A human monoclonal antibody against HBsAg for the prevention and treatment of chronic HBV and HDV infection

Rani Burm, Freya Van Houtte, Lieven Verhoye, Ahmed Atef Mesalam, Sandra Ciesek, Philippe Roingeard, Heiner Wedemeyer, Geert Leroux-Roels, Philip Meuleman

Table of contents

Supplementary materials and methods……..……..2

Fig. S1…….....….....5

Fig. S2 ……..…….....……..6

Fig. S3 …….....….....7

Table S1 …….....……8

Table S2 …….....9

Supplementary references……...……..11

Supplementary materials and methods

Cells

HepAD38, Huh7.5, PLC/PRF/5, HepG2.hNTCP, Huh7.5.hNTCP and HepNB2.7 cells were all cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 1% non-essential amino acids, 1% L-glutamine and antibiotics, unless otherwise specified.

Production of HBV/HDV inocula

The HBV inoculum (genotype D) was produced using the stable tetracycline-regulated HepAD38 cell line as previously described [2]. In brief, tetracycline (1 µg/ml) was supplemented into the culture medium for one week, cells were washed with PBS and further cultured in medium without tetracycline. Three days later, virus-containing supernatant was collected every three to four days and for three consecutive weeks. For the production of HDV (genotype 1), either Huh7.5 cells were co-transfected with plasmids pSVL(D)3 and pT7HB2.7, or alternatively, PLC/PRF/5 cells were transfected with pSVL(D)3, both using TransIT LT1-reagent (Mirus Bio LLC, REF-2300, USA). Virus containing supernatant was collected every two to three days for two weeks. The HBV and HDV supernatants were sterile filtered (0.45 µm) and concentrated using the PEG Virus Precipitation kit (Abcam, REF-ab102538, UK). Finally, the viral load was determined by commercial RealStar® HBV or HDV (RT-)qPCR (Altona Diagnostics, Germany) after automated nucleic acid extraction (NucliSENS® EasyMAG®, Biomérieux, France).

In vitro prevention of HBV and HDV infection and immunofluorescent staining

For *in vitro* prevention of HBV, HepG2.hNTCP cells were plated (day 0) at a density of 20,000 cells/well in Poly-L-Lysine coated (Sigma Aldrich, REF-P9155) 96-well plates (Greiner CELLSTAR, REF-655090). One day later, anti-HBsAg was added in duplicate at 5-fold serial

dilutions ranging from 10 µg/ml (666.67 nM) to 0.128 ng/ml (8.53 pM). Two hours later, HBV inoculum (4,990 IU/cell) in culture medium supplemented with 4% PEG and 2.5% DMSO was applied. After 24 hours of virus incubation (day 2), cells were washed three times with PBS to remove the virus and further cultured in medium supplemented with 2.5% DMSO and the respective concentration of anti-HBsAg. day 8, infection was assessed using immunofluorescent (IF) staining of HBV-core positive cells. Therefore, cells were washed three times with PBS, fixed (4% paraformaldehyde, 20 min.), permeabilized (Nonidet® P40-Substitute, 15 min.), blocked (PBS containing 2% BSA and 10% goat serum, 30 min.) and stained using a polyclonal rabbit anti-HB-Core antibody (38 µg/ml) for 1 hour (DakoCytomation, REF-B0586), followed by 1 hour incubation of an AlexaFluor (AF)-488 labeled goat anti-rabbit IgG secondary antibody at 2 µg/ml (Invitrogen, REF-A11034) and DAPI nuclear staining at 1.42 µl/ml (MolProbes, REF-D3571). DAPI and AF-488 signals were detected using automated spinning disk microscopy using a 40X objective (CSU-X1, Nikon). Per condition, a 20x10 field was captured (in duplicate) and positive cells were automatically counted using ImageJ software v1.53c.

In vitro prevention of HDV infection was similarly conducted with some slight modifications to the above described procedure. First, The hu-mAb was separately incubated with the HDV inoculum (4 IU/cell) for 1 hour prior to addition onto HepNB2.7, HepG2.hNTCP and Huh7.5.hNTCP cells. For IF staining, blocking was performed using 5% skim milk in PBS. As primary antibody, we used ,000 diluted EDTA-plasma from a HDV-cleared patient harboring undetectable HBV DNA and HDV RNA levels at the time of blood donation. Cells were overnight incubated at 4°C. An AF-488 labeled goat anti-human secondary antibody at 2 µg/ml (Invitrogen, REF-A-11013) was then applied for 1 hour. Imaging was performed using the Leica TCS-SPE with 20X objective. Per condition, 3 random pictures were taken (in duplicate).

SDS-PAGE and Western blot analysis

Mouse plasma samples (exactly 0.5µl for all samples) were denatured in the presence of the Bolt™ LDS sample buffer and the Bolt™ Reducing agent (Thermo Fisher Scientific) at 70°C for 10 minutes. After denaturation, samples were submitted to SDS-PAGE gel electrophoresis on a 12% acrylamide gel, using the Novex® mini gel Tank (Thermo Fisher Scientific). Afterwards, proteins were transferred to a polyvinylidene difluoride membrane (0.2 µm pore size; Thermo Fisher Scientific), using the Novex® Blot module (Thermo Fisher Scientific). Blocking of unsaturated sites was performed with 5% (w/v) skim milk powder in Tris-buffered saline supplemented with 0.1% (v/v) Tween-20 (TBS-T) for 2h at room temperature. The membranes were incubated overnight at 4°C with goat anti-HBsAg antibody (70-HG15; Fitzgerald industries; 1/1,000) diluted in blocking buffer. Membranes were washed with TBS-T and subsequently incubated for 2h at room temperature with horse-radish peroxidase-conjugated rabbit anti-goat antibody (31402; Thermo Fisher Scientific; 1/20,000) diluted in blocking buffer. Immunoblots were developed using the SuperSignal™ West Femto Maximum Sensitivity Substrate kit (Thermo Fisher Scientific) and exposed to the ImageQuant LAS4000 chemiluminescent imaging system (GE Healthcare, Diegem, Belgium).

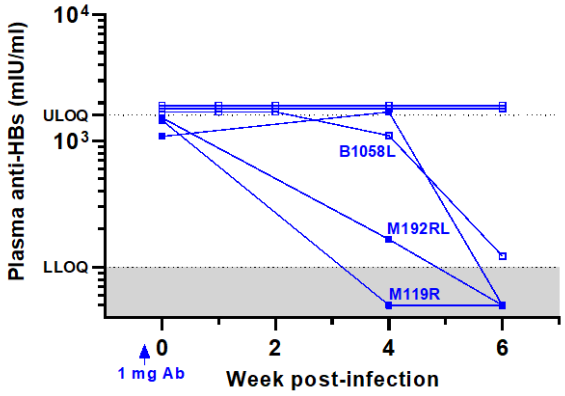

Fig. S1: In vivo prevention of HBV infection. Mice transplanted with hepatocytes from donor C342 (full squares, n=3) or donor HH223 (empty squares, n=3) were passively immunized with 1 mg of hu-mAb (IP) 3 days prior to HBV challenge at week 0 with patient serum (10^6 IU/mouse). Plasma anti-HBsAg levels were determined using quantitative ELISA (Wantai, China) with LLOQ = 100 mIU/ml and ULOQ = 1,600 mIU/ml. All plasma samples collected 1 week prior antibody treatment were tested negative for anti-HBsAg (not depicted).

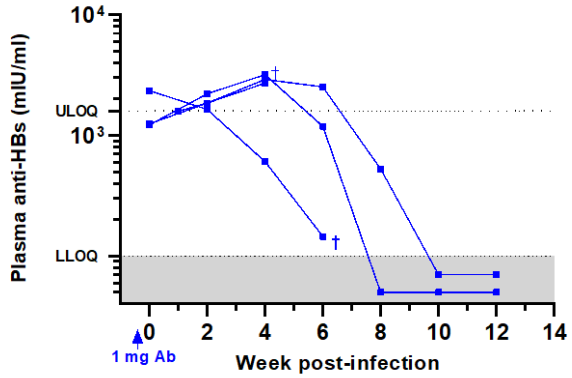

Fig. S2: In vivo prevention of HBV/HDV co-infection. Passive immunization using 1 mg of hu-mAb (IP) was performed 3 days prior to HBV/HDV inoculation in mice engrafted with donor C342 hepatocytes (n=4). Cell-culture derived virus ($5x10^6$ IU HBV and $2.28x10^6$ IU HDV per mouse) was used as viral inoculum. Plasma anti-HBsAg levels were determined using quantitative ELISA (Wantai, China) with LLOQ = 100 mIU/ml and ULOQ = 1,600 mIU/ml. All plasma samples collected 1 week prior antibody treatment were tested negative for anti-HBsAg (not depicted). †: mouse found dead.

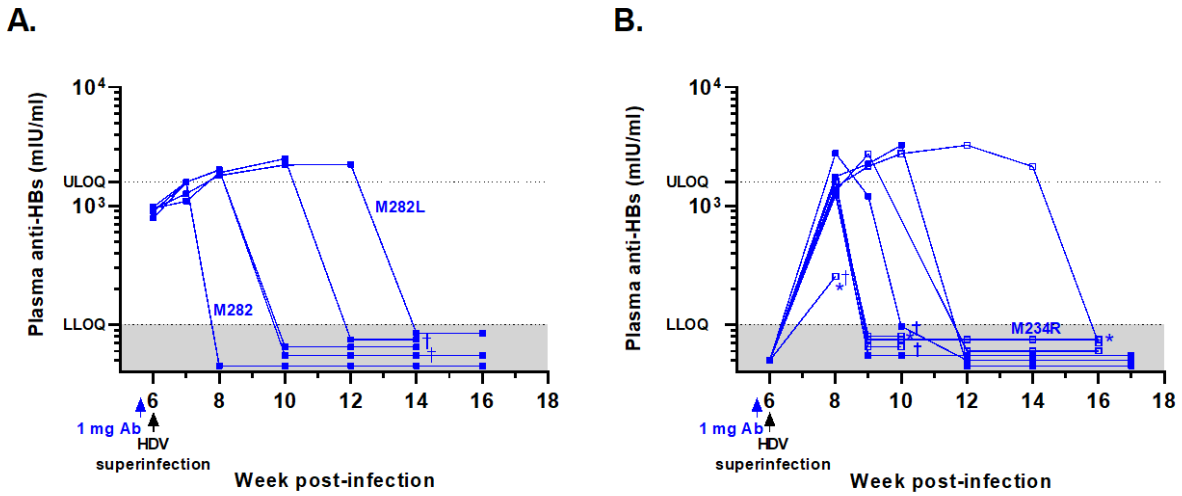

Fig. S3: In vivo prevention of HDV superinfection. (A) Mice engrafted with hepatocytes from donor L191501 were infected at week 0 with patient-derived HBV (10^6 IU/mouse, n=7). Three days prior to HDV superinfection with patient-derived inoculum ($2.55x10^5$ IU/mouse) at week 6, passive immunization in 5 out of 7 mice was performed with 1 mg of hu-mAb (IP). **(B)** Mice engrafted with hepatocytes from donor C342 (n=5, full squares) or C399 (n=9, empty squares) were infected at week 0 with cell culture-derived HBV ($5x10^6$ IU/mouse). Three days prior to HDV superinfection with cell culture-derived inoculum ($2.55x10^5$ IU/mouse) at week 8, passive immunization in 9 (n=3 for C342 mice; and n=6 for C399 mice) out of 14 mice was performed with 1 mg of hu-mAb (IP). Plasma anti-HBsAg levels were determined using quantitative ELISA (Wantai, China) with LLOQ = 100 mIU/ml and ULOQ = 1,600 mIU/ml. All plasma samples collected 1 week prior antibody treatment were tested negative for anti-HBsAg (not depicted). *: patient HDV inoculum, instead of cell culture derived preps, was used for HDV superinfection. †: mouse found dead.

Table S1: Treatment of chronic HBV infection.

	Mean HBV DNA levels (IU/ml)		Mean Log_{10} Reduction			Mann-Whitney U-test	
	Control	**Treated**	**Control**	**Treated**	**Δ Means ± SEM**	**P-value**	**Sign. (P < 0.05)**
0w of therapy (baseline)	1.01E+08 (n=3)	1.04E+08 (n=4)					
1w of therapy	1.96E+08 (n=3)	1.56E+07 (n=4)	0.39 (n=3)	-1.39 (n=4)	-1.78 ± 0.33	0.05714	N.s.
2w of therapy	1.28E+08 (n=3)	1.61E+07 (n=4)	0.18 (n=3)	-1.37 (n=4)	-1.55 ± 0.35	0.05714	N.s.
3w of therapy	1.37E+08 (n=2)	9.56E+06 (n=4)	0.45 (n=2)	-1.64 (n=4)	-2.09 ± 0.60	0.1333	N.s.
4w of therapy (end of therapy)	1.13E+08 (n=2)	9.41E+06 (n=4)	0.42 (n=2)	-1.59 (n=4)	-2.02 ± 0.58	0.1333	N.s.
1w post-therapy cessation	8.16E+07 (n=2)	4.63E+07 (n=4)	0.50 (n=2)	-1.44 (n=4)	-1.95 ± 0.87	0.2667	N.s.
3w post-therapy cessation	2.16E+08 (n=2)	2.64E+08 (n=2)	0.85 (n=2)	-1.18 (n=2)	-2.03 ± 1.99	0.6667	N.s.

Human-liver chimeric mice (hepatocyte donor C342 engrafted, n=7) were chronically infected with HBV for 11 weeks and 4 mice out of these were treated with HBsAg antibody for 4 consecutive weeks: 2 IP injections of 1 mg per week. Control mice (n=3) were infected in the same way with cell-culture derived virus (5 x 10^6 IU HBV per mouse), but were non-treated. Mean plasma HBV DNA levels in mouse plasma are shown at indicated time points. The mean log_{10} reduction is represented and calculated towards one day prior to first antibody injection (0w of therapy-baseline). Mice with undetectable HBV DNA levels were imputed as the LOD for HBV DNA detection, i.e. 3,750 IU/ml. Statistics: n.s (non-significant): p-value > 0.05. SEM: standard error of the mean. Δ: difference of mean log_{10} reduction between treated versus control group. N: number of mice are indicated per time point. W: week.

Table S2: Treatment of chronic HBV/HDV infection.

		Mean viral loads (IU/ml)		Mean Log_{10} Reduction			Mann-Whitney U-test	
		Control	**Treated**	**Control**	**Treated**	**Δ Means ± SEM**	**P-value**	**Sign. (P < 0.05)**
HBV DNA	**0w of therapy (baseline)**	3.09E+08 (n=6)	3.79E+08 (n=7)					
	2w of therapy	5.59E+08 (n=6)	8.32E+06 (n=7)	0.32 (n=6)	-1.64 (n=7)	-1.96 ± 0.17	0.001166	Yes, **
	4w of therapy (end of therapy)	3.73E+08 (n=6)	1.31E+06 (n=6)	0.20 (n=6)	-2.62 (n=6)	-2.82 ± 0.52	0.002165	Yes, **
	2w post-therapy cessation	2.64E+08 (n=6)	2.07E+07 (n=6)	-0.17 (n=6)	-2.14 (n=6)	-1.97 ± 0.64	0.008658	Yes, **
	4w post-therapy cessation	1.81E+08 (n=6)	5.07E+07 (n=4)	-0.57 (n=6)	-2.26 (n=4)	-1.69 ± 0.85	0.06667	N.s.
HDV RNA	**0w of therapy (baseline)**	3.66E+07 (n=6)	2.81E+07 (n=7)					
	2w of therapy	8.99E+06 (n=6)	3.89E+04 (n=7)	0.94 (n=6)	-1.99 (n=7)	-2.92 ± 0.78	0.01399	Yes, *
	4w of therapy (end of therapy)	6.57E+06 (n=6)	2.94E+03 (n=6)	1.37 (n=6)	-2.13 (n=6)	-3.50 ± 1.04	0.008658	Yes, **

	2w post-therapy cessation	9.88E+06 (n=6)	3.03E+04 (n=6)	1.34 (n=6)	-1.86 (n=6)	-3.20 ± 0.88	0.008658	Yes, **
	4w post-therapy cessation	9.58E+06 (n=6)	1.34E+06 (n=4)	1.18 (n=6)	-1.92 (n=4)	-3.10 ± 0.99	0.03810	Yes, *

Human-liver chimeric mice (hepatocyte donor L191501 engrafted, n=13) were chronically infected with HBV for 6 weeks and then superinfected with HDV. At week 10, 7 out of 13 mice were treated with HBsAg antibody for 4 consecutive weeks: 2 IP injections of 1 mg per week. Control mice (n=6) were infected in the same way with cell-culture derived virus (5 x 10^6 IU HBV per mouse and 2.55 x 10^5 IU HDV per mouse), but were non-treated. Mean HBV DNA and HDV RNA levels in mouse plasma are shown at indicated time points. The mean log_{10} reduction is represented and calculated towards one day prior to first antibody injection (0w of therapy-baseline). Mice with undetectable viral levels were imputed as the LOD: 3,750 IU/ml (for HBV DNA) and 187.5 IU/ml (for HDV RNA). Statistics: * p-value < 0.05; ** p-value < 0.01; n.s.(non-significant): p-value > 0.05. SEM: standard error of the mean. Δ: difference of mean log_{10} reduction between treated versus control group. N: number of mice are indicated per time point. W: week.

Supplementary references

1. Depraetere, S., et al., *Human B cell growth and differentiation in the spleen of immunodeficient mice.* Journal of immunology (Baltimore, Md. : 1950), 2001. **166**(5): p. 2929-36.
2. Ladner, S.K., et al., *Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication.* Antimicrobial agents and chemotherapy, 1997. **41**(8): p. 1715-20.