A new high-content screening assay of the entire hepatitis B virus life cycle identifies novel antivirals

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Supplementary materials and methods

Cell culture

HepAD38 cells (provided by Dr. Seeger, Fox Chase Cancer Center, USA) and HepG2-NTCPsec+ cells were generated as described previously [1]. HepG2-NTCPsec+ cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5 mM L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, and 5 µg/mL blasticidin. For HBV infection experiments in 384-well plates, HepG2-NTCPsec+ cells were cultured in infection medium: DMEM, 10% FBS, 5 mM L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, and 2.5% dimethylsulfoxide (DMSO). HepAD38 cells were cultured in DMEM/F-12 supplemented with 10% FBS, 50 U/mL penicillin, 50 µg/mL, 400 µg/mL geneticin, 5 µg/mL insulin, 50 µM hydrocortisone hemisuccinate, 0.3 µg/mL tetracycline, and 400 µg/mL geneticin.

Preparation of infectious HBV from cell culture

For generating HBV particles, HepAD38 cells were cultured for one month in DMEM/F-12 supplemented with 10% FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, 400 µg/mL geneticin, 5 µg/mL insulin, and 50 µM hydrocortisone hemisuccinate. Supernatants were collected once a week, and cells were replenished with fresh medium. Harvested supernatants were filtered with Millipore Sterile Vacuum Filter (0.2 µm) for removing cell debris and mixed with polyethylene glycol (PEG) 8000 to a final concentration of 6%. For precipitation of virus particles, supernatants were centrifuged at 17,600 g for 60 min. The precipitate was re-suspended to the original volume in PBS containing 10% FBS.

HBV preS1 peptide binding and internalization assay

PreS1-cell surface attachment was evaluated by incubating the cells at 37°C for 30 min with Alexa633-labeled peptide spanning amino acids 2–48 of the myristoylated preS1 region (also referred to as preS1 probe), as described previously [2].

Southern blot for HBV cccDNA detection

HBV cccDNA was isolated using the Hirt extraction method with minor modifications, and analyzed by Southern blot hybridization as previously described [3,4]. Briefly, HBV infected cells from one well of 6-well plates were lysed in Hirt extraction buffer (50 mM Tris-Cl pH 7.4, 10 mM EDTA, 150 mM NaCl, and 1% SDS) at 37°C for 1 h. Cell lysates were incubated with 0.5 M KCl overnight at 4°C. After centrifugation, cccDNA in the supernatant was purified by phenol-chloroform extraction (1:1) and ethanol precipitation with 20 µg glycogen. cccDNA samples were dissolved in TE buffer and treated with 0.3 U of plasmid-safe ATP-dependent DNase (Epicentre, Madison, WI, USA) for 10 min at 37°C. Samples were separated by electrophoresis on a 1.3% agarose gel and transferred onto a nylon membrane (HybondTM-XL; GE Healthcare, USA). After UV cross-linking, the membrane was pre-hybridized and hybridized with a 32P-labeled HBV-specific probe, and cccDNA bands were examined using a phosphorimager scanner (BAS-2500; Fujifilm).

Detection of infected cells by IFA

Cells were fixed in 4% paraformaldehyde (w/v) for 2 h at room temperature after washing with PBS using ELx405 automated washer (Biotek, Winooski, Vermont, USA)

and permeabilization with 0.075% Triton X-100 for 6 min. After washing with PBS, cells were blocked in 5% BSA solution (Sigma-Aldrich, MO, USA) overnight. Cells were incubated for 2 h at room temperature in a 1:1500 dilution of polyclonal rabbit anti-HBcAg antibody (DAKO, Agilent, CA, USA) in PBS with 5% BSA. Cells were stained with goat anti-rabbit Alexa Fluor 488 (Invitrogen, MA, USA) for 1 h at a dilution of 1:1500, and nuclei were stained with Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA). Cell images were acquired with the Opera High Content Screening System (PerkinElmer, MA, USA) at 10× magnification with 405 nm excitation/450 nm emission for Hoechst-stained nuclei, and 488 nm excitation/540 nm emission for HBcAg detection. To ensure precise and reproducible read-out conditions, 4 or 6 images per well were taken (approximately 70–40% of the total well area) in a triplicate experimental setup (n=18, 12). The image-based quantification of HBV infection rates was performed in-house using image-mining software to detect cell nuclei and discriminate between HBc positive and negative cells.

Image-based data analysis for dose-response curve analysis of HBV reference compounds

Images were analyzed with in-house image mining software. The absolute numbers of host cell nuclei and viral protein-positive cells were quantified by segmentation, and used to calculate relative numbers (percentage) of infected cells and cell viability. To calculate the test compound infection inhibition, raw data values were normalized to average infection values of uninfected and untreated, infected cells. Average values for infection ratio and cell viability of 6 images per well were calculated in a duplicate or triplicate experimental setup, and used to determine standard deviation (SD) values. EC_{50}

and CC_{50} values were calculated by GraphPad Prism software using sigmoidal dose-response method.

Detection of HBV rcDNA by quantitative real-time PCR

HBV DNA from secreted particles was isolated using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) and analyzed by qPCR as previously described [1]. Briefly, primers 5'-ACTCACCAACCTCCTGTCCT-3', 5'- GACAAACGGGCAACATACCT-3', and a fluorogenic 5'-end nuclease probe 5'FAM-TATCGCTGGATGTGTCTGCGGCGT-TAMRA3' (TaqMan) were selected from a highly conserved region of the S gene. Conserved regions were chosen after careful analysis of 27 full-length HBV sequences belonging to genotypes A to H.

The premix Ex Taq™ PCR master mix (TaKaRa Bio, Otsu, Japan) with 0.2 µM primer, 0.1 µM probe, and 2 µL extracted DNA were prepared and analyzed with a VIIA7 real-time PCR detection system (Thermo Scientific, Waltham, USA).

Reference compounds and chemical libraries

Reference compounds used in these studies included MyrB (PSL GmbH, #7003942), MA 18/07 (provided by Dr. Glebe, University of Giessen, Germany), hepabulin (SK Plasma, # SK-HBIG), CCC-0975 (provided by Dr. Guo, University of Pittsburgh, USA), Bay 41-4109 (MedChem Express, #HY-100029), N-hydroxyisoquinolinedione (provided by Dr. John Tavis, University of Saint Louis, USA), lamivudine (Sigma-Aldrich, # L1295), tenofovir disoproxil fumarate (Selleckchem #S1400). A library of 2,102 small molecules was composed of FDA-approved drugs and experimental bioactives from Tocris Bioscience (Bristol, UK), Selleck Chemicals (TX, USA), LOPAC (Sigma-Aldrich, MO,

USA), Prestwick Chemical (Illkirch, France), and MicroSource Discovery Systems (CT, USA). All compounds were dissolved at 10 mM stocks in 100% DMSO (v/v). For HCS, compounds were dissolved at 2 mM with 100% DMSO (v/v) into 384-well polypropylene storage plates and placed at -20°C until use.

Hit confirmation by DRC analysis

The assays were performed using a similar procedure, as described above. Confirmation of selected hits, compounds were cherry-picked from internal library stocks and to intermediate 384-well polypropylene plates. Compounds were tested in duplication by 10-point serial dilutions, starting at 25 µM in 0.5 % DMSO (v/v). The EC_{50} and CC_{50} values were averaged from the duplicates.

Statistical analysis

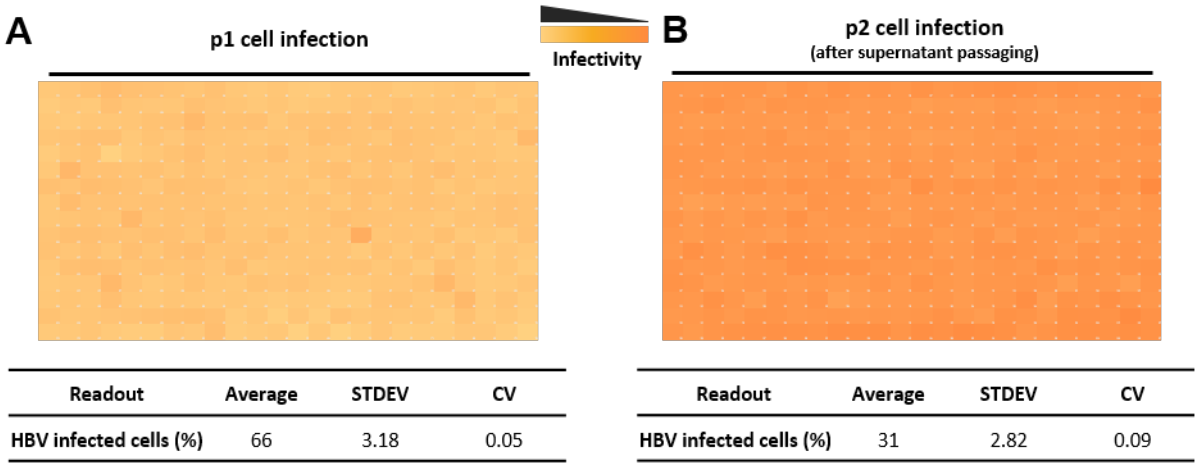
The data in this study were presented as the mean ± SD, and statistical significance was determined by the Student's t-test. *P*-values are indicated by asterisks (***p <0.001, **p <0.01, *p <0.05). Z' factor is used to evaluate the quality of the HTS assay. It was determined using the following formulas [5]: Z' factor = 1 − ([3σc+ + 3σc−]/[µ c+ − µ c−]), where σ, µ, c+, and c− were the standard deviations (SDs) (σ) and averages (µ) of the high (c+) and low (c−) controls. Z' factors between 0.5 and 1 indicated an excellent assay with good separation between controls.

Evaluation of selected hits in HCV, HIV-1, and SARS-CoV-2 infectious cell culture systems

Effect of selected hits on secretion of HCV virus was evaluated as previously described [6]. Briefly, Huh-7.5 cells were electroporated with genotype 2a HCV RNA (JFH1-

E2p7-EGFP), plated, and treated with inhibitors. At 2 days post-treatment, inhibitors were washed out 3-times with 1X DPBS, and cells were incubated for additional 2 days. At 4 days post-electroporation, cell culture supernatants were transferred to naïve target cells (Huh-7.5) and incubated for 3 days. HCV infection was analyzed by calculating GFP positive cells per total cell nuclei. HIV experiments were conducted as described in Ianevski et al. [7]. Briefly, to produce HIV-1, 6×10^6 ACH-2 cells, which possess a single integrated copy of the provirus HIV-1 strain LAI (NIH AIDS Reagent Program), were seeded in 10 mL RPMI-1640 medium supplemented with 10% FBS and Pen–Strep. Virus production was induced by the addition of 100 nM phorbol-12-myristate-13-acetate. The cells were incubated for 48 h, and the HIV-1-containing medium was collected. The amount of HIV-1 was estimated by measuring the concentration of HIV-1 p24 in the medium using anti-p24-ELISA, which was developed in-house. Recombinant purified p24 protein was used as a reference. For inhibitor efficacy and toxicity testing using HIV-1, approximately 4×10^4 TZM-bl cells were seeded in each well of a 96-well plate in DMEM medium supplemented with 10% FBS and Pen–Strep. The cells were grown for 24 h. The medium was then replaced with DMEM containing 0.2% BSA, Pen–Strep, and the compounds in 3-fold dilutions at 7 different concentrations. No compounds were added to the control wells. The cells were infected with HIV-1 (corresponding to 300 ng/mL of HIV-1 p24) or mock. At 48 h post-infection (hpi), the media was removed from the cells, the cells were lysed, and firefly luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI, USA). In a parallel experiment, a CTG assay was performed to measure cell viability. To evaluate inhibitors in SARS-CoV-2 infected cells, approximately 4×10^4 Vero-E6 cells were seeded per well in 96-well plates. The cells were grown for 24 h in DMEM supplemented with 10% FBS and Pen–Strep. The medium

was then replaced with DMEM containing 0.2% BSA, Pen–Strep, and the compounds in 3-fold dilutions at 7 different concentrations. No compounds were added to the control wells. The cells were uninfected (mock) or infected with SARS-CoV-2-mCherry strains at a multiplicity of infection (MOI) of 0.1 [8]. At 48 h post-infection, a CellTiter-Glo (CTG) assay was performed to measure cell viability. Drug efficacy on SARS-CoV-2-mCherry infected cells was measured on PFA-fixed cells using fluorescence as a read-out.



Readout	Average	STDEV	CV
HBV infected cells (%)	66	3.18	0.05

Readout	Average	STDEV	CV
HBV infected cells (%)	31	2.82	0.09

Fig. S1. HBV 2-step infection HTS assay validation. Control run in p1 cells (left) and p2 cells (right) performed with an entire 384-well plate containing 0.5% DMSO (v/v). (A) Heat map analysis shows overall HBV infected cell (%) from high to low (light to dark orange) and shows no systematic errors and no observed edge effects. (B) The value of average, standard deviation, and %CV for HBV infected cell (%) and cell number in p1 and p2 cells.

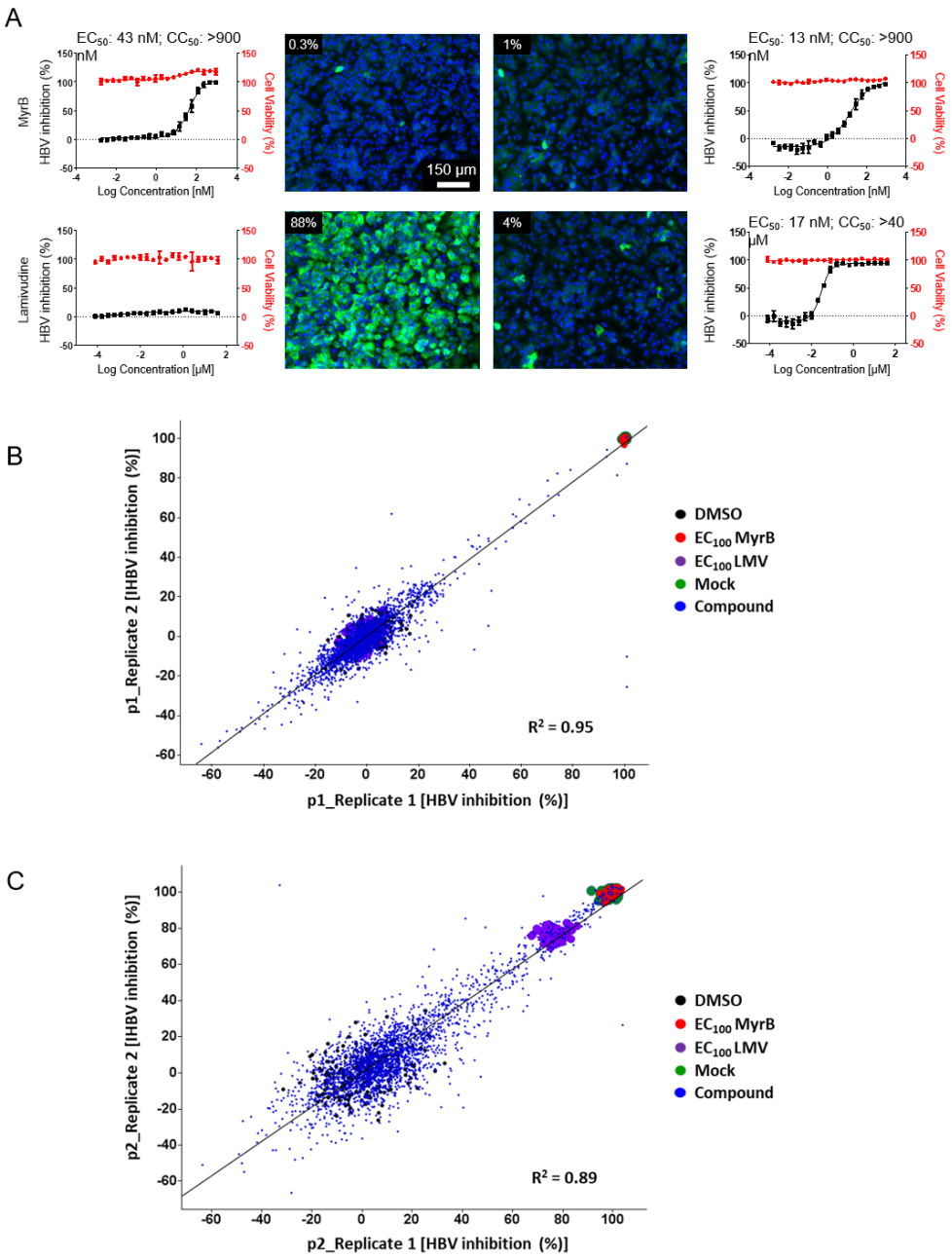


Fig. S2. Screening performance validation. (A) Reference DRC sensitivity analysis with 2-fold serial dilutions of MyrB (900 nM) and LMV (40 µM) in HTS. (B-C) Scatter plot reproducibility analysis in p1 cell and p2 cell in duplicates with controls and compounds. HBV inhibition (%) with compounds (blue), 0.5% DMSO (v/v) (black), 900 nM MyrB (red), 40 µM LMV (purple) as an EC_{100} and Mock (green).

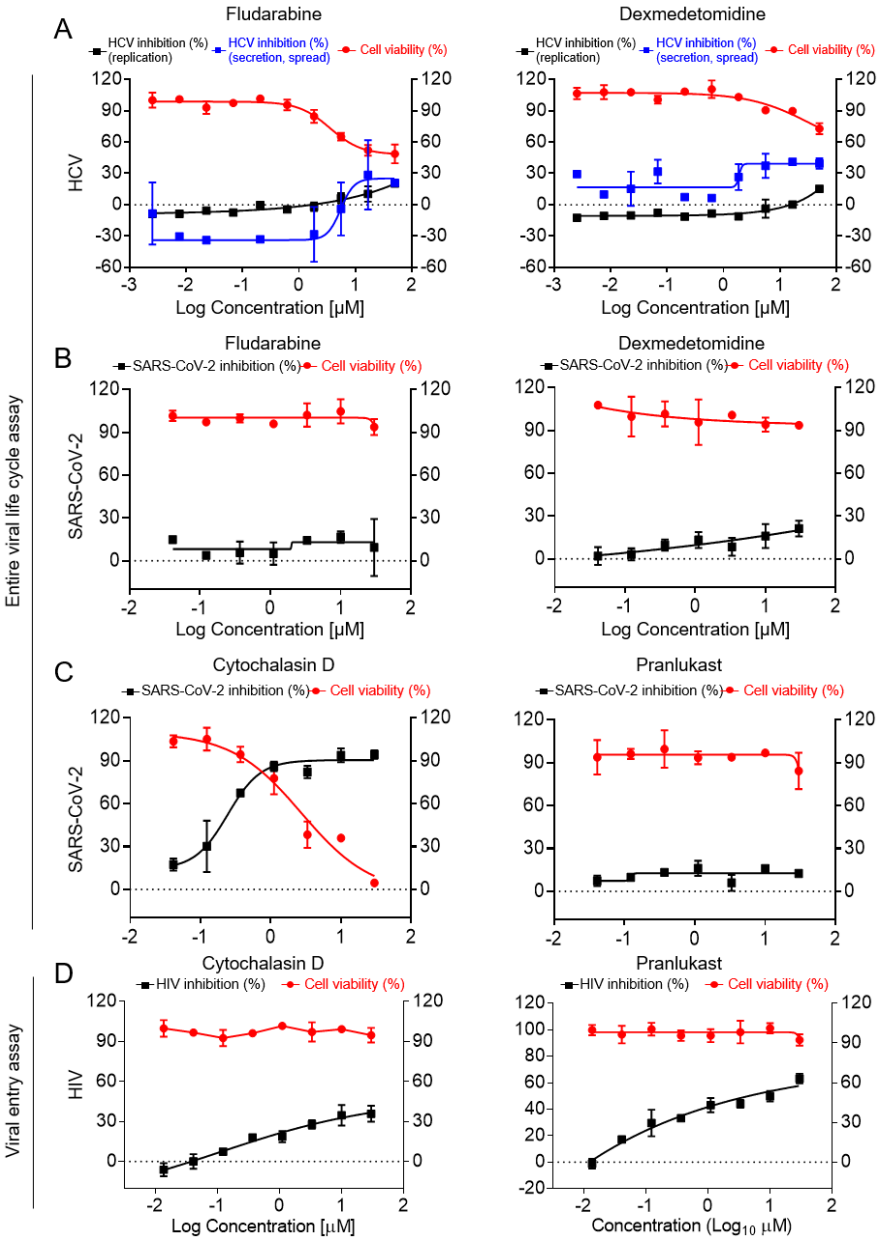


Fig. S3. Evaluation of selected inhibitors in HCV, SARS-CoV-2, and HIV infectious cell culture systems. (A) HCV producer cells were treated with 3-fold serially diluted inhibitors for 72 h and analyzed as described in the Material and Methods section. Antiviral activities of inhibitors before (virus replication) and after (virus secretion) supernatant transfer are shown in black and blue, respectively, and cell viability in red. (C) Vero-E6 cells were treated with 3-fold serially diluted inhibitors and infected with SARS-CoV-2-

mCherry strains at an MOI of 0.1. At 48 h post-infection, drug efficacy on SARS-CoV-2-mCherry infection (in black) and cell viability were measured (in red). (D) TZM-bl cells were pre-treated with 3-fold serially diluted inhibitors and infected with HIV-1 or mock. At 48 hpi, antiviral activities of inhibitors and cell viability were determined shown in black and red, respectively.

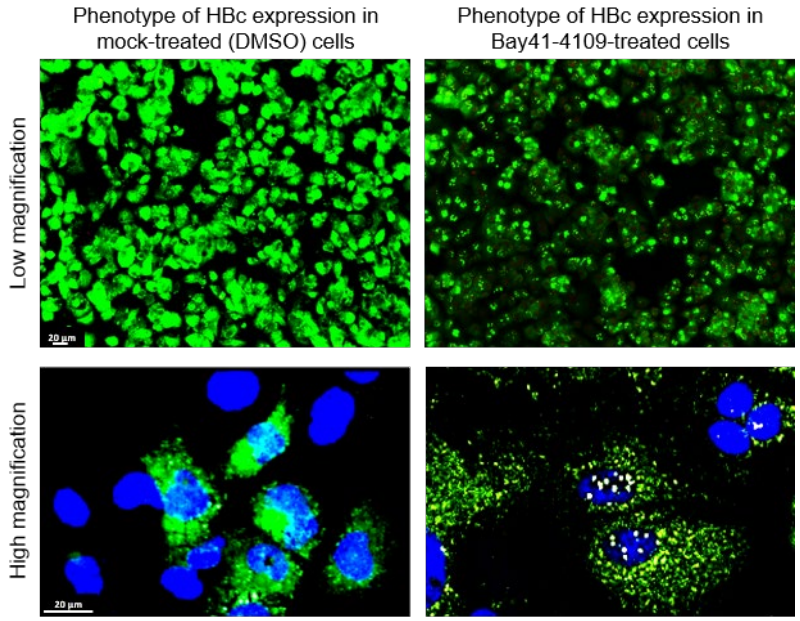


Fig. S4. CpAM induced aggregated HBcAg phenotype detectable by HCS assay. HepG2-NTCPsec+ cells were seeded at day 0 in 384-well plates and pre-incubated with Bay41-4109 inhibitor at 2 h. Cells were inoculation with HBV for 18 h on day 1. After repeated washing on day 2, cells were treated with reference inhibitor and cultured until day 6, followed by IFA of HBc and counterstaining with Hoechst (nuclei). Infected cells were identified microscopically and analyzed, as described in Figure 1. Upper and lower row of images depicts low and high magnification of images, respectively.

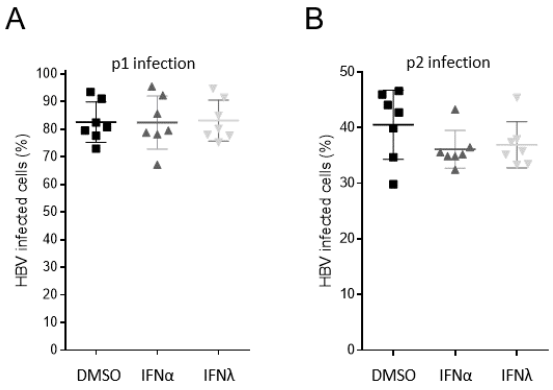


Fig. S5. Evaluation of IFNα and IFNλ in the HBV infectious cell culture system. HepG2-NTCPsec+ cells were infected with HBV and treated with IFNα and IFNλ over several weeks. (A) P1 cells were analyzed for HBcAg expression. (B) Supernatants of infected p1 cells were passaged to naïve target p2 cells for infection, and HBcAg expression analyzed via IFA.

Table S1. HBV 2-step p1/p2 cell infection assay adaptation to HTS laboratory automation. SOP workflow of the 2-step HBV p1/p2 cells infection assay by using automated workstations.

Step	dpi	Parameter	Cells	Description	Automation device
1	-1	Cell plating	p1	Dispense 8,000 HepG2-NTCPsec+cells per 20 µL into 384-well assay plate with DMEM/2.0% DMSO	Thermo Scientific Matrix WellMate Dispenser
2	0	Compound treatment	p1	Add 0.25 µL of 2 mM compound stock (100% DMSO) into 384-well assay plate	CyBi - HummingWell
3		HBV inoculation	p1	Add 2,000 HBV GEq/cell HBV inoculum supplemented with PEG to the p1 cell supernatant (4% final PEG concentration), The compounds final concentration is 10 µM (in 0.5% DMSO)	Thermo Scientific Matrix WellMate Dispenser
4	1	Cell washing	p1	Twice repeated wash with aspirating on the ELx405 automated washer and dispensing with WellMate	BioTek ELx405 automated washer / Thermo Scientific Matrix WellMate Dispenser
5		Compound re-treatment	p1	Add 10 µL of 50 µM compound to reach final top concentration of 10 µM	Apricot Designs - PP384
6	6	Cell plating	p2	Dispense 8000 cells per 20 µL into 384-well assay plate with DMEM/2.5 % DMSO	Thermo Scientific Matrix WellMate Dispenser
7	7	Supernatant transfer	p1 → p2	Transfer 35 µL of supernatant from total 50 µL of p1 cell to p2 cell	Apricot Designs - PP384
8		PEG supplementation	p2	Add 20 µL of PEG media (media 12.5 µL + 40% PEG 7.5 µL) to each well	Thermo Scientific Matrix WellMate Dispenser
9		Assay plate spin	p2	Centrifuged at 400 g, 5 min	
10		Fix	p1	4% PFA (w/v) in 1X PBS for 2 hours	Thermo Scientific Matrix WellMate Dispenser
11	8	IFA staining	p1	Three times repeated wash with aspirating on the ELx405 automated washer and dispensing with WellMate	BioTek ELx405 automated washer / Thermo Scientific Matrix WellMate Dispenser
12		Assay readout	p1	10X magnification, 405 nm/450 nm & 488 nm/540 nm (ex/em)	PE Opera High Content Screening System / Cell::explorer Platform
13		Cell washing	p2	Twice repeated wash with aspirating on the ELx405 automated washer and dispensing with WellMate	BioTek ELx405 automated washer / Thermo Scientific Matrix WellMate Dispenser
14	13	Cell fixation	p2	4% PFA (w/v) in 1X PBS for 2 hours	Thermo Scientific Matrix WellMate Dispenser
15	14	IFA staining	p2	Three times repeated wash with aspirating on the ELx405 automated washer and dispensing with WellMate	BioTek ELx405 automated washer / Thermo Scientific Matrix WellMate Dispenser
16		Assay readout	p2	10X magnification, 405 nm/450 nm & 488 nm/540 nm (ex/em)	PerkinElmer Opera High Content Screening System /cell::explorer platform

Table S2. Robustness of HBV 2-step p1/p2 cell infection assay after adaptation to HTS laboratory automation. HBV infected cell (%), cell number, and coefficient of variation (%CV) were determined for different viral loads to illustrate the robustness of the automated HTS assay in (A) p1 and (B) p2 cell infection.

A

p1 cell	GEq/cell		
	1,000	1,500	2,000
HBV infected cells (%)	55	55	62

B

p2 cell	HBV from p1 cells GEq/cell		
	1,000	1,500	2,000
HBV infected cells (%)	28	46	53

Supplementary references

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