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Measurement of Antiviral Effect and Innate Immune Response during Treatment of Primary Woodchuck Hepatocytes

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Summary

An estimated 350 million people are chronically infected with hepatitis B virus (HBV), and over one million people die each year due to HBV-associated liver diseases, such as cirrhosis and liver cancer. Current therapeutics for chronic HBV infection are limited to nucleos(t)ide analogs and interferon. These anti-HBV drugs in general reduce viral load and improve the long-term outcome of infection but very rarely lead to a cure. Thus, new therapies for chronic HBV infection need to be developed by utilizing liver cell lines and primary cultures and small laboratory animals capable of replicating HBV or surrogate hepadnaviruses for antiviral testing. Natural infection with woodchuck hepatitis virus (WHV), a hepadnavirus closely related to HBV, occurs in woodchucks. Chronic WHV infection has been established over decades as a suitable model for evaluating direct acting antivirals as well as vaccines, vaccine adjuvants and immunotherapeutics because animals are fully immunocompetent. Before HBV-specific compounds are applied to woodchucks, they are usually tested in primary woodchuck hepatocytes (PWHs) replicating WHV at high levels for confirming drug specificity against viral or host targets. Here we describe a protocol for the isolation of PWHs from liver of WHV-infected woodchucks, maintenance in culture, and use in assays for determining antiviral efficacy, safety, and associated host innate immune response of new experimental drugs. Exemplary assays were performed with the nucleoside analog, lamivudine, and the immunomodulator, interferon-alpha.

Keywords

Primary woodchuck hepatocytes; Antiviral treatment; Innate immune response; Woodchuck hepatitis virus; Lamivudine; Interferon-alpha

1. Introduction

Chronic infection with hepatitis B virus (HBV) is responsible for approximately 1.2 million deaths per year worldwide due to HBV-associated liver diseases, including hepatic cirrhosis

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and hepatocellular carcinoma (HCC) [1]. It is estimated that more than 2 billion people have serological evidence of previous or current HBV infection, and that over 350 million individuals are chronic carriers of HBV [1]. The immunologic mechanisms that predispose to the development of chronic HBV infection are not completely understood. However, innate immune response to HBV is believed to play a crucial role in protection against viral infection, and development of an efficient innate immune response is probably needed to avoid viral persistence and subsequent progression to chronic hepatitis B. Furthermore, the contribution of innate immune response to antiviral efficacy observed following therapy with nucleos(t)ide analogs and (pegylated) interferon-alpha (IFN- α) is not well studied. These limitations are mainly due to the lack of immunocompetent, small laboratory animals susceptible to HBV infection and of cell lines suitable for virus propagation.

The Eastern woodchuck (*Marmota monax*) is naturally infected with the woodchuck hepatitis virus (WHV), a hepadnavirus which is closely related to human HBV in regard to genome organization and replication cycle [2]. Neonatal infection of woodchucks with WHV parallels the main route of human (vertical) transmission for chronic HBV infection and displays a disease course similar to that in HBV-infected patients. Thus, chronic WHV infection in woodchucks is a fully immunocompetent model for studying chronic hepatitis B and HBV-induced HCC, and the recent comparison of hepatic transcriptional profiles in woodchucks and humans with acute self-limiting and chronic hepadnaviral infections identified important parallels in the antiviral immune responses [3,4]. Chronic WHV carriers have extensively been used to evaluate efficacy and safety of experimental drugs, therapeutic vaccines and immunomodulators [5]. Such studies have demonstrated the need for more detailed investigation of the innate immune response in woodchucks and to develop new molecular and cellular assays for measuring this response in liver cells. Primary woodchuck hepatocytes (PWHs) can be generated from the liver of chronic WHV carrier woodchucks. The derived cells replicate WHV at rather high levels in culture and retain their morphological characteristics for more than a month [6,7]. Thus, WHV-replicating PWHs represent a cell culture model for various research and antiviral therapeutic applications and allow the measurement of innate immune response during treatment with anti-HBV drugs such as viral polymerase inhibitors and host immunity modulators.

Lamivudine (3TC) is a nucleoside analog licensed for the treatment of chronic HBV infection [8]. The prodrug is converted into its triphosphate form and then competes with the natural cytosine triphosphate for binding at the active site of the HBV polymerase. The incorporation of 3TC at the end of the growing viral DNA strands terminates chain elongation and thereby blocks viral replication. 3TC has been demonstrated to have antiviral efficacy against WHV *in vitro* and *in vivo* [9,10,6].

Interferon-alpha (IFN- α) is a pleiotropic cytokine and used for the treatment of chronic HBV infection as a recombinant protein or in pegylated form [8]. The direct antiviral property of IFN- α results in the expression of various IFN-stimulated genes (ISGs) with antiviral effect functions within the HBV-infected hepatocyte [11]. The immunomodulatory property of IFN- α includes upregulation of immune cells such as natural killer cells and activation of CD8+ T cell function [12]. IFN- α has been demonstrated to have antiviral efficacy against WHV *in vitro* and *in vivo* [13,10,14,15]. For the treatment of PWHs with

IFN- α , a recombinant woodchuck interferon- α 5 protein (rwIFN- α 5) was used (Fletcher S., et al., manuscript submitted).

This chapter describes a method for the isolation of woodchuck PWHs from the liver of chronic WHV carrier woodchucks, their culture and use in assays for determining antiviral efficacy based on the changes of WHV pregenomic (pg) RNA, safety based on cytotoxicity, and associated innate immune response based on the expression changes of selected host genes during treatment with 3TC and rwIFN- α 5. The availability of these *in vitro* assays facilitates the testing of antiviral effects and the characterization of host immune response of larger numbers of new experimental drugs, with applications to the continued modeling and therapy of chronic HBV infection in the woodchuck.

2. Materials

Unless indicate otherwise, prepare all solutions and buffers with DNase- and RNase-free Ultra Pure distilled water (Invitrogen, Life Technologies, NY, USA) at room temperature. Use good sterile handling practice by working under a laminar flow, tissue culture hood or a class II biological safety cabinet. Store all solutions and buffers at 4°C unless indicated otherwise. Follow all appropriate waste disposal regulations when discarding materials in contact with virus and/or drugs.

2.1. Cell Culture Media

1. HBSS, 0.5 mM EDTA: Hanks's Balanced Salts Solution (HBSS) (Gibco, Life Technologies, USA) contains salts and bicarbonate ions. The solution is typically used as a buffer system in cell culture media and supports in combination with ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, MO, USA) the maintenance of an optimal physiological pH (approximately 7.0–7.4).
2. WME, 5% FBS, 10 mM HEPES, 100 μ g/mL Gentamicin: William's Media E (WME) (Gibco) is a medium originally developed for the long-term culture of adult rat liver epithelial cells. Add 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Gibco) and 100 μ g/mL of the aminoglycoside antibiotic Gentamicin (Gibco) to WME. Add heat-inactivated fetal bovine serum (FBS) (Gibco) at 5% (v/v) for use as a reduced serum-supplemented medium that supports growth of PWHs.
3. α MEM, 5% FBS, 10 mM HEPES, 100 μ g/mL Gentamicin: α MEM is a modification of the Minimum Essential Medium (MEM) (Gibco) and contains non-essential amino acids, sodium pyruvate, lipoic acid, vitamin B12, biotin, and ascorbic acid. α MEM is used for diluting out the collagenase enzyme to be applied during liver perfusion (see below). Add HEPES, Gentamicin and FBS to α MEM as described above.
4. α MEM-Collagenase (α MEM, 10 mM HEPES, 0.03% Collagenase): Mix α MEM containing HEPES with 0.03% (w/v) collagenase D (Roche Diagnostics, IN, USA). For a total of 500 mL of medium, add 0.3 g of collagenase powder into a 50 mL conical plastic tube (Falcon, Corning Life Science, Mexico). Add

50 mL of α MEM/HEPES medium to the tube, mix well and sterile filter the solution back into the bottle with the remaining medium using a syringe and a filter containing a 0.22 μ m cellulose acetate membrane (VWR International, PA, USA). As collagenase is active at 37°C, equilibrate α MEM-Collagenase medium in a water bath for at least one hour prior to the collagenase step (see below).

5. Complete medium (WME, 5% FBS, 10 mM HEPES, 100 μ g/mL Gentamicin, 2 μ g/mL Glucagon, 5 mL ITS+1 solution, 2 mM L-Glutamine): It is recommended to prepare two 500 mL bottles of Complete medium for obtaining sufficient amounts of medium during isolation, maintenance, and treatment of PWHs. Add HEPES, Gentamicin and FBS to WME as described above. Dissolve Glucagon (Sigma Aldrich) in 5 mL of acidic water. Acidic water is prepared by adding 1 μ L of 1N HCL (Sigma Aldrich) to 5 mL of water. Sterile filter the Glucagon solution into a 50 mL conical plastic tube using a syringe and a filter containing a 0.22 μ m cellulose acetate membrane. Add 5 mL of WME medium to the Glucagon glass bottle, dissolve any remaining powder, and sterile filter into the plastic tube. Divide the 10 mL Glucagon/WME solution between the two bottles of Complete medium. Add 5 mL of ITS+1 solution (Discovery Labware, MA, USA) and 5 ml of L-Glutamine (100X) (Gibco) to each bottle.

2.2. Antiviral Drug and Immunomodulator

1. 3TC (Tokyo Chemical Industry America, OR, USA): Weigh out the powder using an analytical scale and dissolve in water for preparing a 100 mM stock solution. Store the stock solution at -20°C until use. Dilute the stock solution further at 1:400 or 1:1000 using Complete medium for obtaining working solutions of 250 μ M and 100 μ M. Use the working solutions immediately.
2. rwINF- α 5 (Hoffmann-La Roche, Basel, Switzerland): Dilute the stock solution (1 mg/mL) at 1:100 using Complete medium for obtaining an intermediate solution (10 μ g/mL). Store the intermediate solution at -20°C until use. Dilute the intermediate solution further at 1:100, 1:200 or 1:1000 using Complete medium for obtaining working solutions of 0.01 μ g/mL, 0.005 μ g/mL and 0.001 μ g/mL. Use the working solutions immediately.

2.3. Cell Culture Plates

1. Reconstitute rat tail collagen (Roche Diagnostics) under a sterile hood. Mix 10 mL of water with 20 μ L of glacial acetic acid (Fisher Scientific, PA, USA) within a 50 mL conical plastic tube. Use a syringe and a filter containing a 0.22 μ m cellulose acetate membrane for sterile filtration of the mixture into a fresh tube.
2. Add the entire 10 mL of water/acid solution to the collagen by using a new syringe with a needle for sticking through the rubber stopper of the glass bottle.
3. Mix the collagen with the water/acid solution overnight at 4°C in the cold room using a rotating device.
4. The next morning, add 10 μ L of collagen solution to each well of a 48 well (flat bottom) cell culture plastic plate (Cellstar) (Greiner Bio-One, Germany) under a

sterile hood. Use a sterile pipette plastic tip (Gilson, WI, USA) for spreading homogenously the collagen solution on the surface of the well bottom.

5. Allow the plate to dry under the airflow of the hood for at least one hour before plating PWHs (*see* Note 1).

2.4. Cytotoxicity Assay

1. CellTiter-Glo One Solution (Promega, WI, USA).
2. μ Clear opaque 96-well plates (Greiner Bio-One).

2.5. RNA Isolation

1. TRI Reagent RT (Molecular Research Center, OH, USA) is a pre-prepared solution that combines phenol and guanidine thiocyanate in a mono-phase solution and facilitates effective inhibition of RNase activity. A biological sample such as liver or hepatocytes is homogenized or lysed in TRI Reagent RT and the homogenate/lysate then separated into aqueous and organic phases by bromoanisole within the BAN Phase Separation Reagent (Molecular Research Center) following centrifugation. After the phase separation, RNA remains in the aqueous phase while DNA and proteins are sequestered into the interphase and organic phases.
2. 1.5 mL plastic micro centrifuge tubes (VWR)
3. Isopropanol (BDH Chemicals, PA, USA).
4. 75% Ethanol (BDH Chemicals).
5. Kimberly-Clark Professional Wypall L40 wipes (VWR)
6. Ultra Pure water (Hardy Diagnostics, CA, USA).

2.6. Complementary (c) DNA Synthesis

1. High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA).
2. PCR tube strips and caps (USA Scientific, FL, USA).
3. Reverse WHV primer (position 2579 to 2556 in WHV7 genome) as described [16]: 5'-TGG CAG ATG GAG ATT GAG AGC-3' (Integrated DNA Technologies, IA, USA).

2.7. Real-Time Polymerase Chain Reaction (PCR)

1. TaqMan Gene Expression Master Mix kit (Applied Biosystems).
2. MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems).
3. MicroAmp Adhesive Film (Applied Biosystems).

¹Collagen-prepared plates can be stored at 4°C for approximately one week by wrapping into a sealed plastic bag. If using such stored plates, allow the collagen to re-equilibrate to room temperature by placing under a hood for at least one hour.

4. WHV primers and probe (these and other primers and probes below were obtained from Integrated DNA Technologies):
 - 4.1 Forward primer (position 2504 to 2524 in WHV7 genome) as described [16]: 5'-AGA AGA CGC ACT CCC TCT CCT-3'.
 - 4.2 Reverse primer (see above).
 - 4.3 Probe (position 2531 to 2556 in WHV7 genome) as described [16]: 5'-/56-FAM/AGA AGA TCT CAA TCA CCG CGT CGC AG/3BHQ_1/-3'.
5. Interferon regulatory factor 9 (IRF9) primers and probe:
 - 5.1 Forward primer: 5'-CCC AGA CTG ACC GTG TAT AAA G-3'.
 - 5.2 Reverse primer: 5'-AGC CAC TCT CCA AAC AGA AC-3'.
 - 5.3 Probe: 5'-/56-FAM/AAC AAT GGC TGT AGT TCC TCA GAG GG/3IABkFQ/-3'.
6. Interferon-induced guanosine-5'-triphosphate- (GTP-) binding protein MX1:
 - 6.1 Forward primer: 5'-GCT GGA CGA AGG AAA GGC-3'.
 - 6.2 Reverse primer: 5'-AGT GGC AGG GAT TTA CAG ATG-3'.
 - 6.3 Probe: 5'-/56-FAM/AGC TCA GTG GTC AGT CTC TCC GC/3IABkFQ/-3'.
7. 18S rRNA:
 - 7.1 Forward primer: 5'-GTA ACC CGT TGA ACC CCA TT -3'.
 - 7.2 Reverse primer: 5'-GGG ACT TAA TCA ACG CAA GC -3'.
 - 7.3 Probe: 5'-/56-FAM/GCA ATT ATT CCC CAT GAA CG/3IABkFQ/-3'.

2.8. Equipment

1. Analytical scale such as the Mettler-Toledo ML54 Analytical Balance (Mettler-Toledo, OH, USA).
2. Rotating device such as the BioMixer (Benchmark, NJ, USA).
3. Culture pan, temperature-adjustable such as the VWR Tissue Flotation Water Bath (VWR).
4. Flow rate-adjustable, peristaltic mini-pump, tubing and cannula (Fisher Scientific).
5. Speed- and time-adjustable rocking instrument such as the Timed Rock N Roller Mixer (Laboratory Instruments, MD, USA).
6. Refrigerated centrifuge such as the Eppendorf Centrifuge 5810 R (Brinkman Instruments, NY, USA).

7. Cell incubator such as the Binder CO₂ Incubator (Binder, NY, USA).
8. Microscope such as the Biostar Inverted Biological Microscope (Reichert-Jung, NY, USA).
9. Orbital shaker such as the BioMixer (Benchmark Scientific, NY, USA).
10. Luminescence reader such as the Centro LB960 Microplate Luminometer (Berthold Technologies, Germany).
11. Micro Centrifuge such as the Microfuge 22R Centrifuge (Beckmann Coulter, CA, USA).
12. Spectrophotometer such as the Nano DropTM 8000 Spectrophotometer (Thermo Scientific, MA, USA).
13. PCR instrument such as the 2720 Thermal Cycler (Applied Biosystems).
14. Real-time PCR instrument such as the 7500 Real Time PCR System (Applied Biosystems).

2.9. Others

1. Sterile scalpels (VWR).
2. Sterile gauze (Tyco Healthcare, MA).
3. Cell strainer (Falcon).
4. Hemocytometer such as the Bright-Line Hemocytometer (Hausser Scientific, PA, USA).
5. Trypan Blue solution (Sigma Aldrich).

3. Methods

3.1. Isolation and Maintenance of Primary Woodchuck Hepatocytes (PWHs)

Primary cultures of non-proliferating woodchuck hepatocytes are derived from the liver by using the collagenase perfusion technique as described previously [7]. This method allows converting the entire liver into a suspension of intact hepatocytes and provides a high initial cell yield, with up to 98% cell viability under optimal conditions. Perfusion results into the swelling of liver lobes due to the expansion of extracellular space and into the digestion of intracellular collagenous tissue by collagenase. The expansion/digestion then leads to cell dissociation [17].

1. Following euthanasia of a woodchuck chronically infected with WHV, remove aseptically the entire liver and place into a sterile glass beaker containing HBSS/0.5 mM EDTA buffer on wet ice (Fig. 1).
2. Place the liver into a temperature-adjustable culture pan under a sterile hood.
3. Perfuse each lobe for approximately 10 minutes with ice-cold HBSS/0.5 mM EDTA using a flow rate-adjustable, peristaltic pump and appropriate tubing and

cannula for the removal of blood (Fig. 2) (*see* Note 2). A flow rate of approximately 3.5 mL/minute is recommended for perfusion.

4. When the liver starts blanching, perfuse the lobes further with a total of 100 mL WME/HEPES/Gentamicin/FBS medium for restoring $\text{Ca}^{+}/\text{Mg}^{+}$ ions using the peristaltic pump.
5. Discard the medium from the culture pan and continue to perfuse the liver with a total of 300 mL of 37°C warm α MEM-Collagenase medium. Administer the medium through each lobe for approximately 10 minutes using the peristaltic pump. Once the 300 mL of medium are used, α MEM-Collagenase medium is recycled from the culture pan which is adjusted to keep the temperature at 37°C.
6. After the collagenase perfusion, cut the liver in smaller pieces with a sterile scalpel and mince the tissue pieces with sterilized tweezers.
7. Incubate the liver pieces in α MEM-Collagenase medium for one hour at 37°C by placing the culture pan on a speed- and time-adjustable rocking instrument to support thorough dissociation of hepatocytes.
8. Filter the resulting hepatocyte solution into 50 mL conical plastic tubes by using sterile gauze placed over the tube opening. Place plastic tubes with filtered hepatocytes on wet ice. Dependent on the organ size, use approximately 6–8 plastic tubes per liver.
9. Pellet hepatocytes at $50 \times g$ for 4 minutes at 4°C by using a refrigerated centrifuge and discard the supernatant.
10. Suspend cell pellets in fresh, ice-cold α MEM/HEPES/Gentamicin/FBS medium and concentrate hepatocytes into 4 plastic tubes.
11. Pellet again hepatocytes at $50 \times g$ for 4 minutes at 4°C, discard the supernatant, and suspend cell pellets in fresh, ice-cold α MEM/HEPES/Gentamicin/FBS medium. Repeat this washing step at least 3 more times to remove cellular debris.
12. After the final wash, suspend cell pellets in WME/FBS/HEPES/Gentamicin medium.
13. Remove remaining cellular debris by filtering the hepatocyte suspension through a 70 μm cell strainer into new tubes (Fig. 3).
14. Count a hepatocyte aliquot with a hemocytometer using Trypan Blue solution for determination of viability. Adjust the cell concentration to 2×10^5 viable cells/mL with WME/FBS/HEPES/Gentamicin medium.
15. Plate 500 μL of hepatocyte solution into each well of a 48-well plate pre-coated with rat tail collagen (Fig. 4).

²If not already performed immediately after euthanasia, remove the gallbladder and any surrounding connective or fat tissue with a sterile scalpel and forceps.

16. Place the plates into an incubator and allow hepatocytes to attach to the pre-coated well bottom for at least four hours at 37°C and 5% CO₂.
17. Confirm cell attachment with a microscope, discard the old medium and replace with 500 µL of Complete medium per well.
18. Following incubation of cells for 2 days at 37°C and 5% CO₂, use hepatocytes for the desired experimental application, including antiviral treatment and host immune response determination.

3.2. Treatment of PWHs with Antiviral Drugs and Immunomodulators

1. Remove medium from hepatocytes and add 500 µL of Complete medium per well containing 3TC (e.g., at concentrations of 100 µM and 250 µM) and rwIFN-α5 (e.g., at concentrations of 0.001 µg/mL, 0.005 µg/mL and 0.01 µg/mL) to selected wells. Add 500 µL of Complete medium to control wells.
2. Incubate plates at 37°C and 5% CO₂ for the desired duration of treatment (e.g., for 1–14 days/6–336 hours).
3. Remove medium every second day (every 48 hours) and replace with 500 µL of fresh Complete medium per well containing 3TC, rwIFN-α5 or placebo (i.e., only Complete medium).

3.3. Test of Drug-associated Cytotoxicity in PWHs

For determining the percentage of viable hepatocytes during treatment with 3TC, rwIFN-α5 and placebo, a cytotoxicity assay such as the CellTiter-Glo One Solution Assay is applied. Cytotoxicity is usually tested at pretreatment (0 hours) and thereafter at days 1 (24 hours), 3 (72 hours), 5 (120 hours), 7 (168 hours), 10 (240 hours) and 14 (336 hours) of treatment. The manufacturer's instructions are followed.

1. Thaw the CellTiter-Glo One Solution overnight at 4°C.
2. The next morning, equilibrate the solution to room temperature and then place the bottle in a water bath at 22°C. Prior to use, mix the solution gently by inverting the bottle several times.
3. In the meantime, equilibrate PWHs to room temperature by placing the plate under a sterile hood for 30 minutes.
4. Remove the cell supernatant and add 100 µL of CellTiter-Glo One Solution to each well (*see* Note 3).
5. Place the plate for 2 minutes on an orbital shaker for inducing cell lysis.
6. For stabilizing the luminescence signal, incubate the plate at room temperature by placing under a hood for one hour.

³Wear gloves when removing the seal of the CellTiter-Glo One Solution bottle for avoiding any contamination with adenosine triphosphate (ATP).

7. Remove 100 μ L of supernatant from the lysed cells and transfer into the wells of an opaque 96-well plate.
8. Measure the luminescent signal by placing the plate into a luminescence reader instrument.
9. Obtain luminescence data and calculate the change in viability of treated PWHs from untreated and/or placebo-treated control PWHs set at 100%.
10. Plot cell viability percentages for each drug, concentration and treatment duration for determining cytotoxicity during treatment with antiviral drugs and immunomodulators (Fig. 5).

3.4. Isolation of RNA from PWHs

For determining changes in WHV replication activity such as the concentration of intracellular WHV pgRNA, cellular RNA is isolated during treatment of PWHs with 3TC, rwIFN- α 5 or placebo. Cellular RNA is also utilized for assaying changes in the expression of selected host innate immune response genes. Isolation of cellular RNA is usually performed at pretreatment (0 hours) and thereafter at days 1 (6, 12 and 24 hours), 3 (72 hours), 5 (120 hours), and 7 (168 hours) of treatment.

1. Remove supernatant from each well (and store at -80°C if needed for subsequent applications).
2. Add 500 μ L of TRI Reagent RT per well and leave the solution in contact with hepatocytes at room temperature for at least 10 minutes to allow optimal cell lysis.
3. Transfer the entire lysis solution into a labelled 1.5 mL plastic micro centrifuge tube (*see* Note 4).
4. Add 25 μ L of BAN Phase Separation Reagent to the lysate and mix by vortexing for 15 seconds.
5. Spin the sample for 15 minutes at 4°C at full speed using a refrigerated centrifuge (do not exceed $20,000 \times g/14,000$ rpm).
6. Without disturbing the interphase, transfer up to 250 μ L of liquid from the aqueous phase into a fresh micro centrifuge tube.
7. Add 250 μ L of ice-cold isopropanol. Vortex and incubate at room temperature for 10 minutes.
8. Spin the sample for 15 minutes at 4°C at full speed. As RNA is insoluble in isopropanol, a pellet will be visible after the centrifugation. Discard carefully the supernatant without disturbing the pellet.
9. Add 1 mL of ice-cold 75% ethanol to the micro centrifuge tube.

⁴If cellular RNA does not need to be isolated immediately, store the cell lysate within a micro centrifuge tube at -80°C . Storage will allow processing the sample up to several months later.

10. Wash the pellet by spinning for 5 minutes at 4°C at full speed. Carefully discard the supernatant without disturbing the pellet. Repeat the ethanol wash one more time.
11. Following the last ethanol wash, remove all ethanol from the micro centrifuge tube with a pipette or by using a small paper tissue such as a folded Kimberly-Clark wipe.
12. Let the pellet air dry at room temperature for 5 minutes.
13. Add 12 µL of RNase-free water to the sample and mix well to solubilize the RNA pellet.
14. For determination of RNA concentration within the sample, use a spectrophotometer for measuring the 260, 280 and 230 optical density values for calculation of concentration and judgement of purity of isolated cellular RNA.
15. Store RNA sample at –80°C for later processing or use immediately for the synthesis of cDNA (see below) (see Note 5).

3.5. cDNA Synthesis for Assay of WHV pgRNA

The synthesis of the first strand of cDNA from cellular total RNA is performed by using the reverse WHV primer. This primer binds to a region within the WHV core and polymerase open reading frames (ORFs) which is only present in the WHV pgRNA/precore mRNA [16].

1. Add 50 ng of cellular RNA in a total volume of 8 µL per tube of a PCR tube strip.
2. Add 0.2 µL of reverse primer (100 µM).
3. Incubate the RNA/primer solution at 65°C for 5 minutes in a PCR instrument or thermal cycler.
4. Following incubation, immediately place the PCR tube strip on wet ice for 5 minutes.
5. Utilizing the High-Capacity cDNA Reverse Transcription kit, add 2 µL of RT buffer (10X), 0.8 µL of deoxynucleotide (dNTP) mix (100 mM; 25X), 1 µL of multiscribe reverse transcriptase enzyme (50 U/µL) and 8 µL of RNase-free water to the RNA/primer solution.
6. Perform the cDNA reaction in a PCR instrument or thermal cycler using the following conditions: 10 minutes at 25°C, 120 minutes at 37°C and 5 minutes at 85°C; thereafter cool to 4°C.
7. Store cDNA sample at –80°C for later processing or use immediately for PCR amplification (see below).

⁵Although not required when using TRI Reagent RT and BAN Phase Separation Reagent per the manufacturer's instructions, digestion of the isolated RNA with DNase I before use in the subsequent cDNA synthesis for removal of residual DNA contamination is recommended.

3.6. cDNA synthesis for Assay of Host Innate Immune Response Gene Expression

The synthesis of the first strand of cDNA from cellular total RNA is performed by using random primers from the High-Capacity cDNA Reverse Transcription kit.

1. Add 1 µg of cellular RNA in a total volume of 14.2 µL per tube of a PCR tube strip.
2. Incubate the RNA solution at 65°C for 5 minutes in a PCR instrument or thermal cycler.
3. Following incubation, immediately place the PCR tube strip on wet ice for 5 minutes.
4. Utilizing the High-Capacity cDNA Reverse Transcription kit, add 2 µL of RT buffer (10X), 0.8 µL of dNTP mix (100 mM; 25X), 2 µL of random primer mix (10X), 1 µL of multiscribe reverse transcriptase enzyme (50 U/µL) and 6 µL of RNase-free water to the RNA solution.
5. Perform the cDNA reaction in a PCR instrument or thermal cycler using the following conditions: 10 minutes at 25°C, 120 minutes at 37°C and 5 minutes at 85°C; thereafter cool to 4°C.
6. Dilute the cDNA sample 1:5 with RNase-free water.
7. Store cDNA sample at –80°C for later processing or use immediately for PCR amplification (see below).

3.7. Amplification of WHV pgRNA

Real-time polymerase chain reaction (PCR) is a technology to amplify very small amounts of DNA/cDNA by applying steps involving denaturation, annealing and extension, thereby generating millions of copies of a particular target DNA sequence. For determining changes in WHV replication activity during drug treatment, a primer and probe set is used that specifically amplifies cDNA representing WHV pgRNA [16].

1. Add 5 µL of cDNA sample per well of a 96-well PCR plate. Use triplicate wells for each sample to account for any variability.
2. Add 5 µL of a standard dilution series of a (linearized) plasmid containing one copy of the WHV7 DNA genome [16,9] per well of the same PCR plate. Use triplicate wells for each plasmid concentration ranging from 2×10^1 to 2×10^7 WHV copies/mL.
3. Utilizing the TaqMan Gene Expression Master Mix kit, add 10 µL of master mix and 4.6 µL of DNase-free water to each well.
4. Add 0.18 µL of WHV forward and reverse primers (each 100 µM) and 0.05 µL of WHV probe (100 µM) to each well.
5. Perform the amplification on a real-time PCR instrument using the following conditions: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 1 minute at 65°C.

6. Following completion of the amplification, obtain Ct values for WHV pgRNA samples and plasmid dilutions. Average Ct values from triplicate wells (see Note 6).
7. Calculate the copy numbers of WHV pgRNA in all samples by using the slope and intercept parameters derived from the standard curve for the plasmid dilutions.
8. Plot copy numbers of WHV pgRNA for each drug, concentration and treatment duration for determining antiviral efficacy during treatment with 3TC or rwIFN- α 5 (Fig. 6).

3.8. Amplification of Host Immune Gene Expression and Normalization with 18S rRNA

For determining changes in host innate immune response during drug treatment, the expression of selected immune genes is analyzed, including IRF9 and MX1. Upregulated MX1 expression is associated with the resolved outcome of acute WHV infection and with the antiviral efficacy mediated by immunomodulators in chronic WHV carrier woodchucks [3,4,18]. IRF9 is involved in the Jak/STAT pathway that is activated when (pegylated) IFN- α binds to its receptor resulting in the induction of antiviral ISGs such as MX1 and 2', 5'-oligoadenylate synthetase (2'5'-OAS) [19]. Primer and probe sets that bind to a region within the cDNA representing woodchuck IRF9 and MX1 are used for amplification. For normalization of cellular RNA concentration in different samples, a primer and probe set is utilized that specifically amplifies cDNA representing 18S rRNA. This primer and probe set binds to a region within the human 18S rRNA cDNA sequence which is highly conserved in the 18S rRNA molecule of different species, including the woodchuck.

1. Add 5 μ L of diluted cDNA sample per well of a 96-well PCR plate. Use triplicate wells for each sample.
2. Utilizing the TaqMan Gene Expression Master Mix kit, add 10 μ L of master mix and 4.6 μ L of DNase-free water to each well.
3. For amplification of IRF9 or MX1, add 0.18 μ L of the respective forward and reverse primers (each 100 μ M) and 0.05 μ L of the respective probe (100 μ M) to selected wells.
4. For amplification of 18S rRNA, add 0.18 μ L of 18S rRNA forward and reverse primers (each 100 μ M) and 0.05 μ L of 18S rRNA probe (100 μ M) to selected wells.
5. Perform the amplification on a real-time PCR instrument using the following conditions: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 1 minute at 65°C.
6. Following completion of the amplification, obtain Ct values for IRF9 and MX1 and for 18S rRNA. Average Ct values from triplicate wells (see Note 6).

⁶For comparison of Ct values obtained by different PCR amplifications, it is recommended to define parameters such as threshold (e.g., 0.05) and baseline (e.g., cycles 3–9 or 3–15) which are then applied to each PCR run.

7. Normalize IRF9 and MX1 expression to 18S rRNA expression by using the formula 2^{-C_t} , where C_t indicates the difference in the threshold cycle between 18S rRNA and target gene (i.e., IRF9 or MX1).
8. Plot the normalized expression of IRF9 or MX1 for each drug, concentration and treatment duration for determining induction/changes of host innate immune response during treatment with 3TC and rwIFN- α 5 (Fig. 7).

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Fig. 1.
Liver of a chronic WHV carrier woodchuck.

Fig. 2.
Perfusion of a liver lobe with ice-cold HBSS/0.5 mM EDTA within a temperature-adjustable culture pan.

Fig. 3.
Concentrated woodchuck hepatocytes in Complete medium following removal of cellular debris.

Fig. 4.
Plating of 1×10^5 viable cells into each well of a 48-well plate pre-coated with rat tail collagen.

Fig. 5.

Changes in cell viability following treatment of PWHs from a chronic WHV carrier woodchuck with an antiviral drug (red bars) for 7 days (168 hours) and 14 days (336 hours). Cell viability for placebo-treated control PWHs (green bars) was set at 100% and is shown for comparison at both time points. Compared to the placebo control, changes in viability of antiviral treated PWHs were minimal indicating no drug-associated cytotoxicity during prolonged treatment.

Fig. 6.

Changes in WHV pgRNA copy number following treatment of PWHs from a chronic WHV carrier woodchuck with 3TC at concentrations of 100 and 250 µM (red bars) or rwIFN-α5 at concentrations of 0.001, 0.005, or 0.01 µg/mL (blue bars) for 1 day (6, 12 and 24 hours), 3 days (72 hours), 5 days (120 hours) and 7 days (168 hours). WHV pgRNA copy numbers for untreated/placebo-treated control PWHs (green bars) are shown for comparison at pretreatment (0 hours) and at each other time point. Compared to the placebo control, the highest concentration of 3TC reduced the copy number of intracellular WHV pgRNA by approximately 2 log₁₀ after 7 days (168 hours) of treatment. Compared to the placebo control, all concentrations of rwIFN-α5 reduced the copy number of WHV pgRNA by more than 1 log₁₀ between 1 and 5 days (24–120 hours) and the antiviral effect appeared more pronounced than that of 3TC during this time.

Fig. 7.

Induction of (a) IRF9 and (b) MX1 gene expression following treatment of PWHs from a chronic WHV carrier woodchuck with 3TC at concentrations of 100 and 250 μM (red bars) or rwIFN-α5 at concentrations of 0.001, 0.005, or 0.01 μg/mL (blue bars) for 1 day (6, 12 and 24 hours), 3 days (72 hours), 5 days (120 hours), and 7 days (168 hours). Gene expression in untreated/placebo-treated control PWHs (green bars) are shown for comparison at pretreatment (0 hours) and at each other time point. Compared to the placebo control, 3TC at both concentrations appeared to induce IRF9 expression after 1 day (6 hours) and more so after 5 days (120 hours) of treatment. Induction of MX1 expression by 3TC was sporadic but appeared somewhat upregulated after 5 days (120 hours) of treatment. Compared to the placebo control, all concentrations of rwIFN-α5 induced IRF9 and MX1 expression after 1 day (6–24 hours) and especially after 5 days (120 hours) of treatment and the expression levels were more pronounced than those mediated by 3TC. Significant expression of IRF9 and MX1 correlated well with the reductions in WHV pgRNA copy numbers (Fig. 6) suggesting an important role of both genes in the *in vitro* antiviral effects mediated by rwIFN-α5.