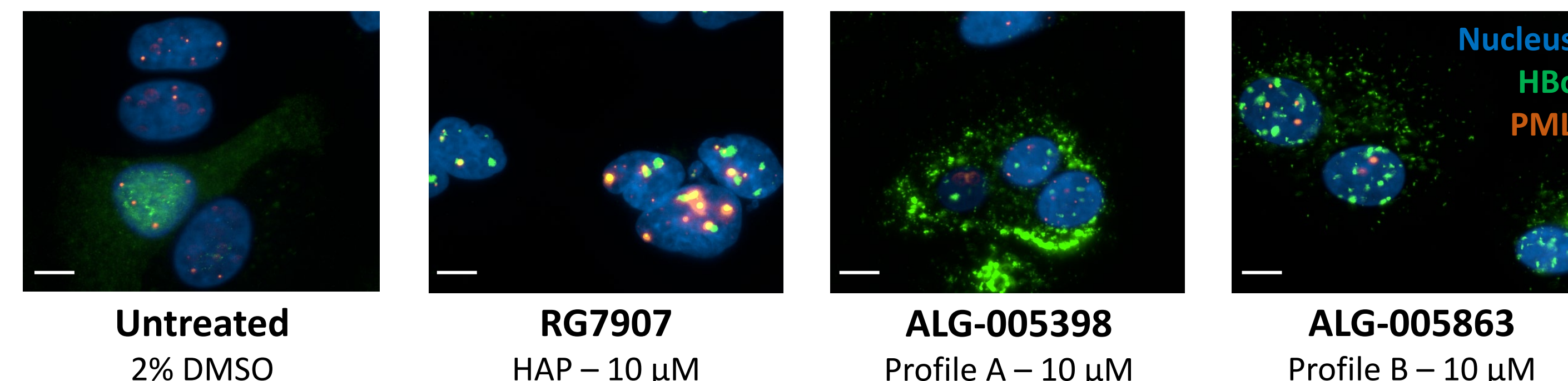


Figure 5: Immunofluorescent staining for HBc and PML bodies in CAM1-treated HepG2.117 cells showed a clear co-localization of PML bodies and HBc aggregates for HAP RG7907, but not for profile A and B compounds ALG-005398 and ALG-005863. DMSO was added to a final concentration of 2%, final compound concentrations were 10 μM. Bars are 10 μm.

Profile A ALG-006162 shows a sustained HBsAg reduction in AAV-HBV mice

We previously reported on the *in vivo* efficacy of ALG-005398, which reduced HBV DNA, HBsAg and HBeAg in the AAV-HBV mouse model, especially when its pharmacokinetics were boosted with ritonavir. ALG-006162 is an optimized analogue of ALG-005398 with improved ADME properties, eliminating the need for ritonavir boosting while retaining typical profile A characteristics. ALG-006162 dosing at 55 mg/kg BID p.o. induced an immediate HBV DNA decline and a somewhat delayed reduction in serum HBsAg and HBeAg. This was accompanied by a minimal ALT flare. HBsAg and HBeAg reductions were sustained after end of treatment, while HBV DNA levels rebounded to some extent. Immunohistological staining for HBc and HBsAg showed a pronounced loss of HBV-positive hepatocytes.

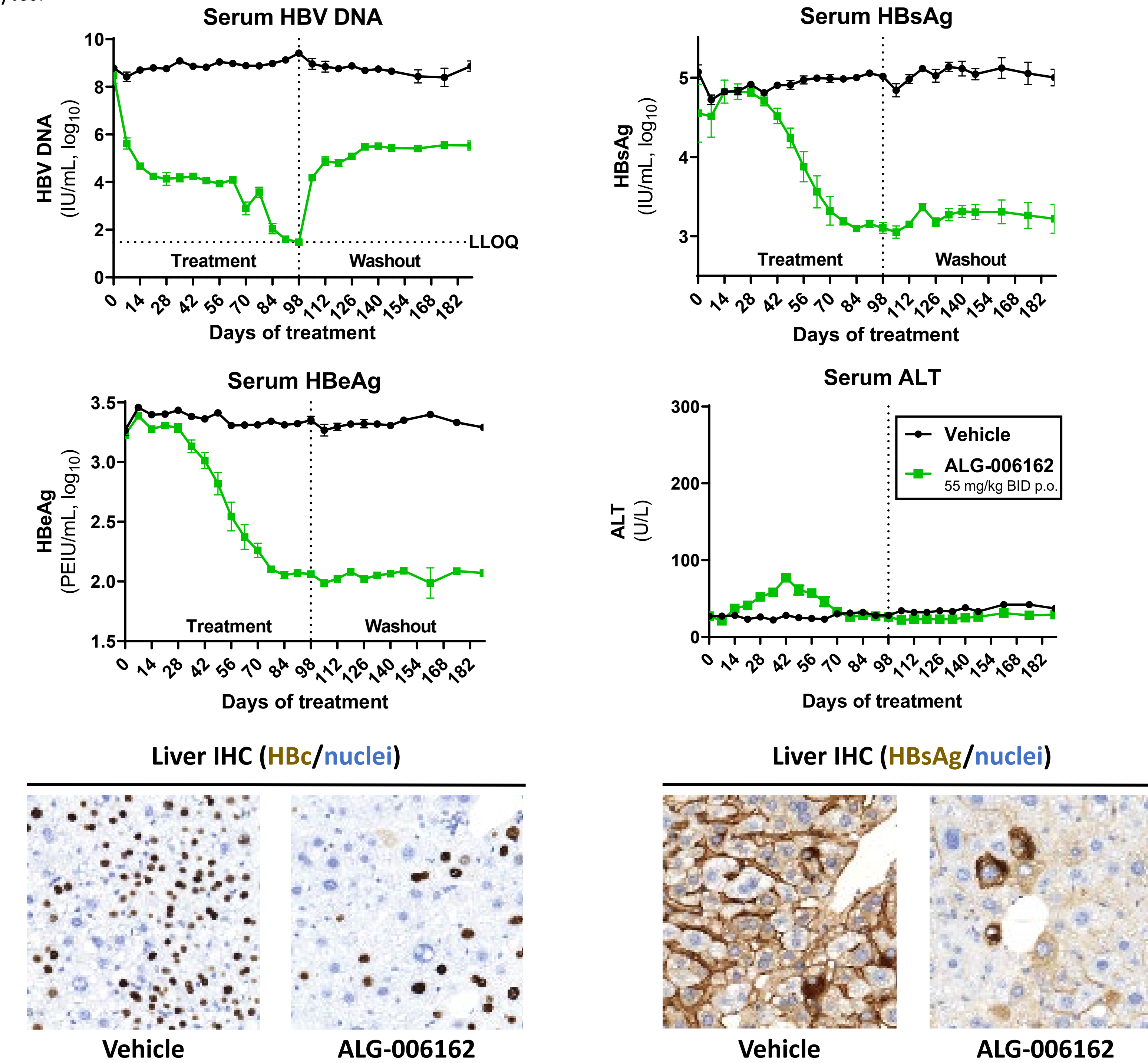


Figure 6 – Top: Kinetics of serum levels of HBV DNA, HBsAg, HBeAg and ALT in AAV-HBV mice during treatment, with statistically significant declines for HBV DNA, HBsAg and HBeAg. Values represent mean ± SEM for 4 animals per group. **Bottom:** Immunohistological HBc and HBsAg staining in AAV-HBV mouse livers at the end of the washout period. LLOQ, lower limit of quantification.

Conclusions

Non-HAP CAM1s have profiles that are clearly distinct from known HAP CAM1s such as RG7907. The reported data provides evidence that their underlying mechanisms of action governing *in vivo* HBsAg reduction may also be differentiated. Since optimized non-HAP CAM1s have suitable ADME and tox profiles, they represent an attractive class of molecules for further development as a part of potential functional cure regimens for chronic hepatitis B, offering a potential advantage over HAPs.

References: [1] Zhou et al 2018 J Hepatol, 68: S770. [2] Zhou et al 2019 J Hepatol, 70: e98. [3] Zoulim et al 2022 Nature Rev Gastroenterol Hepatol, doi: 10.1038/s41575-022-00700-z. [4] Yuen et al 2021 Lancet Gastroenterol Hepatol, 6: 723-32. [5] Zhang et al 2021 Clin Infect Dis, 73: 175-82. [6] Debing et al 2021 AASLD TLM, Abstract 818. [7] Yang et al 2014 Cell Mol Immunol, 11: 71-8. [8] Corcuera et al 2018 Antiviral Res, 158: 135-42.

Financial disclosures: All authors with affiliations ¹, ² or ⁴ are /were directly or indirectly employed by Aligos Therapeutics Inc.