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Optimization of a cellular HBV infection model for use in high-throughput drug screening

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Summary. – Hepatitis B virus (HBV) is a partially double-stranded DNA virus that specifically targets hepatocytes. It is considered a major health issue due to its high prevalence and the life-threatening consequences of chronic infection, including liver cirrhosis and hepatocellular carcinoma. Despite widespread vaccination against HBV, millions of people live with chronic HBV infection. Existing antiviral therapies fail to achieve full HBV elimination, so most patients with the disease require lifelong treatment. The search for new antiviral therapy strategies is hindered by the limited availability of *in vitro* HBV infection models that are able to support the full HBV life cycle. Therefore, the development and optimization of cellular models are crucial to the search for drugs effective against HBV. In this study, we optimized an *in vitro* HBV infection model consisting of two cell lines: HepAD38 cells, which are able to produce infectious HBV; and HepG2-NTCP cells, which are susceptible to HBV infection. We showed that prolonged production of HBV in the "donor" cells and HBV inoculation of the "acceptor" cells simultaneously with seeding improves the established procedure. This modified protocol was proven effective in experiments involving compounds with known activity against HBV, suggesting its utility for future high-throughput screening.

Keywords: HBV; HBV *in vitro* models; HepG2-NTCP; HepAD38

Introduction

Hepatitis B virus (HBV) is a major health issue due to its high prevalence and the consequences of chronic infection, including liver cirrhosis and hepatocellular carcinoma (HCC). The WHO estimates that 257 million people, or 3.5% of the global population, are living with chronic HBV infection (World Health Organization, 2017). The only treatment options currently available for chronic HBV infection are two types of anti-viral therapy agents: interferon-α and nucleoside analogs. Pegylated interferon-α treatment has a finite treatment duration, does not lead to drug resistance, and may result in viral eradication. However, it is expensive, associated with low rates of sustained virologic response (only 25–30%), and often accompanied by adverse side effects (Rijckborst *et al*., 2011). Nucleoside analogs suppress viral replication by inhibiting viral reverse transcriptase but are unable to eliminate the virus (Deres *et al*., 2003). There is therefore an urgent need for new treatment approaches aimed at different stages of the viral cycle and capable of depleting or inactivating covalently closed circular DNA (cccDNA).

The most challenging features of HBV are its tissue tropism and its species-specific infection. Aside from chimpanzees and treeshrews (Hu *et al*., 2019), only humans possess liver cells that have been shown to be susceptible

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Abbreviations: cccDNA = covalently closed circular DNA; HBV = hepatitis B virus; HBeAg = HBV e-antigen; IC50 = half maximal inhibitory concentration; NTCP = sodium-taurocholate co-transporting polypeptide

to HBV infection. This greatly restricts the models that could be used to investigate the virus life cycle and infection progression.

In vivo models shed light on how the immune response to HBV infection influences the development of hepatitis, hepatocellular carcinoma, and liver cirrhosis. Due to ethical concerns, research on chimpanzees is very restricted, so mouse models are widely used instead. These include transgenic mice that express HBV proteins, mice transfected with HBV vectors into the liver, and chimeric mice with humanized livers. The latter model makes it possible to study not only the immune tolerance and response to the infection, but also the cccDNA formation and the liver injury caused by HBV infection (Hu *et al*., 2019). However, the impact of other factors such as host age and sex, viral load, and transmission route on the HBV infection process and the HBV immune response can only be studied in animal models naturally susceptible to the infection. For this reason, ducks and woodchucks, being natural hosts of duck hepatitis B virus and woodchuck hepatitis B virus, respectively, may be chosen as alternative models for HBV infection. As these infections have some features in common with human HBV, including the progression of the infection, such animal models aid in the development of new vaccines as well as in the study of the immune response, hepatocellular carcinoma formation and cccDNA biogenesis (Nassal, 2015; Roggendorf *et al*., 2015). Although they have a number of advantages, these models are not suitable for high-throughput screening assays to discover and test new potential drugs applicable to antiviral therapy. This task calls for effective cellular models of HBV infection. After decades of research, several cell lines capable of producing HBV were generated by modifying the human liver hepatocellular carcinoma cell line HepG2: HepG2.2.15 (Sells *et al*., 1987), HepAD38 (Ladner *et al*., 1997), and more recently HepDE19 (Guo *et al*., 2007) with tetracycline-regulated replication of HBV and Flag-epitope-containing HepBHAe82, suitable for testing anti-cccDNA drugs (Cai *et al*., 2016). These cell lines serve as virus producers, but they are insufficient for studying viral entry and several other HBV cycle stages (relaxed circular DNA (rcDNA) processing and formation of cccDNA). For this purpose, cell cultures susceptible to HBV infection are needed. Primary human hepatocyte cultures (PHH), which are naturally susceptible to HBV, were shown to be able to support the full HBV cycle, including viral entry (Shlomai *et al*., 2014). The same goes for stem cell-derived human hepatocyte-like cells (iHeps). Immortalized HepaRG cells with hepatocyte-like morphology can be infected with HBV if they have been cultured under specific conditions (Gripon *et al*., 2002). However, maintaining and producing these cell lines requires sophisticated techniques and is very time-consuming. Different variations of hepatoma cell lines with induced expression of sodium-taurocholate co-transporting polypeptide (NTCP), which was found to be an essential HBV receptor (Yan *et al*., 2012), do not require such delicate handling in order to achieve sufficient levels of infection.

In this study, we optimized a model involving two hepatocellular carcinoma cell lines – one responsible for producing HBV viral particles, the other susceptible to HBV infection – and made it suitable for high-throughput drug-screening approaches.

Materials and Methods

Cell cultures. Human hepatoblastoma cells with stable HBV production controlled using a Tet-Off system (HepAD38) and human hepatoma cells overexpressing the HBV viral receptor NTCP (HepG2-NTCP) were grown in a DMEM/F12 medium (PanEco, Russia) supplemented with 2 mM L-Glutamine (Gln) (PanEco, Russia), 10% FBS (HyClone, USA) and a 1% antibiotic-antimycotic solution (Gibco, USA). For the selection of virus-producing cells, 250 μg/ml G418 (Geneticin, Gibco, USA) was added into the HepAD38 growth medium.

Virus collection and concentration. HepAD38 cells were grown in collagen-covered T-175 flasks using a DMEM/F12 medium supplemented with 10% FBS, 1% antibiotic-antimycotic solution, glutamine, and G418. Growth media were collected every two days, from the 7th day to the 59th day. (For the optimal collection days, see the results section.) The collected supernatants were stored at 4°C (for no more than a week). Cell debris was discarded after centrifugation (4,000 x *g* 30 min at 4°C). PEG 8000 (Promega, USA) (final concentration 7%**,** m/V) was added to every collected supernatant, and the suspension obtained was incubated at 4°C overnight on a shaker. The HBV virus precipitate was separated after centrifugation (4,000 x *g*, 1 h at 4°C). After the supernatant was removed, the pellet was suspended in an OPTI-MEM (Gibco, USA) medium at 1/100 of the original sample volume. Aliquoted cell culture-derived HBV samples were stored at -70°C.

HBV infection of HepG2-NTCP cells. HepG2-NTCP cells were seeded in a collagen-covered 96-well plate in DMEM/F12 (50 μl per well). (For the optimal cell density, see the results.) For infection following preliminary cellular adherence: After 18 hours, the cell medium was replaced with OPTI-MEM supplemented with 2% DMSO, 4% PEG 8000, and 2.5 μg/ml polyethylenimin (PEI) (30 μl per well), and 15 μl of HepAD38 cell culture-derived HBV, diluted in OPTI-MEM with 2% DMSO, was added. For infection without preliminary cellular adherence: HepAD38 cell culture-derived HBV inoculum was diluted in DMEM/F12 with 1% DMSO and added at the same time that the HepG2-NTCP cells were seeded. 24 hours after infection, the medium containing HBV was removed. The cell plate was rinsed with 100 μl of DPBS and filled with 250 μl DMEM/F12 with 10% FBS per well.

Then the cells were incubated for 6 days at 37°C with 5% CO_2. Cell supernatants were used for HBeAg detection. For the testing assay, the compounds were diluted in serum-free DMEM/F12, and a 1% DMSO solution was used as a control sample. The compounds were added on the infection day (5 µl per well) before the HBV mixture was added. For the cells that underwent simultaneous seeding and infection, the compounds were diluted in the culture medium, and then the HBV was added. For the cells that were pre-seeded and allowed to incubate for 18 h, the medium was removed, the compound was added into the well, and then the mixture with HBV was added. In both cases (simultaneously seeded, and pre-seeded), the day after infection, the medium was removed and replaced with fresh medium, and the test compounds were added again (225 µl of DMEM, and 25 µl of the compound). The optimal incubation time is stated in the results.

HBV DNA detection. Total DNA from HepAD38 cells was extracted using a PureLink Pro 96 Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. HBV DNA detection was performed with 1 µl of eluate using real-time PCR with primers and a TaqMan probe, as described elsewhere (Bottecchia *et al.*, 2008).

HBeAg detection. HBeAg detection in the HepG2-NTCP cell supernatant was performed using a commercial ELISA kit (DS-EIA-HBeAg, Diagnostic Systems, Russia) according to the manufacturer's protocol. The absorbance was measured on a microplate reader Varioskan Flash (Thermo Scientific) at a wavelength of 450 nm.

Viability assay with resazurin. For the viability assay, 100 µl per well of a 40% solution of resazurin diluted in DPBS was added to a plate with cells after infection. After 1 and 2 hours, respectively, of incubation at 37°C, absorption at 570 nm and 600 nm, respectively, was measured on a microplate spectrophotometer Varioskan Flash (Thermo Scientific).

Results

Optimization of HBV collection and concentration from the virus-producing HepAD38 cell line

The first step in developing our *in vitro* model was the production and concentration of the HBV-containing inoculum. The HepAD38 cell line is commonly used for secreting HBV virions. According to the standard protocol, supernatant should be collected from the cell line every other day starting from the 10th day after the cell culture reaches the monolayer confluency (Li *et al.*, 2020). We found that concentrated samples collected after one week of incubation since the infection of HepG2-NTCP showed insignificant levels of HBV DNA. On the other hand, after the 17th day of incubation, virion production greatly increased (Fig. 1). Moreover, supernatants

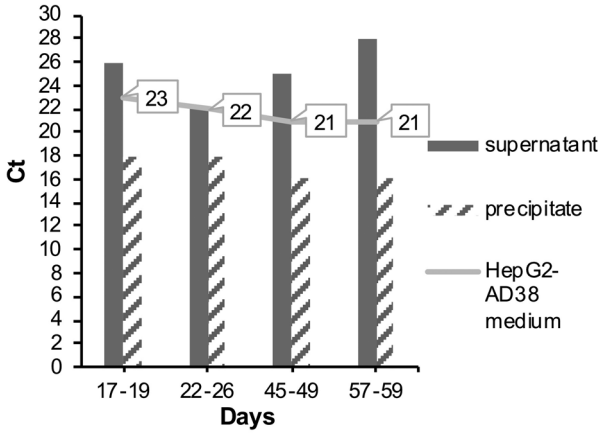

Fig. 1

Threshold cycle (Ct) values for HBV DNA in medium samples collected on different days of the HepAd38 culturing process
Ct values for the HegG2-AD38 medium are given in squares.

collected after one month showed no decrease in viral production, indicated by persistently low values of the threshold cycle number in PCR. No pronounced peak was observed in virus production during the experiment. On the contrary, virus production levels were stable until the very end of the observation period (day 59). At the same time, the precipitation efficiency in the samples after the 45th day slightly increased, as the difference in Ct values of the precipitate and the supernatant grew (Fig. 1, days 45–49, 57–59).

Another important issue arose in the concentration process, which included overnight incubation with 4% PEG 8000 and subsequent centrifugation (for the complete procedure, see the corresponding section in Materials and Methods) qPCR analysis showed that an increase in the duration of centrifugation from 30 min to 1 h improved virion concentration in the final aliquots, as samples which were centrifugated for less than 1 h showed Ct > 21 (with Ct 22–25 in the precipitate alone after centrifugation (data not shown)).

Optimal cell density for effective HBV infection detection

To find the optimal cell density for effective HBV infection, three different cell densities were examined: 3,000, 6,000, and 9,000 cells per well. Smaller numbers of cells were not tested, as any fewer were not enough to guarantee confluency on the day after the inoculation.

Cells were seeded on 96-well plates, infected after 18 h with 5.8 $\log_{10}$ (lg) copies (a non-diluted viral sample) of cell culture-derived HBV per well, and incubated for 24 h at 37°C. After the plates had been washed with DPBS, the

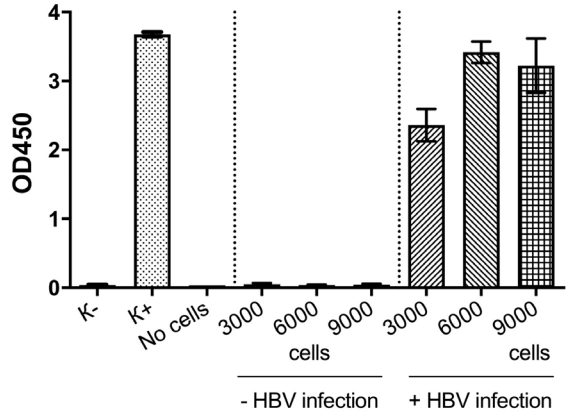

Fig. 2

Effect of HepG2-NTCP cell density on infection level measured by HBeAg production

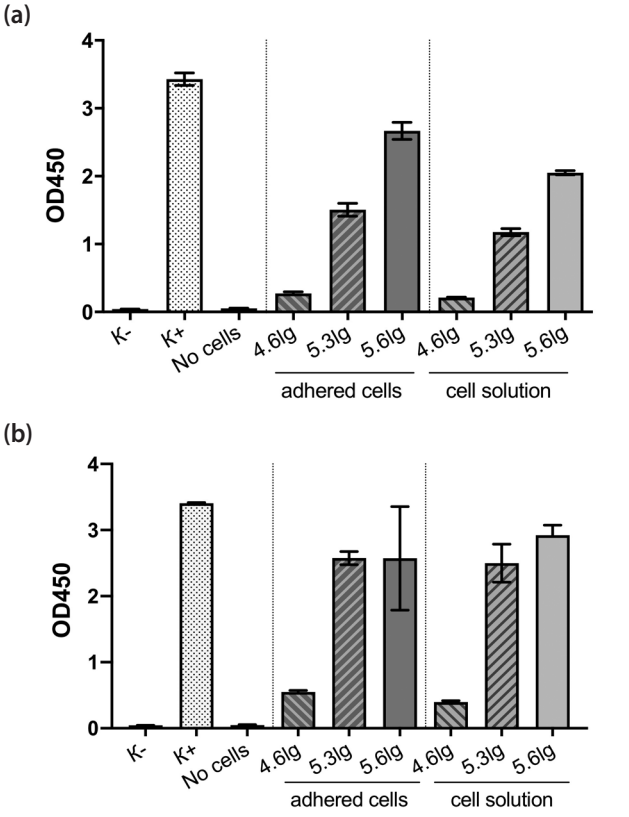

Fig. 3

Optimal infection conditions for HepG2-NTCP cells
Measurements of HBeAg production 6 days **(a)** and 8 days **(b)** after HBV infection. The amount of HBV used for inoculation is expressed in lg copies per well.

medium was replaced with DMEM/F12 supplemented with 10% FBS. The cell medium was collected after one week and tested for HBeAg, a nonstructural secretory HBV protein, in ELISA. We observed an increase in HBeAg production in response to a higher density of HepG2-NTCP cells (Fig. 2). This could be explained by the presence of a larger quantity of HBV-susceptible cells available for infection. However, there was no significant increase in HBeAg optical density in wells that had HepG2-NTCP density greater than 6,000 cells per well. Therefore, 6,000 cells per well was sufficient to reach a robust infection level.

Effective HBV concentration and incubation time for infection of HepG2-NTCP

To examine the possibility of using less concentrated inoculum for effective infection, we tested a series of dilutions of collected and concentrated HBV samples on 96-well plates with HepG2-NTCP cells susceptible to HBV infection after incubation with collected cell culture-derived HBV within 6 or 8 days after infection. We also tried two methods of infecting the HepG2-NTCP cells. One plate was inoculated with HBV after 18 h of incubation, when cells reached full adherence, while the other plate was inoculated with HBV at the same time that the cells were seeded. After 24 h of incubation with the HBV-containing medium, the cells were washed with DPBS and then maintained under standard conditions.

We tested the following concentrations of HBV in OPTI-MEM with a 2% DMSO solution: 4.6 lg, 5.3 lg, and 5.6 lg viral genome copies per well (~6,000 cells). The success of the HBV infection was assessed using ELISA to measure the optical density of the HbeAg produced by the infected HepG2-NTCP cells.

A sharp increase in HBeAg production, reflecting the number of cells successfully infected with HBV, was observed in wells that had been inoculated with 5.3 lg and 5.6 lg of HBV genome copies per well (Fig. 3a,b). Moreover, for all dilutions of HBV inoculum tested, the production of HBeAg was more ample on the 8th day after infection than on the 6th day. No significant differences in HBeAg levels were observed between plates inoculated with HBV on the 2nd day after seeding and plates inoculated simultaneously with seeding, i.e. before adhesion. Therefore, inoculating the cells while seeding did not lead to a decrease or loss in productivity of HBV infection.

Testing of the optimized in vitro system for future compound screening

To validate the developed assay described above, we tested serial dilutions of Bay 41-4109 (CAS No. 298708-81-3)

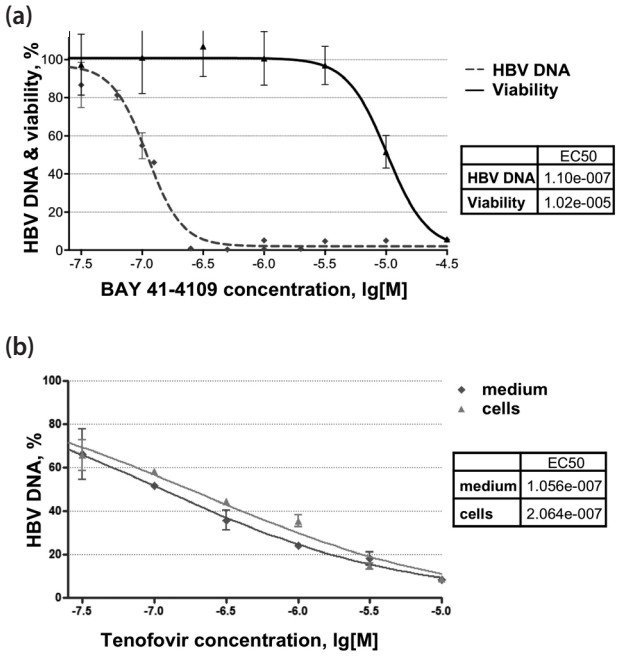

Fig. 4

Cell viability and HBV DNA inhibition curves after BAY 41-4109 treatment (a) and HBV DNA inhibitor curves in cells for Tenofovir (b)

The level of HBV DNA is displayed using a relative scale: the ratio of HBV DNA in cells treated with the inhibitor to the average HBV DNA in the untreated group, multiplied by 100. The HBV DNA quantity was derived from qPCR results. Logarithmic x-axis is used for tested concentrations.

and Tenofovir. Bay 41-4109 is a known non-nucleosidic inhibitor of viral replication that disrupts maturation of HBV nucleocapsids (Deres *et al*., 2003) and has a half maximal inhibitory concentration (IC50) of 0.05 μM (Stray and Zlotnick, 2006). In the HBV DNA detection assay, our model confirmed the Bay 41-4109 activity with similar IC50 (~0.11 μM, see Fig. 4a) but showed a cytotoxicity two orders of magnitude greater (CC50 ~10 μM).

Tenofovir is a nucleoside analog approved by the FDA for treatment of chronic hepatitis B. The concentrations used in our experiment (from 31 nM to 10 μM) were previously shown to be nontoxic for HepG2 (CC50 ~399 μM from Cihlar *et al*., 2002). Tenofovir inhibited HBV production, with IC50~0.21 μM (see Fig. 4b), which is consistent with the results obtained by Schinazi *et al*. in 2012 (IC50~0.34 μM).

Discussion

Cell lines for *in vitro* models of HBV infection are crucial for high-throughput screening assays. Such systems should be reproducible and easy to maintain. At present, the most common variant is *in vitro* HBV infection of modified HepG2 cell lines using cell culture-derived viral particles. In this process, HepG2 cells transfected with a plasmid containing the HBV genome (such as HepAD38 or HepDE19) serve as HBV donors, while cell cultures susceptible to HBV serve as acceptors. The HepG2-NTCP cell line is widely used for the latter purpose. HepG2-NTCP cells are more convenient than HepaRG cells, because they are stably susceptible to HBV and do not require a time-consuming cellular differentiation period (Schulze *et al*., 2012). The HepG2-NTCP infection process requires the presence of at least 4% polyethylene glycol (PEG). One of the possible reasons for this is that treatment with PEG may promote the spread entry of HBV to uninfected HepG2-NTCP cells (Michailidis *et al*., 2017). HepG2-NTCP cells could be used to study the different stages of HBV infection and to identify, which chemicals target key stages of the virus life cycle, including HBV cccDNA formation, thus enabling the development of novel antivirals against the infection (Wose Kinge *et al*., 2020).

In this study, we refined a model of HBV infection using the hepatocellular carcinoma cell line HepG2-NTCP and confirmed its efficacy and applicability for screening assays. Not only did we optimize the necessary conditions for virus concentration and consequent infection, we also found several possible improvements. In a series of experiments, we demonstrated that the medium collected from the HBV donor cell culture HepAD38 after incubation for more than a month stably produced HBV and contained concentrations of HBV DNA optimal for infection of HepG2-NTCP cells. This means that this cell line is able to consistently produce active HBV inoculum for a prolonged period of time. Furthermore, we observed that inoculating the HepG2-NTCP cell line with HBV simultaneously with cell seeding results in levels of infection comparable to those observed when inoculation is given 18 h after seeding. Therefore, HepG2-NTCP cells are susceptible to HepAD38-derived HBV without preliminary full cellular adherence. General protocols suggest infecting HepG2-NTCP at least 3 h after seeding (Li *et al*., 2020). Omitting this waiting period would reduce the time needed for experiments, which would be especially beneficial if the assay were to be done in a high-throughput manner. Since this *in vitro* model reproduces the early stages of HBV infection, our findings could help optimize the screening of potential inhibitors of viral entry.

Furthermore, we validated this optimized protocol by conducting an experiment using compounds with known antiviral activity. The known non-nucleosidic inhibitor of viral replication in our *in vitro* model acted as expected, similar to observations in other models (Stray and Zlotnick, 2006; Schinazi *et al*., 2012). At the same time, Bay

41-4109 appeared to be less active in the HBeAg detection assay (~70% at 1 μM; data obtained in only one experiment). This could be explained by assuming that the HBeAg detection assay might be more sensitive to inhibitors acting in the early stages (such as viral entry inhibitors).

In conclusion, we demonstrated that current protocols for an *in vitro* model of HBV infection based on two cell lines, the HBV-producing HepAD38 line and the susceptible HepG2-NTCP line, could be optimized by prolonged virus production in the "donor" cells and by HBV inoculation of "acceptor" cells simultaneously with seeding. This modified protocol was proven to be effective in experiments using compounds with known activity against HBV, suggesting its utility for future high-throughput screening.

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