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## Novel Hepatitis B Virus Capsid Assembly Modulator Induces Potent Antiviral Responses In Vitro and in Humanized Mice

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1 **Novel Hepatitis B Virus Capsid Assembly Modulator Induces Potent**  
2 **Antiviral Responses *in Vitro* and in Humanized Mice**

3

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15 Running title: Efficacy of HBV Capsid Assembly Modulator

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## 23 **Abstract**

24 Hepatitis B virus (HBV) affects an estimated 250 million chronic carriers worldwide. Though several  
25 vaccines exist, they are ineffective for those already infected. HBV persists due to the formation of  
26 covalently-closed circular DNA (cccDNA) – the viral minichromosome – in the nucleus of hepatocytes.  
27 Current nucleoside analogs and interferon therapies rarely clear cccDNA, requiring lifelong treatment. Our  
28 group identified GLP-26, a novel glyoxamide derivative that alters HBV nucleocapsid assembly and prevents  
29 viral DNA replication. GLP-26 exhibited single-digit nanomolar anti-HBV activity and inhibition of HBeAg  
30 secretion, and reduced cccDNA amplification in addition to a promising pre-clinical profile. Strikingly, long  
31 term combination treatment with entecavir in a humanized mouse model induced decrease in viral loads and  
32 viral antigens that was sustained for up to 12 weeks after treatment cessation.

33

## 34 **Introduction**

35 Despite the availability of effective vaccines, epidemiologic data estimate that approximately 250 million  
36 people are chronically infected with hepatitis B virus (HBV) (more than the HIV and HCV carriers combined)  
37 and are at high risk for development of hepatitis, cirrhosis and hepatocellular carcinoma (1). Current anti-  
38 HBV treatment options include pegylated interferon alpha2a (pegIFN) and/or nucleoside analogs that require  
39 lifetime use to suppress the virus. Two key events in the HBV replication cycle involve first the generation of  
40 cccDNA transcriptional template, either from input viral DNA or newly replicated capsid-associated DNA,  
41 and second, reverse transcription of the viral pre-genomic (pg) RNA to form HBV DNA genomes that are  
42 encapsidated into de novo viral particles (2). HBV persists in long-lived hepatocytes due to the establishment  
43 and maintenance of cccDNA in the nucleus of host cells (3) where it is not targeted by current therapies and  
44 serves as a viral reservoir (4). Since hepatocytes have a long half-life, elimination of cccDNA by hepatocyte

45 turnover can be considered as a means of viral clearance only if the cccDNA is disrupted or silenced while  
46 replication of new HBV is stopped. HBV rebounds after cessation of treatment with currently approved  
47 nucleoside analog inhibitors. To address this issue, novel antiviral agents are now being investigated including  
48 entry inhibitors, hepatitis B surface antigen (HBsAg) inhibitors and capsid assembly modulators (CAM) (5).  
49 HBV capsid assembly plays an essential role in many steps of the viral replication cycle (6). Notably, HBV  
50 capsid is responsible for trafficking relaxed circular DNA (rcDNA) to the nucleus thereby establishing and  
51 maintaining cccDNA levels as a “refill” mechanism. Further, the HBV capsid protein is found in the nucleus  
52 of hepatocytes and interacts with host factors responsible for transcriptional regulation (7). Therefore, it is  
53 hypothesized that targeting disruption of the nucleocapsid could impact cccDNA stability and potentially lead  
54 to eradication of HBV (8). Based on the promise of sustained antiviral activity, several CAM have been  
55 studied such as GLS-4 (1) (phase II) (9), RG-7907 (Roche, phase I), AT-130 (2) (10), DVR-23 (11), NVR 3-  
56 778 (3) (12) (Novira/JnJ, phase IIa), AB-423 (13) and AB-506 (14) (Arbutus), JNJ-379 (Phase IIa) (15) and  
57 ABI H0731 (16) (Assembly Bioscience, Phase 1a) (**SI Appendix, Fig. S1**). Structurally these compounds are  
58 heteroaryldihydro-pyrimidines (HAPs), phenylpropenamides (PP) or sulfamoylbenzamides (SBA). Here we  
59 report the discovery and the preclinical characterization of GLP-26, a novel CAM with a unique  
60 glyoxamidopyrrolo backbone, obtained through chemical optimization of early SBA derivatives identified by  
61 our team (17).

62 GLP-26 (**Fig. 1**) is an HBV capsid assembly modulator displaying substantial effects at low nanomolar ranges  
63 on both HBV DNA replication and HBV e antigen (HBeAg) secretion, with greater than 1 log reduction of  
64 cccDNA amplification along with promising pre-clinical profile. Most interestingly, sustained decreases in  
65 HBeAg and HBV surface antigen (HBsAg) levels were observed in an HBV-infected humanized mouse  
66 model treated with GLP-26 in combination with entecavir up to 3 months after drug cessation.

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## 70 **Results**

### 71 **GLP-26 is a non-toxic inhibitor of HBV DNA, HBeAg and cccDNA production *in vitro*.**

72 The *in vitro* anti-HBV activity of GLP-26 was determined by measuring secreted HBV DNA from HepAD38  
73 cells and from infected primary human hepatocytes (PHH). GLP-26 displayed potent antiviral activity, with  
74 EC<sub>50</sub> of 0.003 μM and 0.04 μM in HepAD38 cells (**Table 1**) and PHH (**Table 2**), respectively. GLP-26 did  
75 not show toxicity up to 100 μM in human hepatoma cell lines (HepG2) nor in a panel of other relevant cell  
76 types (**SI Appendix, Table S1**) yielding a wide selectivity index (SI, HepG2 cells > 33,333). It is noteworthy  
77 that GLP-26 was 25-120x more potent in these assays than GLS4, a CAM currently evaluated in the clinic. In  
78 addition, GLP-26 did not show signs of mitochondrial toxicity at concentrations up to 50 μM and no increase  
79 in lactic acid production (% of lactic acid/ % of nuclear DNA) was observed at concentrations up to 25 μM,  
80 which is well above the EC<sub>50/90</sub> antiviral values (**SI Appendix, Table S2**). As a correlate to cccDNA levels,  
81 GLP-26 was evaluated for inhibition of HBeAg production in HepAD38 cells (18). GLP-26 effectively  
82 inhibited HBeAg secretion with an EC<sub>50</sub> = 0.003 μM, which was over 50 times more potent than GLS4 (EC<sub>50</sub>  
83 = 0.16 μM, *data not shown*). As expected, the nucleoside analog 3TC had minimal effect on inhibition of  
84 HBeAg secretion (**Fig. 2A**). The same system was used to investigate the effects of agents on cccDNA using  
85 RT-qPCR. Both GLP-26 and GLS-4 equally exhibited potent inhibition of HBV cccDNA amplification, with  
86 > 1 log reduction relative to untreated mock control (**Fig. 2B**).

### 87 **GLP-26 stabilizes HBV capsid particles and induces their accumulation in the cytoplasm.**

88 Direct binding of GLP-26 to HBV capsid protein was evaluated using a fluorescent thermal shift assay by  
89 measuring changes in the thermal stability of capsids upon complexation with GLP-26 (19). The HBV capsid

90 protein fragment 1-149aa (Cp149) was expressed and isolated as dimers as previously described, and capsid  
91 particles were formed from Cp149 dimers by decreasing pH and increasing salt concentration. GLP-26  
92 reproducibly increased the melting temperature of HBV Cp149 capsids ( $T_m = 87 \pm 0.3^\circ\text{C}$ ) to a greater extent  
93 than GLS4 ( $T_m = 85 \pm 0.3^\circ\text{C}$ ) (**Fig. 3A**). Fitting the titration of GLP-26 to HBV Cp149 capsids provides a  $K_d$   
94  $= 0.7 \pm 1.5 \mu\text{M}$  which was ~60x fold lower than GLS4 ( $K_d = 41 \pm 13 \mu\text{M}$ ) indicating that GLP-26 binds to and  
95 stabilizes HBV capsids.

96 The effect of GLP-26 on cellular localization of capsids was determined by confocal microscopy in HepAD38  
97 cells (**SI Appendix, Fig. S3**). In the absence of drug (**Fig. 3B**), 50% of the cells contained HBV core proteins  
98 corresponding to HBV capsids in both the nucleus and the cytoplasm. In contrast, treatment with GLP-26 for  
99 24 h emptied the nucleus and led to an accumulation of capsid particles exclusively in the cytoplasm (**Fig. 3C**)  
100 while with GLS4, capsids formed large aggregates spread in the cell (**Fig. 3D**) as previously reported for this  
101 type of HAP derivatives. (20, 21, 22, 23)

#### 102 **GLP-26 induces the formation of firm HBV capsid particles.**

103 The effects of GLP-26 on capsid assembly were observed using negative-stain electron microscopy (TEM).  
104 To determine the effects on the assembly process, Cp149 dimers were first incubated with drug followed by  
105 addition of salt to initiate assembly. In the absence of drug, HBV capsids formed regular icosahedrons with a  
106 diameter of approximately 40 nm (**Fig. 4A-B**). Addition of GLP-26 to Cp149 dimers followed by assembly  
107 generated clusters of small and misshapen particles in contrast to the large, aberrant capsid morphologies  
108 observed induced by GLS4 (**SI Appendix, Fig. S4**). To determine the effects post-assembly, images of pre-  
109 formed HBV capsid particles treated with compounds were collected. Addition of GLS4 to pre-formed  
110 capsids resulted in larger, broken assemblies, similar in appearance to cracked egg shells (**SI Appendix, Fig.**  
111 **S4**). Unlike GLS4, fewer particles were observed upon addition of GLP-26, and those that remained exhibited  
112 smaller and firmer morphologies like “hard boiled eggs” (**Fig. 4E-F**).

113 **GLP-26 demonstrated synergistic antiviral activity with nucleoside analogs *in vitro*.**

114 Anticipating that HBV CAMs such as GLP-26 will be administered in combination with existing direct acting  
115 agents, we evaluated its interaction with entecavir (ETV), a potent nucleoside analog inhibitor of HBV  
116 replication. For the median-effect analysis, the drugs were combined at a 5:1 ratio (GLP-26 + ETV) based on  
117 their EC<sub>50</sub> values. These two agents resulted in a combination index (CI) of 0.6 (**SI Appendix, Table S3**)  
118 indicating that GLP-26 interacted synergistically with ETV in the HepAD38 system.

119 **GLP-26 has a favorable *in vitro* and *in vivo* pharmacokinetic profile.**

120 In preparation for *in vivo* applications, the stability of GLP-26 was evaluated. GLP-26 demonstrated favorable  
121 stability profiles in plasma with half-lives > 24 h in human, mouse, and dog and a t<sub>1/2</sub> of ~ 8.5 h in rat plasmas  
122 (**SI Appendix, Table S4**). Liver microsome stability was also satisfactory with t<sub>1/2</sub> of 71 min and 7.6 h in  
123 mouse and human respectively (**SI Appendix, Table S6**).

124 The pharmacokinetic characteristics of GLP-26 were evaluated in CD-1 mice where it displayed favorable  
125 oral bioavailability (**SI Appendix, Figure S5**) with AUC<sub>0-7h</sub><sup>obs</sup> of 1,587 and 1,306 hr.ng/mL by oral (PO) and  
126 intravenous (IV) routes respectively. The t<sub>1/2</sub> was much longer from PO administration (> 6 h) compared to  
127 that from IV (1.5h), providing prolonged concentrations high above the *in vitro* EC<sub>50</sub>. Due to the favorable  
128 oral absorption profile and long half-life, we decided to deliver GLP-26 *in vivo* using oral dosing.

129 **GLP-26 decreases HBV DNA, HBsAg and HBeAg levels in HBV-infected humanized mice.**

130 To evaluate GLP-26 activity *in vivo*, HBV-infected BRGS-uPA mice with chimeric humanized livers  
131 (HUHEP mice) (24, 25) were treated with either GLP-26 alone (60 mg/kg/day) or in combination with ETV  
132 (0.3 mg/kg/day) by oral administration (**Fig. 5A-D**). At the start of treatment all mice had serum hAlb levels  
133 above 100 µg/ml and serum HBV DNA levels above 10<sup>6</sup> copies/ml. Over a period of 10 weeks, the untreated  
134 cohort increased HBV DNA (>1 log<sub>10</sub>) and HBsAg (0.5-2 log<sub>10</sub>) with no significant change in HBeAg  
135 expression. Treatment with GLP-26 alone led to decreases in viral loads (1-3 log<sub>10</sub>), HBsAg (0.3-2 log<sub>10</sub>)

136 and HBeAg (0.3-1 log<sub>10</sub>). In comparison, mice treated with ETV alone had a 2.7-3.3 log<sub>10</sub> decrease in HBV  
137 DNA (one mouse had levels below limits of detection); however, the ETV-treated group showed minimal  
138 decreases in HBsAg and HBeAg (0.7 log<sub>10</sub> and 0.2 log<sub>10</sub>, respectively).

139 **GLP-26 and ETV act synergistically to reduce viral DNA and antigens with long-term sustained activity**  
140 **post-treatment in HBV-infected humanized mice.**

141 Based on our *in vitro* results showing a synergistic effect between capsid assembly modulator GLP-26 and  
142 nucleoside analog ETV, a similar combination was evaluated *in vivo*. HBV-infected mice were treated  
143 concomitantly with both agents for 10 weeks. In the six mice treated with combination ETV (0.3 mg/kg/day)  
144 + GLP-26 (60 mg/kg/day), viral loads strongly decreased with a mean -4 log<sub>10</sub> HBV DNA and half the mice  
145 had undetectable viremia at the end of treatment (**Fig. 5A**). Furthermore, all the mice had significantly  
146 decreased viral antigen loads – mean -1.8 log<sub>10</sub> HBsAg and -1 log<sub>10</sub> HBeAg (**Fig. 5B-C**).

147 As half the mice in the ETV + GLP-26 group had viral loads below the limit of detection after 10 weeks, viral  
148 kinetics were monitored for 11-12 weeks post-treatment cessation. During the rebound phase, viral loads  
149 returned rapidly in HBV-infected HUHEP mice that had received ETV alone, consistent with previous results  
150 (26). However, in the ETV+GLP-26 combination treatment group, of the three mice that had undetectable  
151 viremia, two remained aviremic for several weeks. HBV DNA was undetectable in one mouse for 5 weeks  
152 and the other for 11 weeks off treatment (**Fig. 5A**). Interestingly, even if HBeAg levels remained stable or  
153 slightly increased in most mice during the rebound phase, reduction of HBeAg up to -2 log<sub>10</sub> was observed in  
154 two mice (**Fig. 5C**). HBsAg levels decreased substantially even after treatment cessation with the exception of  
155 two mice. One mouse showed undetectable levels of HBsAg (lower limit of detection = 0.1 IU/ml) despite  
156 being weakly viremic (**Fig. 5B**).

157

158 **Discussion**



159 Current treatments for chronic HBV are limited to nucleoside polymerase inhibitors and/or PEG-interferon.  
160 These strategies rarely achieve functional cure and development of novel therapeutic agents interfering with  
161 other essential steps of the viral replication cycle are needed. GLP-26 is a novel nontoxic CAM that displays  
162 low nanomolar activity against HBV in both HepAD38 and PHH cells. GLP-26 is highly specific for HBV  
163 and did not show activity against a panel of viruses including Dengue virus, West Nile virus, Chikungunya  
164 virus, Zika virus and HIV-1 up to 30  $\mu\text{M}$  (**SI Appendix, Table S6**). Unlike heteroaryldihydropyrimidine  
165 derivatives (HAP) such as GLS4, GLP-26 binds to the capsid and induces formation of tight, intact particles.  
166 These biochemical outcomes are similar to those observed for the alternate class of CAMs that includes the  
167 propenamides (AT-130) and sulfamoylbenzamides (SBA, such as AB-423 or NVR-3-778) derivatives.  
168 GLP-26 decreases cccDNA *in vitro* and decreases HBeAg (a biomarker of cccDNA) levels *in vitro* and *in*  
169 *vivo*. HBV maintains cccDNA levels by recycling mature relaxed circular (rc) DNA to the nucleus (27). This  
170 process relies on the proper biophysical properties of HBV capsid protein for rcDNA formation and nuclear  
171 transport. Since GLP-26 affects capsid assembly and possibly transport into the nucleus, the agent likely  
172 disrupts cccDNA maintenance from this cycle leading to an overall reduction in cccDNA levels.  
173 GLP-26 in combination with ETV potently decreased HBsAg and HBeAg levels in a humanized mouse model  
174 of infection both during and more importantly after treatment. It is worth noting that previous studies have  
175 shown decreased HBV viral antigens during combination treatments (28), yet continued antiviral effects on  
176 these markers after treatment have not been yet reported for other CAMs from the same class (AB-423 and  
177 JNJ-632 respectively) (13, 29). The mechanisms resulting in sustained response could arise from the very  
178 potent antiviral activity of GLP-26 (10-100 times more potent than AB-423 and JNJ-632) combined with a  
179 prolonged exposure from oral administration leading to sustained efficacious levels of GLP-26. Since HBsAg  
180 seroconversion is more likely with low levels of HBsAg (30), these observations suggest there is potential for  
181 seroconversion when these biomarkers are decreased with GLP-26 treatment. In addition, the model used in

182 this study did not reconstitute the mice with humanized immune systems, and we anticipate improved activity  
183 and seroconversion in immunocompetent animal models or humans upon treatment with GLP-26.  
184 Overall, GLP-26 displayed favorable metabolic stability with high oral bioavailability and no adverse effects  
185 were observed after oral administration for up to 10 weeks, highlighting the relative safety of this compound.  
186 Optimization of the treatment period, oral dosing and drug combinations (ETV, pegIFN, etc.) will be essential  
187 to deliver a more pronounced and lasting antiviral effect in animal models and eventually in humans.  
188 **Conclusion:** We identified GLP-26 as a highly potent and promising HBV CAM. Direct effects of GLP-26 on  
189 HBV capsid assembly was established using electron microscopy, confocal microscopy and thermal shift  
190 assays. GLP-26 inhibited HBV DNA, HBeAg and cccDNA amplification and did not display any toxicity *in*  
191 *vitro*. Oral bioavailability in mice and stability in both plasma and liver microsomes strengthen an already  
192 excellent preclinical profile. Combination treatment of GLP-26 with ETV in a humanized mouse model of  
193 HBV infection delivered sustained antiviral response up to 12 weeks after treatment cessation offering the  
194 hope that similar effects can be reached in humans with this novel CAM.

195

## 196 **Materials and Methods**

197 **Synthesis of compounds.** Synthesis and characterization of GLP-26 is detailed in Supplementary Information  
198 (SI) Section. GLS-4 was prepared according to the chemistry and methods previously described (31). Both  
199 compounds had a purity > 95% as determined by proton <sup>13</sup>NMR and HPLC analysis. Entecavir (ETV),  
200 lamivudine (3TC), tenofovir disoproxil fumarate (TDF) were purchased from commercial vendors and  
201 confirmed > 95% purity using standard analytical methods such as mass spectrometry and NMR.

202 **Cytotoxicity assays.** *In vitro* cytotoxicity was determined using the CellTiter 96 non-radioactive cell  
203 proliferation colorimetric assay (MTT assay, Promega) in primary human peripheral blood mononuclear cells

204 (PBMC), human T lymphoblast (CEM) and human hepatocellular carcinoma (HepG2) cell lines. Toxicity  
205 levels were measured as the concentration of test compound that inhibited cell proliferation by 50% ( $CC_{50}$ ).

206 **HBV Assay in HepAD38.** The HBV assay was performed in HepAD38 cells as previously described (32).  
207 Briefly, HepAD38 cells were seeded onto 96-well plates and incubated for two days at 37°C in a humidified  
208 5% CO<sub>2</sub> atmosphere. On day two, medium with tetracycline (Tet) was removed and cells were washed with  
209 1X PBS. Antiviral drugs were prepared in medium without Tet and added in duplicate at various  
210 concentrations. After a total of seven days incubation, total DNA was extracted using DNeasy 96 Tissue kit  
211 (Qiagen), and HBV DNA was amplified by real-time PCR (18). Antiviral activity was measured by  
212 determining the average threshold cycle for the HBV DNA amplification with the compounds (alone or in  
213 combination), which was subtracted from the average cycle of the untreated-tetracycline control ( $\Delta CT$ ). Drugs  
214 were first tested individually for effective concentration, which inhibited 50% and 90% of HBV DNA  
215 replication ( $EC_{50}$  and  $EC_{90}$ ) using CalcuSyn software program (Biosoft, Ferguson, MO, USA).

216 **Evaluation of HBeAg secretion.** The effect of GLP-26 on the levels of cccDNA amplification was assessed  
217 using the HepAD38 cells to measure HBeAg as a cccDNA-dependent marker (33). In this system, HBV  
218 replication is controlled with tetracycline: its presence in the medium blocks pre-genomic (pg) RNA  
219 synthesis, and in its absence, synthesis of pgRNA and HBV DNA replication occur. In addition, when cells  
220 are re-treated with Tet, new cccDNA formation is restored and HBe Ag production can be measured as a  
221 reporter for levels of intracellular cccDNA. HepAD38 cells were incubated with or without test compounds  
222 for seven days in medium without Tet, and another seven days in medium with Tet when cccDNA formation  
223 and virus production relies exclusively on restored cccDNA and not on the transgene. Supernatants were  
224 harvested at day-14, clarified by centrifugation at 2,550 x g for 5 min, and stored at -70°C until use. Levels of  
225 HBeAg secreted in the culture medium were measured by using HBeAg ELISA kit (BioChain Institute Inc).

226 Hayward, CA) according to the manufacturer's protocol. The concentration of compound that reduced levels  
227 of secreted HBeAg by 50% (EC<sub>50</sub>) was determined by linear regression.

228 **Evaluation of Intracellular HBV DNA and cccDNA Levels.** DNA was extracted from HepAD38 cells for  
229 cccDNA detection. On day 14 of the experiments, DNA was purified from cells using a commercially  
230 available kit (Plasmid Miniprep Kit, Qiagen) or a modified Hirt extraction (34). All samples were treated with  
231 plasmid-safe adenosine triphosphate (ATP)-dependent deoxyribonuclease (PSAD) (Epicentre, Lucigen  
232 Corporation, Middleton, WI) at 37°C for 18 h, followed by incubation at 70°C for 30 min to inactivate PSAD.  
233 For HBV cccDNA amplification, we used TaqMan primers as previously shown (35) to specifically amplify  
234 cccDNA (forward primer: 5' ACTCTTGGACTCBCAGCAATG3', reverse primer: 5'-  
235 CTTTATACGGGTCAATGTCCA-3', and probe: 5'-FAM-  
236 CTTTTTCACCTCTGCCTAATCATCTCWTGTTCA-TAMRA-3') using the LightCycler 480 instrument  
237 (Roche).

238 **Anti-HBV Evaluation in Primary Human Hepatocytes (PHH).** PHH were seeded on collagen-coated 48-  
239 well plates at 1.4x10<sup>5</sup> cells/well in InVitroGro CP (BioIVT) medium for three hours, and then replenished  
240 with maintenance HI medium - InVitroGro HI plus Torpedo antibiotic mix (BioIVT). After 24 h, cells were  
241 incubated with HBV inoculum at multiplicity of infection (MOI) of 1,000 genome equivalent per cell in  
242 maintenance HI medium containing 4% PEG-8000. The HBV inoculum was removed 24 h post infection, and  
243 the cultures were maintained in HI medium for four days. Infected PHH cells were then incubated with the  
244 indicated concentrations of test compounds for seven days, when the medium with or without compound was  
245 replenished. After a total of 10 days, supernatants were harvested and HBV DNA production was quantified  
246 by qRT-PCR using the following HBV specific primers: HBV-AD38-qF1 (5'-CCGTCT GTG CCT TCT CAT  
247 CTG-3'), HBV-AD38-qR1 (5'- AGT CCA AGA GTY CTC TTATRY AAG ACC TT-3'), and HBV-AD38-  
248 qP1 (5'-FAM-CCG TGT GCA /ZEN/CTT CGCTTC ACC TCT GC-3'BHQ1).

249 **Electron Microscopy.** Samples of HBV Cp149 dimers and capsids were prepared as previously described  
250 (17). Samples were fixed onto a charged carbon grid and stained by uranyl acetate contrast agent for 15 min.  
251 EM images were collected using a JEOL JEM-1400 electron microscope operating at 120 kV at 25,000 -  
252 35,000 x magnification (Emory University Robert P. Apkarian electron microscopy core facility).

253 **Thermal Shift Fluorescence Assay.** Samples were prepared containing 2  $\mu$ M HBV Cp149 capsids and  
254 varying concentrations of compounds (1-80  $\mu$ M) with < 1% DMSO to a final volume of 40  $\mu$ L. Sypro orange  
255 was added to each well at 2  $\mu$ L of 1:50 dilution. Each measurement was made in triplicate across two samples.  
256 Sypro orange fluorescence was monitored continuously as temperature scanned from 45-95°C at a rate of  
257 1°C/min on a Light Cycler 480 (Roche).

258 **Confocal microscopy.** Experiments were performed on HepAD38 cells. Cells were maintained in DMEM  
259 supplemented with 10% fetal bovine serum (Gibco, France) and 1% antibiotics (penicillin/streptomycin:  
260 Gibco, France) at 37°C in 5% CO<sub>2</sub>. HepAD38 cells were seeded for 6 hours, washed and incubated in DMEM.  
261 Next, 1% v/v DMSO or 100  $\mu$ M of GLP-26 (in 1% v/v DMSO) were added to this medium. After 24 h of  
262 treatment, the medium was refreshed and cells were incubated for additional 24 h. HepAD38 cells were fixed  
263 with 4% paraformaldehyde/PBS, permeabilized with 0.2% triton/PBS and blocked for 45 min with 0.4% of  
264 BSA. Cells were then incubated with a human anti-HBc antibody (36) and after successive washing in PBS,  
265 with Alexa fluor 488 goat anti-human (ThermoFisher scientific). The last wash contained DAPI and cells  
266 were kept in PBS at 4°C until observation. Fluorescence confocal images were taken using a confocal  
267 microscope LEICA SP8 gSTED equipped with 63x PL APO 1.40 CS2 Oil, a laser diode at 405 nm for DAPI  
268 and an argon laser at 488 nm for Alexa 488.

269 **Drug Combination Study.** Drug interactions were analyzed using CalcuSyn (Biosoft, Ferguson, MO, USA)  
270 computer software For the median-effect analysis, the drugs were combined at a 5:1 ratio (GLP-26 + entecavir

271 - ETV) based on their EC<sub>50</sub> values. Five to six concentrations of each single drug, or in combination, were  
272 performed in at least two independent experiments (37).

273 **Stability in Mouse, Rat, Dog and Human Plasma.** One mL of mouse, rat, dog or human plasma containing  
274 5 mM MgCl<sub>2</sub> were used for the stability assay. Propantheline bromide at 10 μM was used as a positive  
275 control. The reaction was started by adding 10 μL of 1 mM stock solution of GLP-26 to give a final  
276 concentration of 10 μM and incubated at 37°C. At selected times (0, 0.25, 0.5, 1, 2, 4, 6 and 24 h), 100 μL  
277 aliquots were taken and the reaction was stopped by mixing with 400 μL of ice-cold acetonitrile. The samples  
278 were centrifuged and 100 μL of the supernatant was mixed with 100 μL of LC-MS mobile phase then  
279 subjected to LC-MS/MS analysis.

280 **Stability in Mouse and Human Liver Microsomes.** The reaction mixture was prepared in a total volume of  
281 1.5 mL containing 5 mM of MgCl<sub>2</sub>, 100 mM of potassium phosphate buffer (pH 7.4), 1 mg/mL mouse or  
282 human liver microsome and 1 μM compound. The reaction was initiated by adding 1 mM NADPH to the  
283 mixture and incubated at 37°C. At selected times (0, 5, 15, 30, 45, 60 and 90 min), 200 μL aliquots were taken  
284 and the reaction stopped by mixing with 200 μL of 70% ice-cold methanol. The samples were centrifuged and  
285 supernatant were subjected to LC-MS/MS analysis. Propranolol at 10 μM was used as a positive control.

286 **Pharmacokinetic Studies:** GLP-26 (3 mg/mL) in PBS containing 20% DMSO and 20% PEG-400, was given  
287 by intravenous (IV) injection (15 mg/kg) and orally (PO) 30 mg/kg to female CD-1 mice. At the given time  
288 points (0.5 h, 2 h, 4 h, and 7 h), blood samples were collected using heparinized capillaries. Samples were  
289 centrifuged at 15,000 g for 10 min. Subsequently, blood plasma was collected and frozen at -80°C until  
290 analysis using LC-MS/MS (ACQUITY UPLC BEH C18 Column, 130Å, 1.7 μm, 3 mm X 30 mm and Turbo-  
291 Ionspray™ Interface in the negative ion-mode on AB Sciex 5500Qtrap). IACUC approval was obtained  
292 prior to initiation of these mouse studies.

293 **Generation of HUHEP Mice, HBV Infection and Treatment.** BALB/c Rag2<sup>-/-</sup>IL-2Rγc<sup>-/-</sup>NOD.*sirpa* uPA<sup>tg/tg</sup>  
294 (BRGS-uPA) mice were intrasplenically injected with 7 x 10<sup>5</sup> freshly thawed human hepatocytes (BD  
295 Biosciences, Corning) to generate HUHEP mice as previously described (25). Liver chimerism of HUHEP  
296 mice was evaluated with a species-specific human albumin (hAlb) ELISA (Bethyl Laboratories) on plasma  
297 samples as previously described (25). HUHEP mice with ≥100 μg/ml hAlb were intraperitoneally infected  
298 with 1x10<sup>7</sup> HBV genome equivalents as previously described (24). HBV-infected mice with >10<sup>6</sup> HBV DNA  
299 copies/ml were treated *per os* with either ETV 0.3 mg/kg/day (Baraclude, BMS), or GLP-26 60 mg/kg/day  
300 (dissolved in PEG400, Sigma), or the combination of ETV+GLP-26 at the same doses, delivered in MediDrop  
301 Sucralose (Clear H<sub>2</sub>O) continuously for 10 weeks. For the rebound phase, mice were returned to regular  
302 drinking water. Animals were housed in isolators under pathogen-free conditions with humane care.  
303 Experiments were approved by an institutional ethical committee at the Institut Pasteur (Paris, France) and  
304 validated by the French Ministry of Education and Research (MENESR # 02162.02).

305 **Virological Measurements in HUHEP Mice.** HBV DNA was extracted from plasma and quantified by  
306 qPCR as previously described (24). HBeAg was quantified with an ELISA chemiluminescent immunoassay  
307 kit (Autobio, China), and HBsAg was quantified with the MONOLISA HBsAg Ultra kit (Bio-Rad) following  
308 manufacturer's protocols.

309

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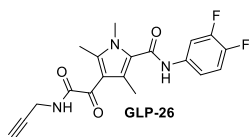
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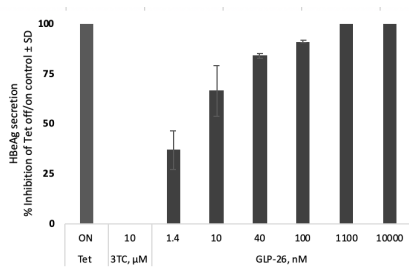


445

446 **Figure 1.** Structure of GLP-26.

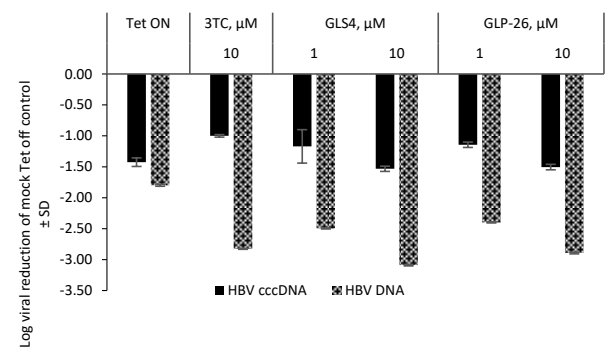
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448 A.



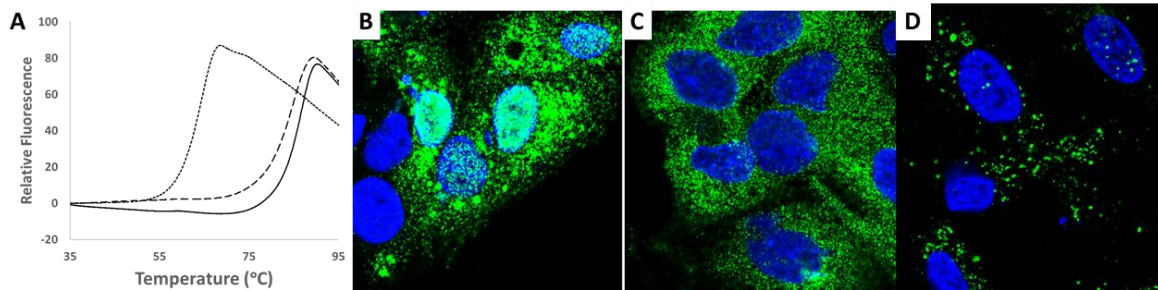
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450 B.



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452 **Figure 2. Decrease of cccDNA markers in HepAD38 cells by GLP-26.** A) Percent inhibition of HBeAg  
 453 secretion by ELISA; Cells with or without drugs were incubated in the absence of tetracycline for 7 days,  
 454 followed by addition of Tet to culture of both untreated and drug-treated cells for another 7 days (7-14 days).  
 455 Tet ON, cells cultured in the presence of tetracycline for 14 days. Inhibition (%) of HBeAg secretion was  
 456 determined relative to untreated Tet off/on control. and B) The levels of HBV DNA and cccDNA were  
 457 quantified by qPCR and log viral reduction was determined relative to untreated mock Tet off control. All  
 458 values represent the average of at least two independent experiments and samples were performed in duplicate  
 459 ± SD.

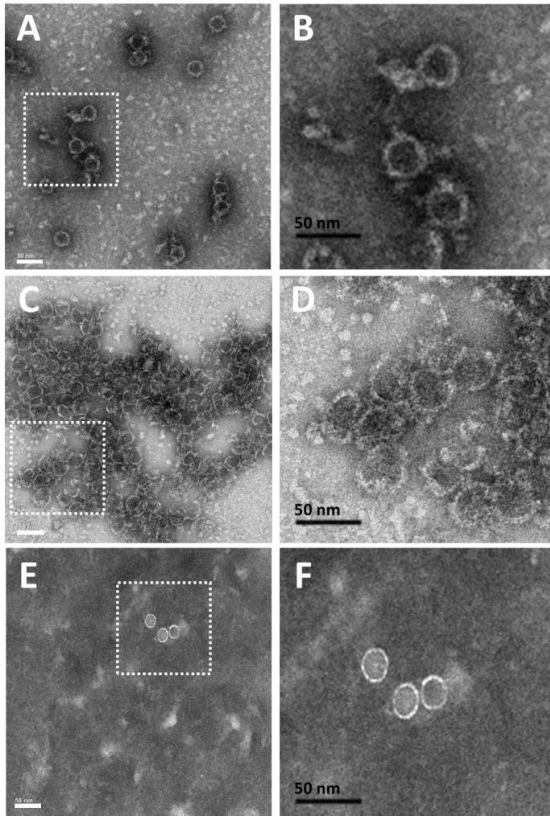


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462 **Figure 3. Effects of GLP-26 binding to HBV capsids.** A) Thermal shift fluorescence assay thermograms for  
 463 vehicle (dotted line), GLS4 (dashed line) and GLP-26 (solid line) treated HBV Cp149 capsids. Confocal  
 464 immunofluorescence microscopy images showing HBV Core protein (in green) distribution in HepAD38  
 465 hepatocytes after 24 h for B) vehicle (DMSO) , C) GLP-26 (1  $\mu$ M). D) GLS-4 (1  $\mu$ M). Nuclei are DAPI  
 466 stained (blue).

467



468

469 **Figure 4. Effects of GLP-26 on the HBV Cp149 capsid morphology determined by negative-stain**  
470 **electron microscopy.** A and B) HBV Cp149 capsid particles treated with vehicle and. C and D) HBV Cp149  
471 treated with GLP-26 (25  $\mu$ M) prior to assembly initiation. E and F) Pre-formed HBV-Cp149 capsid particles  
472 treated with GLP-26 (25  $\mu$ M). Black bars represent 50 nm scale.

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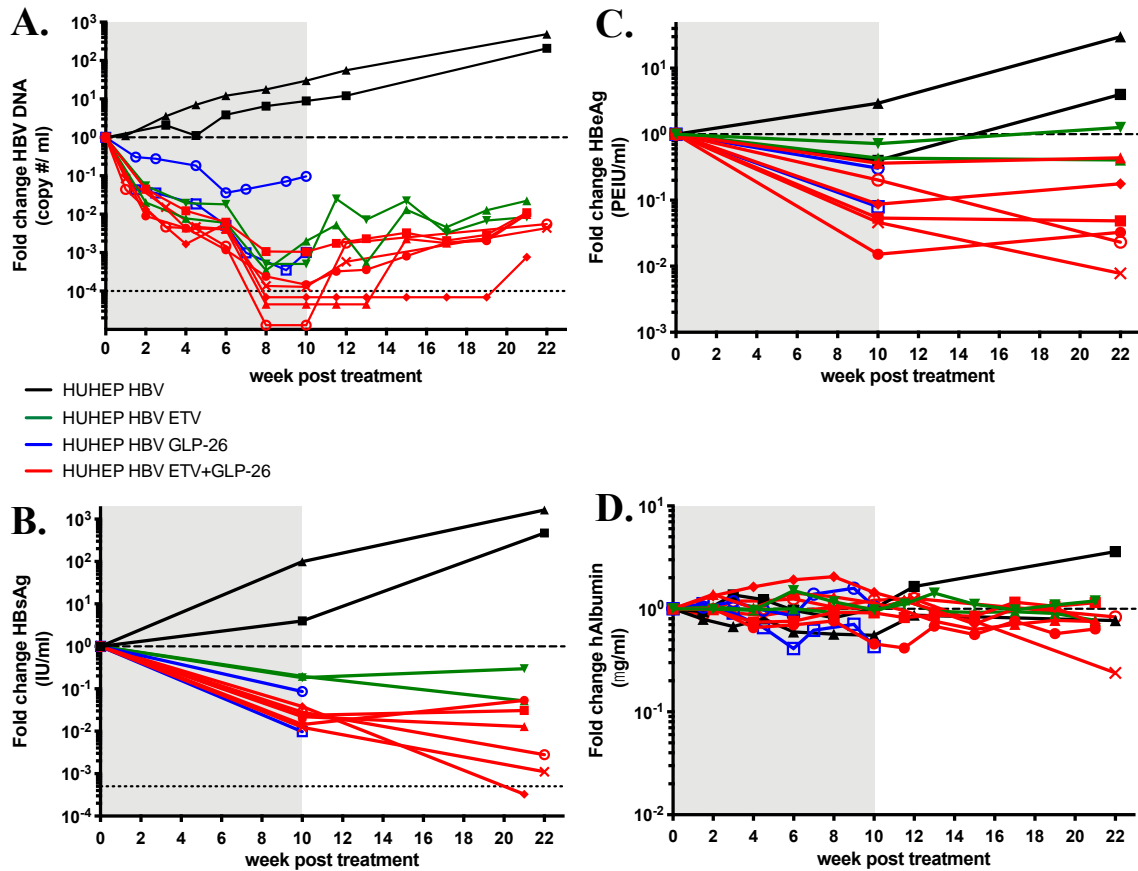
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481 **Figure 5. Sustained antiviral activity of GLP-26 in HBV-infected humanized mice by oral**  
 482 **administration.** Effect of entecavir (ETV) 0.3 mg/kg/day, GLP-26 60 mg/kg/day or the combination of ETV  
 483 + GLP-26 at the same doses on A) HBV DNA, B) HBsAg, C) HBeAg, and D) human Albumin in HBV-  
 484 infected HUHEP mice. Treatment period is indicated in the grey shaded area followed by a rebound period,  
 485 the lower limit of detection is shown as a thin dotted horizontal line in A) and B). The thick dotted horizontal  
 486 line shows the reference point of one to evaluate fold changes. Each full line represents the longitudinal

487 results from an individual HBV-infected HUHEP mouse either untreated (black line), ETV (green line), GLP-  
 488 26 (blue line), or ETV+GLP-26 (red line) treated.

489

490 **Table 1.** Anti-HBV activity of GLP-26 in HepAD38 cells.

Drug <sup>a</sup>	Anti-HBV Activity ( $\mu\text{M}$ )	
	EC <sub>50</sub>	EC <sub>90</sub>
<b>GLP-26</b>	0.003 $\pm$ 0.002	0.014 $\pm$ 0.002
<b>GLS4</b>	0.08 $\pm$ 0.02	0.28 $\pm$ 0.06
<b>3TC</b>	0.41 $\pm$ 0.36	1.65 $\pm$ 0.92
<b>ETV</b>	0.0006 $\pm$ 0.0003	0.011 $\pm$ 0.002
<b>TDF</b>	0.005 $\pm$ 0.0004	0.070 $\pm$ 0.010

491 <sup>a</sup>3TC: Lamivudine; ETV: Entecavir. TDF: Tenofovir disoproxil fumarate

492 All values represent the average of at least two independent experiments and samples were performed in  
 493 duplicate  $\pm$  SD.

494

495 **Table 2.** Anti-HBV activity of GLP-26 in primary human hepatocytes (PHH).

496

Drug	Anti-HBV Activity ( $\mu\text{M}$ )	PHH
	EC <sub>50</sub>	CC <sub>50</sub> ( $\mu\text{M}$ )
<b>GLP-26</b>	0.04 $\pm$ 0.01	>10
<b>GLS4</b>	4.34 $\pm$ 1.62	>10
<b>TDF<sup>a</sup></b>	0.27 $\pm$ 0.23	>10

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<sup>a</sup>TDF: Tenofovir disoproxil fumarate. All values represent the average of at least two independent experiments and samples were performed in duplicate.

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