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A screening assay for the identification of host cell requirements and antiviral targets for hepatitis D virus infection

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ABSTRACT

Hepatitis delta virus (HDV) is a minimalistic satellite virus of hepatitis B virus (HBV). HBV/HDV co-infection, i.e. "hepatitis D", is the most severe form of viral hepatitis. No effective therapy for HDV infection is available partly due to the fact that HDV is a highly host-dependent virus devoid of any potentially drugable enzyme encoded in its small genome. In this study we present a semi-automated method to evaluate HDV infection and replication under the influence of different drugs. We utilized a Huh-7/hNTCP cell culture based system in a 96-well plate format, an automated microscope and image acquisition as well as analysis with the CellProfiler software to quantify the impact of these drugs on HDV infection. For validation, three groups of potential anti-HDV agents were evaluated: To target ribozyme activity of HDV RNA, we screened ribozyme inhibitors but only observed marked toxicity. Testing innate antiviral mediators showed that interferons alpha-2a and beta-1a had a specific inhibitory effect on HDV infection. Finally, we screened a library of 160 human kinase inhibitors covering all parts of the human kinome. Overall, only inhibitors targeting the tyrosine kinase-like group had significant average anti-HDV activity. Looking at individual substances, kenpaullone, a GSK-3β and Cdk inhibitor, had the highest selective index of 3.44. Thus, we provide a potentially useful tool to screen for substances with anti-HDV activity and novel insights into interactions between HDV replication and the human kinome.

1. Introduction

Hepatitis D is the most severe form of viral hepatitis. Globally over 15 million people are – in addition to being infected with hepatitis B virus (HBV) – also infected with hepatitis delta virus (HDV) a minimalistic satellite virus of HBV (Hughes et al., 2011). This coinfection increases the risk of developing end stage liver disease and hepatocellular carcinoma (Fattovich et al., 2000, 1987). Currently, hepatitis delta is treated with pegylated interferon alpha-2a but cure rates are below 20% (Heidrich et al., 2014).

HDV is the smallest virus known to infect man and has a very strong host cell dependency. The single-stranded RNA HDV genome with a size of about 1700 nt encodes for no viral enzyme (Wang et al., 1986). The delta antigen (HDAg), HDV's only protein gene product, is present in two major forms: the small, 195 amino acids (aa) S-HDAg and the large L-HDAg which carries 19 aa extra at the C-terminus (Weiner et al., 1988). S-HDAg redirects the host RNA polymerase to interact with HDV RNA to replicate the virus genome (Greco-Stewart et al., 2009; Kuo et al., 1989; Yamaguchi et al., 2001). Replication occurs via a rolling circle mechanism where multimers of HDV RNA are produced (Macnaughton et al., 2002) and then cleaved by an intrinsic ribozyme activity of the HDV RNA

Abbreviations: AGC, named after protein kinases A, G, and C families; BET, bromo-and extra-terminal domain; CDK, cyclin-dependent kinase; CK-1, cell kinase 1; CLK, CDK-like kinase; Crk, CT10 regulator of kinase; CT10, chicken tumor virus No.10; ERK, extracellular signal-regulated kinases; FCS, fetal calf serum; fms, McDonough feline sarcoma; GSK-3, glycogen synthase kinase 3; HBV, hepatitis B virus; HDV, hepatitis D virus; IRAK, interleukin-1 receptor-associated kinase; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinase; MyrB, myristoylated B; PK, protein kinase; STE, sterile; Syk, spleen-associated tyrosine kinase; SI, selective index; TK, tyrosine kinase; TKL, TK-like.

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(Kuo et al., 1988; Wu et al., 1989). HDV uses the surface proteins of HBV (L-, M,- and S-HBsAg) and thus they both share the same entry receptor, human sodium taurocholate cotransporting polypeptide (hNTCP) (Yan et al., 2012).

Given the lack of virus-encoded drug targets it seems reasonable to look for host factors that the virus depends on and that may be amenable to therapeutic interventions. Only a few novel anti-HDV compounds are in mid-stage clinical development: the synthetic preS1 peptide myrcludex B (MyrB), targeting hNTCP is a promising drug candidate that is currently in clinical trial phase 2 (Bogomolov et al., 2016). MyrB targets entry of HBV/HDV (Lütgehetmann et al., 2012; Petersen et al., 2008; Volz et al., 2016). Lonafarnib, a farnesyltransferase inhibitor initially developed for anticancer treatment (Morgillo and Lee, 2006), has been tested as an HDV assembly inhibitor (Bordier et al., 2003) and is for this purpose in clinical trial phase 2 (Koh et al., 2016).

The human kinome comprises 518 human kinases that can be divided into different groups (Manning et al., 2002). Also the HDAg is considered a phosphoprotein and phosphorylation of HDAg plays a vital part in HDV life cycle (Mu et al., 1999). Conversely, misfolded HDV ribozyme dimers have been reported to activate protein kinase R (PKR) (Heinicke and Bevilacqua, 2012). Yet, on what specific host encoded kinases HDV depends for execution of its replication cycle is largely unknown.

In this study we present a medium-sized semi-automated screening tool for HDV infection in Huh-7/hNTCP cells. We used this assay to screen interferons, ribozyme inhibitors and a library of 160 kinase inhibitors for anti-HDV activity.

2. Methods

2.1. Drugs

For screening of human protein kinase inhibitors the InhibitorSelect™ 384-Well Protein Kinase Inhibitor Library 1 (Cat. No. 539743, Calbiochem, Merck, Darmstadt, Germany) was used. Detailed information on the drugs is given in the manufacturer's protocol. The substances were supplied diluted in DMSO and further diluted in cell culture medium. The ribozyme inhibitors toyocamycin (T3580), tubercidin (T0642), and 5-fluoruridine (F5130; all from Sigma Aldrich Chemie GmbH, Munich, Germany) were initially diluted in DMSO and further diluted in cell culture medium. The interferons alpha-2a (SRE0013, Sigma-Aldrich Chemie GmbH), beta-1a (IF014, Merck), and lambda3 (PHC0894, Thermo Fisher Scientific, Waltham, MA; USA) were diluted and applied in 1X PBS (0.1% BSA). The farnesyltransferase inhibitor lonafarnib (Selleckchem, Munich, Germany) was first diluted in DMSO and further diluted in cell culture medium. The synthetic preS1-peptide derived from the L-HBsAg, MyrB, was kindly provided by Stephan Urban (Heidelberg University, Heidelberg, Germany). It was originally diluted in distilled water and further in cell culture medium.

2.2. Synthesis of a novel monoclonal anti-HDAg antibody

For this project, a monoclonal anti-HDAg antibody, HDAg#280, was generated by Synaptic Systems GmbH (SySy, Goettingen, Germany) based on the complete amino acid sequence of S-HDAg, HDV genotype 1 (GenBank accession No. U88619.1) (Dingle et al., 1998). In short, at SySy three Balb/c mice were immunized with a peptide encoding the complete sequence of S-HDAg (U88619.1). The mice were sacrificed and B lymphocytes were extracted from lymph nodes and fused with myeloma cells (murine cell line P3-X63-Ag8) to obtain hybridoma cells. After subcloning of hybridoma cells the clone with the best signal-to-noise ratio was selected for antibody production. Testing of pools of antibody candidates was performed by the authors.

2.3. Cell lines and cell culture

The previously reported human hepatoma cell line HuH-7/hNTCP (Pereira et al., 2015) and newly established Huh-7.5/hNTCP cells both stably overexpress hNTCP under the selection of blasticidin (10 μg/mL; Life Technologies/Thermo Fisher Scientific). All cells were cultured in Dulbecco's modified eagle medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS), L-glutamine, nonessential amino acids, penicillin, and streptomycin (Invitrogen) under standard incubation conditions at 37 °C and 5% CO_2. Huh-7 cells were originally established in 1982 (Nakabayashi et al., 1982). None of the authors were involved in collecting these cells.

2.4. In vitro production of HDV particles

HDV particles were produced following the protocol of Sureau (2010). Huh-7/hNTCP cells were seeded in a 6-well plate 24 h prior transfection so that they reached 80% confluency on the day of transfection. Then, supernatant was changed to 1 mL/well DMEM (3% FCS plus supplements). The plasmids pSVLD3 and pT7HB2.7 encoding for a trimer of the HDV genome, and HBV preS1, preS2 and S genes, respectively, were mixed 1:2 in OptiMEM (Life Technologies/Thermo Fisher Scientific) and FuGENE® HD reaction reagent (Promega GmbH, Mannheim, Germany) was added in a dilution 1:3.5 and applied according to the manufacturer's protocol (www.promega.com/techserv/tools/FugeneHdTool/). In total, 3.3 μg DNA were added per well. After 16 h supernatant was removed and the cells were supplied with fresh medium, 2 mL/well. Medium change was done every other day until on days 7, 9 and 12 supernatant, containing virus particles, was harvested and pooled. Supernatants were filtered through 0.45 μm pore size filters (Filtropur S, Sarstedt, Göttingen, Germany) and stored at 4 °C.

2.5. PEG precipitation of HDV supernatant

Collected HDV-supernatant was concentrated using polyethylene glycol (PEG) precipitation as previously reported (von Hahn et al., 2011). In short, 50 mL of supernatant was spun down and the pellet discarded. Then, 42.5 mL of supernatant was mixed with PEG8000 (final concentration 6%) and incubated with agitation overnight at 4 °C. The next day after centrifugation at 11,325 × g for 1 h the supernatant was discarded and the pellet resuspended in 1X PBS with 12.5% FCS and incubated with agitation for at least 6 h at 4 °C. After low speed centrifugation the supernatant was collected and stored at −80 °C until further use.

2.6. Infection of Huh-7/hNTCP cells with in vitro HDV

Huh-7/hNTCP cells were seeded in the 96-well-plate format at a density of 1.5 × 10^4 cells/well 24 h prior to infection. PEG-precipitated HDV was diluted 1:20 in DMEM (3% FCS plus supplements) and mixed with PEG8000 (final concentration 4%) and supplemented with drugs where required. 50 μL virus-mix were added per well. The outer rows and columns of a 96-well plate were omitted from experimental procedure to avoid “edge effect” phenomena. After six hours (cell entry phase) the virus was removed and the cells were supplied with DMEM (3% FCS plus supplements), 100 μL/well with the same drugs at the same concentration as before. Cells were then incubated for 5 days (viral replication phase). Cells were fixed and analyzed after 5 days.