Development of an Automated High-Throughput Screening Assay for Inhibition of HBV Infection *In Vitro*

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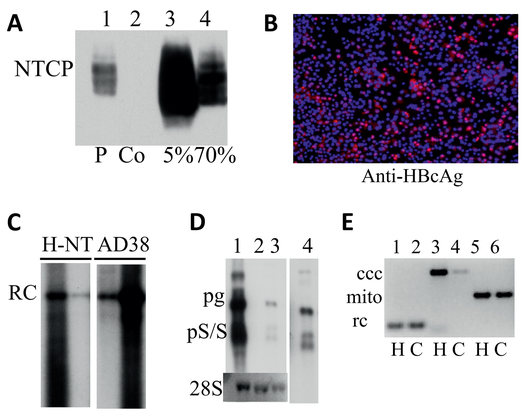
Abstract

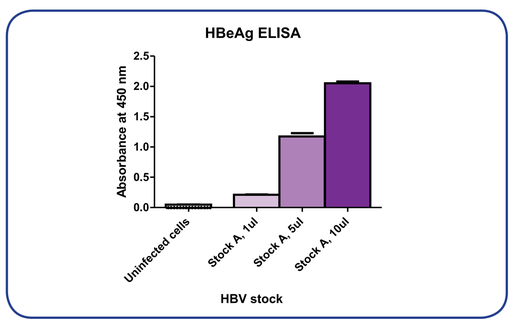
We developed and implemented a high-throughput screen (HTS) to identify small molecules that inhibit HBV infection *in vitro*. A human hepatoma cell line HepG2, stably expressing the sodium taurocholate cotransporting polypeptide (NTCP), has been constructed and characterized. HepAD38 cell culture-generated HBV was used to infect HepG2/NTCP cells. Efficient HBV replication in infected cells was demonstrated with cell ELISA specific for intracellular large, middle and small surface antigens, as well as with immunofluorescence and immunohistochemistry. In addition, efficient HBeAg and HBsAg secretion was observed, using ELISA. Using this assay, we screened a set of 50,000 compounds from ChemDiv's proprietary compound library. This set included novel compounds from an ongoing synthetic program. The library consisted of compounds with high Fsp3 score, spiro, caged structurally rigid compounds, peptidomimetics, shape and recognition element compounds, and medium- and macrocyclic compounds.

The HTS was automated and run on a Biomek robotic workstation. HepNTCP cells in the 96-well plates were infected with HBV, treated with test compounds at 10 μM for 7 days, and HBV replication was measured using ELISA for secreted HBeAg. Compound cytotoxicity was determined in parallel. Several distinct chemistry series were identified, with EC_{50} values in the low micromolar range and CC_{50} values >30 μM. Investigations on the mechanism of HBV inhibition are in progress.

Production of HepG2/NTCP cells



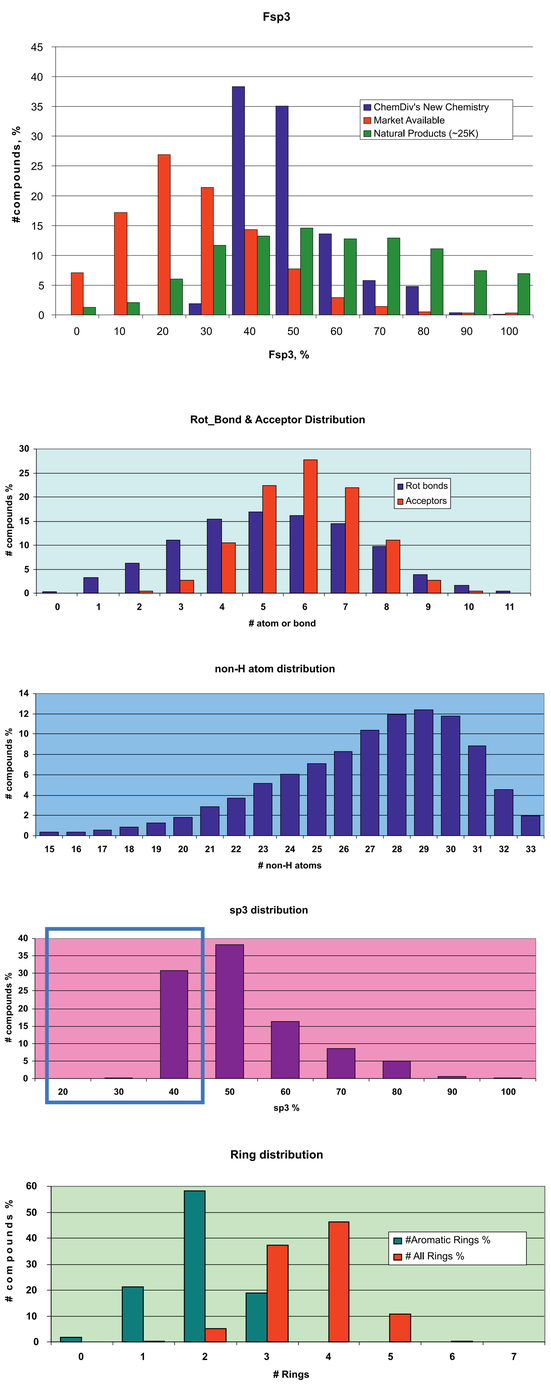
A) HepG2 cells were infected with a lentivirus expressing NTCP and GFP and sorted by FACS to enrich for populations with high NTCP levels. Lane 1, cell pool prior to FACS, lane 2, control HepG2 cells, lane 3 and 4, 5% and 70% fraction of FACS sorted cells. B) HepG2/NTCP cells were infected with HBV derived from HepAD38 cells and processed for IF analysis with an HBcAg specific antibody eight days after infection. C) Analysis of core DNA extracted from HepG2/NTCP (H-NT) cells infected with HBV. DNA was extracted 14 days after HBV infection (lanes 1 and 2). Cells were cultured without (lane 1) and with entecavir (ETV, 10 micrograms/ml)) (lane 2). DNA extracted from HepAD38 cells served as a control (lanes 3 and 4). Lane 3 was loaded with 1/10th of the amount of DNA used for lane 2. D) Northern blot analysis of total RNA extracted from HepG2/NTCP cells infected with HBV (lane 3) and HepAD38 cells as a control (lane1). Uninfected cells (lane 2) and ribosomal 28S RNA served as a control for the amount of RNA loaded onto each well. Lane 4 is a longer exposure of lane 3. E) PCR analysis of HBV DNA extracted from infected HepG2/NTCP cells with the Hirt procedure. H; total cell extracts, C; cytoplasmic DNA, ccc; cccDNA specific primers overlapping the cohesive overlap in rcDNA, mito; mitochondrial DNA specific primers, rc; primers for amplification of rcDNA.



Secreted HBeAg is detected by ELISA in the HepG2/NTCP cell cultures, infected with HBV *in vitro* with different multiplicity of infection.

HTS Compound Library

A set 50,000 of proprietary ChemDiv compound library had been selected. The selection includes compounds with drug-like properties, including diversity set from ongoing "Escape from Flat Land" chemistry program. "Escape from Flat Land" program include novel molecules with higher Fsp3 score. Increase in Fsp3 score leads to improvement of physchem parameters, better stability and may improve *in vivo* profile of the molecules. Also, we are achieving better diversity and get access to chiral natural-like compounds. Novel chemistry ensures high IP potential for indentifies hits.

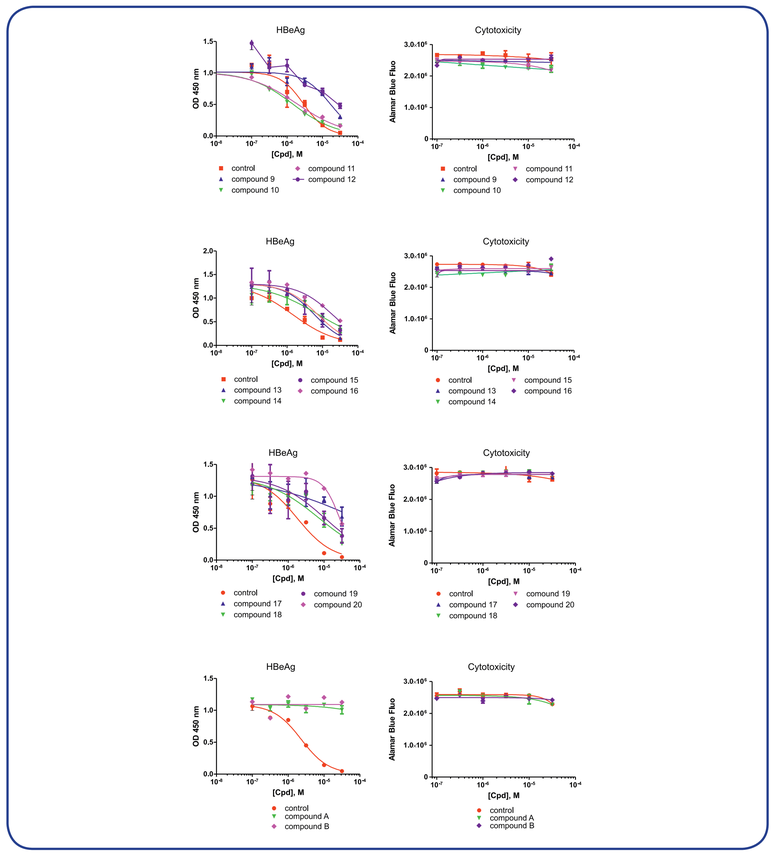


HTS workflow

HBV was concentrated from the medium of HepAD38 cells. HepG2/NTCP cells were infected with HBV in the presence of test compounds at 10 μM. Following overnight incubation, the HBV inoculum was washed out with PBS and replaced with fresh medium and test compounds at 10 μM. Seven days post infection, supernatants were removed and tested using the HBeAg ELISA. Cell viability in the corresponding wells was determined using an AlamarBlue assay. Percent HBV inhibition and cell viability was calculated for each test compound. An automated HTS was carried out in 96-well format, using a Biomek FX robotic station.

HTS results

A total of ~36K compounds were screened to date. Compounds with >50% HBV inhibition and > 80% cell viability at 10 μM were scored as primary hits. For some of these compounds, the EC_{50} and CC_{50} values were determined. As a result, 15 novel HBV inhibitors from at least 5 distinct chemistry series with EC_{50} ranging from 0.5 to 10 μM and CC_{50} > 30 μM were identified.



The EC_{50} and CC_{50} value determination for primary hits, using the HBeAg ELISA and the Alamar Blue viability assay (representative data).

Compound ID	EC_{50}, μM	CC_{50}, μM
control	1.6 +/- 0.2 (n=7)	>30
1	6.5	25
8	9.8	25
9	5.3	>30
10	0.43	>30
11	0.48	>30
12	8.8	>30
13	5.9	>30
14	7.7	>30
15	7.3	>30
16	19	>30
17	~30	>30
18	7.3	>30
19	0.21	>30
20	27	>30
A	>30	>30
B	>30	>30
C	2.2	14

The EC_{50} and CC_{50} values for confirmed hits, as determined using the HBeAg ELISA and the Alamar Blue Cytotoxicity Assay (representative data).

Conclusions

- A fully automated 96-well HTS assay for HBV inhibition in the *in vitro*-infected HepG2/NTCP cell culture was developed.
- A total of ~36K compound library was screened to date.
- A number of novel chemistry series of selective HBV inhibitors were identified, the screening campaign is ongoing.
- The molecular mechanism-of-action studies with the newly-identified HBV inhibitors are in progress.