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The natural compound silvestrol inhibits hepatitis E virus (HEV) replication *in vitro* and *in vivo*

Daniel Todt[a,b,1], Nora Moeller[b,1], Dimas Praditya[b], Volker Kinast[b], Martina Friesland[b], Michael Engelmann[b], Lieven Verhoye[c], Ibrahim M. Sayed[c,d], Patrick Behrendt[b,e], Viet Loan Dao Thi[f,g], Philip Meuleman[c], Eike Steinmann[a,b,*]

[a] Ruhr-University Bochum, Faculty of Medicine, Department of Molecular and Medical Virology, Germany
[b] Institute for Experimental Virology, TWINCORE Centre for Experimental and Clinical Infection Research, a Joint Venture Between the Medical School Hannover (MHH) and the Helmholtz Centre for Infection Research (HZI), Feodor-Lynen-Str. 7, 30625, Hannover, Germany
[c] Laboratory of Liver Infectious Diseases, Department of Clinical Chemistry, Microbiology and Immunology, Center for Vaccinology, Ghent University, Ghent, Belgium
[d] Microbiology and Immunology Department, Faculty of Medicine, Assuit University, Assuit, Egypt
[e] Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Germany
[f] Department of Virology, University Hospital, Heidelberg, Germany
[g] Laboratory of Virology and Infectious Disease, The Rockefeller University, New York, NY, USA

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ABSTRACT

Hepatitis E virus (HEV) is the causative agent of hepatitis E in humans and a member of the genus *Orthohepevirus* in the family *Hepeviridae*. HEV infections are the common cause of acute hepatitis but can also take chronic courses. Ribavirin is the treatment of choice for most patients and type I interferon (IFN) has been evaluated in a few infected transplantation patients *in vivo*. However, no effective and specific treatments against HEV infections are currently available.

In this study, we evaluated the natural compound silvestrol, isolated from the plant *Aglaia foveolata*, and known for its specific inhibition of the DEAD-box RNA helicase eIF4A in state-of-the-art HEV experimental model systems. Silvestrol blocked HEV replication of different subgenomic replicons in a dose-dependent manner at low nanomolar concentrations and acted additive to ribavirin (RBV). In addition, HEV p6-based full length replication and production of infectious particles was reduced in the presence of silvestrol. A pangenotypic effect of the compound was further demonstrated with primary isolates from four different human genotypes in HEV infection experiments of hepatocyte-like cells derived from human embryonic and induced pluripotent stem cells. *In vivo*, HEV RNA levels rapidly declined in the feces of treated mice while no effect was observed in the vehicle treated control animals. In conclusion, silvestrol could be identified as pangenotypic HEV replication inhibitor *in vitro* with additive effect to RBV and further demonstrated high potency *in vivo*. The compound therefore may be considered in future treatment strategies of chronic hepatitis E in immunocompromised patients.

1. Introduction

Hepatitis E virus (HEV) is the causative agent of hepatitis E in humans and is classified as a member of the genus *Orthohepevirus* within the *Hepeviridae* family. Worldwide, hepatitis E is the leading cause for acute viral hepatitis. With approximately 20 million people infected with HEV causing approx. 3.3 million cases of acute illness leading to nearly 44.000–70.000 deaths per year, this pathogen has substantial impact on humans life and needs to be socioeconomically considered (Wedemeyer et al., 2012). At least four human-pathogenic HEV genotypes exist (gt1-4). Genotype 1 and 2 solely infect humans and are mainly present in developing areas causing water-borne outbreaks. Pregnant women harbor a high risk for a fatal outcome during HEV gt1 infection with mortality rates up to 30% in the last trimester (Kamar et al., 2017). In contrast, gt 3 and 4 are zoonotic pathogens with their main reservoir in pigs, wild boars and deer (Sayed et al., 2015). Having contact with these animals or consumption of contaminated meat-products are major risk factors for acquiring an HEV infection. The

* Corresponding author. Department of Molecular and Medical Virology, Ruhr-University Bochum, Universitätsstr. 150, 44801 Bochum, Germany.
E-mail address: eike.steinmann@rub.de (E. Steinmann).
[1] These authors contributed equally to the work.

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latter genotypes are responsible for most of infections in industrialized nations. HEV gt 3 infections in humans are usually self-limiting, but can evolve also to chronicity in immunosuppressed individuals with the risk for the development of liver cirrhosis and eventually hepatic decompensation with the need for liver transplantation (Behrendt et al., 2014). Current therapies used to treat HEV infections including ribavirin and interferon-alpha have severe side effects and are contra-indicated in pregnant woman. Furthermore, virus isolates have been recently identified that possess lower ribavirin sensitivity leading to higher treatment failure rates (Debing et al., 2014, 2016; Todt et al., 2016a, 2016b). Therefore, efficacious and safe antiviral treatments against HEV are urgently needed.

In a screening approach for novel HEV inhibitors, we identified the natural compound silvestrol. Silvestrol is a structurally unique cyclopenta[b]benzofuran that can be isolated from the plant *Aglaia foveolata* belonging to the family of *Meliacae* (Kim et al., 2007). It is described as a highly efficient, non-toxic and specific inhibitor of the DEAD-box RNA helicase eIF4A (Bordeleau et al., 2008), which is part of the eIF4F complex that drives cap-dependent translation initiation in eukaryotes (Silvera et al., 2010). In this study, we show that silvestrol inhibits the replication of different HEV gts in a dose-dependent manner at low nanomolar concentrations. This anti-HEV activity was further observed in infection experiments with laboratory and primary isolates in human liver cells and *in vivo* using humanized mice. These results identify silvestrol as a novel natural compound that blocks HEV replication and could therefore be part of an antiviral strategy for the treatment of HEV infections.

2. Materials and methods

2.1. Compounds and reagents

Silvestrol was received from MedChem Express (Monmouth Junction, NJ, USA). Ribavirin (RBV) was received from Sigma Aldrich, St. Louis, MO, USA. All compounds were stored and diluted according to manufacturer's recommendations.

2.2. Cell culture

The human liver cell lines HepG2 and Huh7.5 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (GE Healthcare), 100 µg/mL of streptomycin and 100 IU/mL of penicillin (Invitrogen), 2 mM L-glutamine and 1% nonessential amino acids (Invitrogen) (DMEM complete). The HepG2/C3A subclone was cultured in Eagle's minimum essential medium (MEM with glutamine, Invitrogen), 10% ultra-low IgG FCS (Invitrogen), 2 mM L-glutamine, 100 µg/mL gentamicin, 1 mM sodium pyruvate and nonessential amino acids (Invitrogen). HepG2 and HepG2/C3A cells were further grown on rat collagen-coated (SERVA Electrophoresis GmbH, Heidelberg, Germany) culture plates. Cells were kept at 37 °C in a 5% (vol/vol) CO_2 incubator.

2.3. HEV constructs and in vitro transcription

A plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, gt3; GenBank accession no. JQ679013) and four constructs harboring a subgenomic HEV sequence coupled to a *Gaussia* luciferase reporter gene were used to generate HEV *in vitro* transcripts as previously described (Drave et al., 2016). One of the constructs derives from the gt1 Sar55/S17 strain (based on clone pSK E2, GenBank accession no. AF444002) and two other constructs are based on the Kernow-C1 p6 genome of which one contains the polymerase mutation G1634R (Todt et al., 2016a). The other gt3 *Gaussia* luciferase coupled construct derives from the G3-HEV83-2-27 virus (gt3; GenBank accession no. AB740232) and was a kind gift of the laboratory of Takaji Wakita. Capping of all constructs was performed using Ribom7G Cap Analog (Promega, Madison, WI, USA). A HCV JFH1 subgenomic replicon was used as reference construct as described (Anggakusuma et al., 2015).

2.4. HEV primary isolates

Primary isolates gt1 strain Sar-55 (1.21×10^8 viral RNA copies/mL) and gt2 Mexico-14 (1.65×10^6 viral RNA copies/mL) in 10% fecal suspension from infected macaques and Kernow-C1 P1 supernatant from HepG2 cells (2.3×10^6 viral RNA copies/mL), were a kind gift from Suzanne U. Emerson (Emerson et al., 2004). Primary isolates gt3 strain US-2 (1.6×10^4 viral RNA copies/mL) and gt4 strain TW6196E (9.39×10^3 viral RNA copies/mL) in 10% fecal suspension from infected pigs were a kind gift from Xiang-Jin Meng (Virginia Tech) (Feagins et al., 2008).

2.5. Generation of human pluripotent stem cell-derived hepatocyte-like cells and HEV infection

ESC or iPSC were differentiated into HLCs as previously described (Wu et al., 2018).

2.6. Infection of humanized mice

Human liver chimeric mice (humanized mice) were generated by transplantation of primary human hepatocytes (donor HH223, Corning) into homozygous uPA$^{+/+}$-SCID mice, as previously described (Meuleman et al., 2005). Humanized mice were inoculated via intrasplenic route with a filtered fecal suspension containing $2{,}8 \times 10^5$ IU of the genotype 1 HEV strain Sar-55 (generously provided by Dr. Suzanne Emerson, NIH, USA). Four weeks after inoculation, mice were treated with a daily intraperitoneal dose of 0.3 mg/kg silvestrol, formulated in 30% 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich), or vehicle only. Stool samples were collected on a daily basis and viral RNA was detected and quantified in mouse 10% stool suspensions using a previously described RT-qPCR (Sayed et al., 2017).

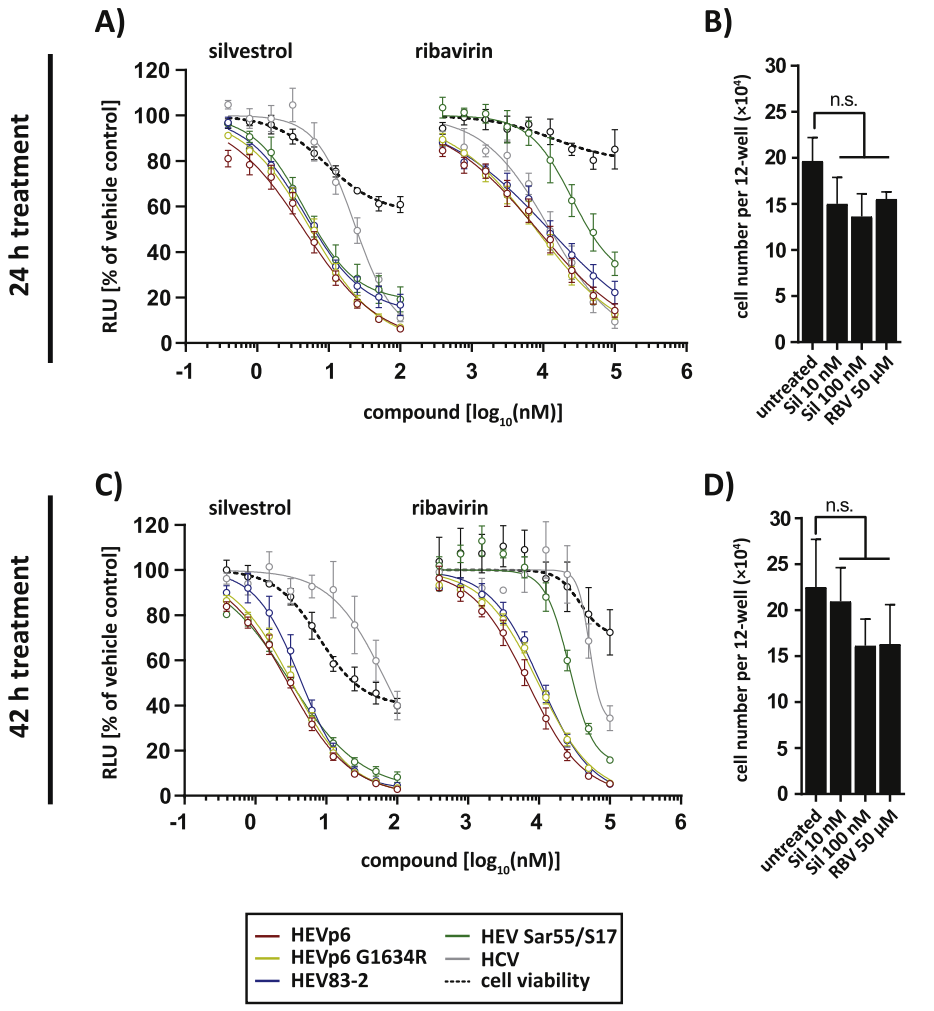
2.7. Statistical analysis

Dose-dependent inhibition of replication was plotted using GrahPad Prism v7.03 for Windows (La Jolla California USA, www.graphpad.com) and IC_{50}, IC_{90} and CC_{50} were calculated using the four-parameter log-logistic model. Statistical significance of differences of means was calculated using one-way ANOVA followed by Dunnett's multiple comparison test were indicated. P values < 0.05 were considered significant (n.s. non-significant).

3. Results

3.1. Antiviral activity of silvestrol against subgenomic HEV replication

HEV and other viral mRNAs contain highly structured 5′-UTRs that need to be translated by the cellular translation machinery in order to promote viral protein synthesis. Silvestrol is a specific inhibitor of the host encoded eIF4A, an RNA helicase that promotes 5′ cap-dependent translation initiation (Gingras et al., 1999). To analyse the effect of silvestrol against HEV, we first transfected the human hepatoma cell line Huh7.5 with different HEV subgenomic replicons, in which a part of the ORF2 was replaced by a *Gaussia* luciferase reporter gene. These include the HEV gt 3 construct p6 (Shukla et al., 2011), a variant (G1634R) thereof with increased replication fitness (Debing et al., 2014; Todt et al., 2016a), the gt3-based wild boar isolate 83-2-27 (Shiota et al., 2013) and the gt 1 strain Sar55 harboring an S17 insertion in the ORF1 like the p6 strain (Shukla et al., 2011). As depicted in Fig. 1A, incubation of silvestrol for 24 h resulted in a dose-dependent inhibition of HEV replication with inhibitory concentrations 50 (IC_{50})



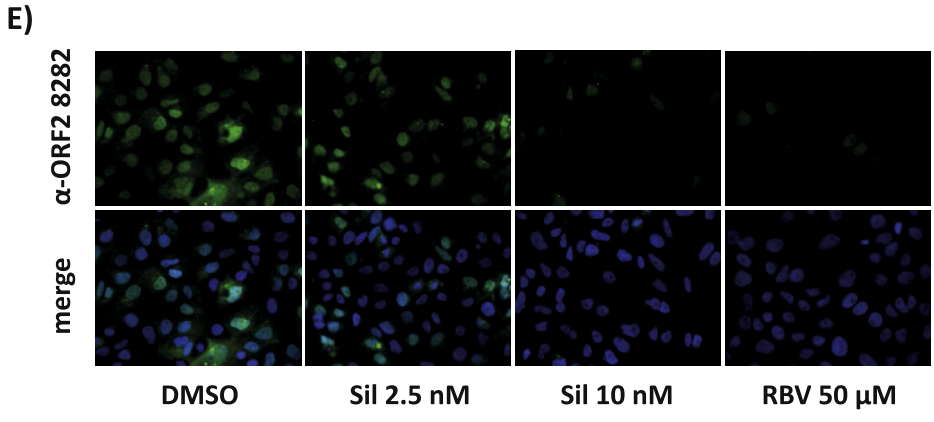


Fig. 1. Silvestrol inhibits Hepatitis E virus (HEV) replication. Depicted is the dose-dependent inhibition of replication of transfected HEV replicons in Huh7.5 after silvestrol or ribavirin (continuous x-axes) treatment for 24 h (**A**) and 42 h (**C**). Reduction in replication was determined via reporter luciferase read-out and normalized to the respective DMSO treated control (y-axes). Three genotype 3 replicons and one genotype 1 replicon were employed and compared to a control replicon based on hepatitis C virus JFH1 (solid lines, see legend for color coding). Cell viability was monitored via MTT assay and is shown as dotted line. In addition, antiproliferative properties of silvestrol (medium concentration: 10 nM and high concentration: 100 nM) were assessed via visual cell counting after trypan blue staining and compared to ribavirin treated (50 µM) and untreated cells (**B, D**) (n.s. non-significant). **E)** Immunofluorescence staining of HEVp6 transfected Huh7.5 cells treated with silvestrol (low concentration: 2.5 nM and medium concentration: 10 nM), ribavirin or DMSO 4 h after transfection for 42 h.

of 4–6.6 nM for the different HEV constructs (Fig. 1A and Table 1). For the known HEV inhibitor RBV, 1000-fold higher concentrations in µM range were needed to inhibit HEV replication (IC_{50} 8–12 µM, Fig. 1A and Table 1). For the gt1 replicon an IC_{50} of 43 µM was observed for RBV (Fig. 1A and Table 1). HCV was employed as a reference virus and was less susceptible to silvestrol as translation initiation occurs cap-independent via an internal ribosomal entry site (IRES) (Fig. 1A). Cell viability was determined by an MTT assay, in which the enzymatic activity started to decrease at higher concentrations, but did not reach a cytotoxic concentration 50 (CC_{50}) in line with reported data (Cencic et al., 2009; Biedenkopf et al., 2017) (Fig. 1A). Additionally, determination of viable cells under silvestrol treatment demonstrated no significant reductions (Fig. 1B). Treatment of silvestrol for 42 h resulted in even lower IC_{50} values for example 2.9 nM for the p6 strain with a CC_{50} value of 24.8 nM (Fig. 1C and Table 1), resulting in a selectivity index of about 8.6. Longer incubation times in the presence of silvestrol resulted in decreased cell viability (data not shown). Overall, RBV and cell viability profiles at 42 h were similar in comparison to the shorter treatment (Fig. 1C and D). Next, the inhibitory effect of silvestrol was evaluated in the full-length p6 clone in Huh7.5 cells. To visualize HEV antigen expression, we performed indirect immunofluorescence staining of the structural capsid protein ORF2. As depicted in Fig. 1E, ORF2 positive cells were highly reduced in the high dose (10 nM) silvestrol and RBV-treated cells (Fig. 1E). In summary, the natural compound silvestrol demonstrates an antiviral activity against different HEV gts at non-toxic concentrations.

Table 1
IC_{50}, IC_{90} and CC_{50} values calculated for silvestrol and ribavirin in Huh7.5 transfected with the indicated replicons.

Replicon constructs	Silvestrol [nM]			Ribavirin [μM]		
	IC_{50} (CI)	IC_{90} (CI)	CC_{50} (CI)	IC_{50} (CI)	IC_{90} (CI)	CC_{50} (CI)
	24 h					
HEVp6	4.43 (3.73–5.25)	64.69 (43.55–95.66)	n.d.	8.16 (6.53–10.24)	244.44 (n.d.)	n.d.
HEVp6 G1634R	5.58 (4.99–6.24)	60.25 (46.68–77.51)		7.86 (6.69–9.23)	171.14 (n.d.)	
HEV 83-2	6.19 (5.28–7.33)	n.d.		11.90 (9.37–15.32)	524.19 (n.d.)	
HEV Sar55/S17	6.65 (5.22–8.73)	n.d.		43.08 (29.94–63.30)	n.d.	
HCV	24.29 (19.99–30.26)	127.80 (n.d.)		12.33 (9.60–15.93)	128.24 (n.d.)	
	42 h					
HEVp6	2.87 (2.61–3.16)	27.28 (21.93–33.87)	24.84 (16.82–39.78)	6.90 (5.98–7.96)	50.38 (36.66–68.73)	n.d.
HEVp6 G1634R	3.29 (2.97–3.64)	28.82 (22.98–36.05)		9.69 (8.84–10.62)	70.99 (57.70–87.05)	
HEV 83-2	4.49 (3.81–5.36)	27.66 (18.45-n.d.)		10.51 (9.27–11.93)	63.84 (48.23–83.94)	
HEV Sar55/S17	3.17 (2.81–3.60)	52.80 (36.92-n.d.)		30.28 (25.67–36.92)	n.d.	
HCV	71.11 (n.d.)	517.83 (n.d)		62.48 (51.15–95.77)	n.d.	

n.d. not determined.
CI 95% confidence interval.

3.2. Inhibition of cell culture- and patient-derived HEV infection by silvestrol

Next, we evaluated the antiviral effect of silvestrol in HEV infection experiments using cell culture-derived infectious HEV (HEV_{CC}) based on the p6 isolate (Dao Thi et al., 2016) in HepG2/C3A cells and patient-derived HEV of gts 1–4 in pluripotent stem cell-derived hepatocyte-like cells (HLCs) (Wu et al., 2018). At first, we evaluated the antiviral activity of silvestrol with the HEVp6 replicon in the HepG2/C3A cell line and observed a shift of the dose-response curves in comparison to Huh7.5 (suppl. Fig. 1). In case of the HEV_{CC} infection assay, treatment setups with silvestrol with a concentration of 10, 20 and 50 nM between 24 h and 48 h, day 6 and day 7 or at both time intervals were carried out (Fig. 2A). The experimental read out was the determination of infectious particle production in focus forming units (FFU/mL) from the cell lysate after the first round of infection (Fig. 2A). The inhibition of HEV replication was most prominent with the 20 nM and 50 nM treatment at the late time point (Fig. 2B). RBV treatment served as a control and demonstrated inhibitory effects in the late treatment and combinatory arms (Fig. 2B). For all silvestrol incubations cell viability was not reduced (Fig. 2C). Next, patient-derived HEV from gts 1–4 were used to infect HLCs and treated 24 h post-infection with silvestrol for 24 h (Fig. 2A). The inhibitory effect of the natural compound for non-adapted HEV isolates in non-carcinoma cell lines was confirmed in all major human pathogenic genotypes (Fig. 2D) without cytotoxicity (Fig. 2E). In conclusion, infection of hepatoma or HLC cells with different HEV isolates can be reduced by the treatment with silvestrol.

3.3. Inhibitory effects of silvestrol in human liver chimeric mice infected with HEV

Recently, several studies demonstrated that human liver chimeric mice can be infected with HEV and are useful tools for studying chronic HEV infection and antiviral drugs *in vivo* (Allweiss and Dandri, 2016; van de Garde et al., 2016; Sayed et al., 2017). To evaluate the antiviral effects of silvestrol *in vivo*, we chose based on previous work (Hwang et al., 2004; Saradhi et al., 2011), a silvestrol dose of 0.3 mg/kg, which was administered intraperitoneally to gt1 HEV (strain Sar-55) infected mice. Two mice were treated on a daily basis for ten days or six days respectively. Two additional served as control and were only treated with the vehicle. As depicted in Fig. 3A, during the first days of treatment a rapid decline in HEV RNA was observed in the feces of all treated mice, while no effect was observed in the vehicle treated control animals (Fig. 3A). No changes in body weight of either animal were noted over the monitored period. Successful engraftment of human hepatocyte into the mouse livers was ensured by assessing human serum albumin levels in blood samples drawn before and after the treatment (supp. Fig. 2). These results implicate a strong antiviral effect of silvestrol in an HEV animal model system.

3.4. Combinatory activity of silvestrol with RBV

As RBV is the treatment of choice as off-label medication in chronically infected patients, we next performed combinatory treatments of silvestrol and RBV. Following electroporation of Huh7.5 cells with HEVp6 RNA genome harboring a *Gaussia* luciferase reporter gene, cells were grown in presence of various doses of silvestrol or RBV or their combinations. Luciferase assay read-out was performed after 24 h or 42 h of treatment. To characterize the interactions of the combined drugs, compounds were mixed at various molar ratios about matching their equipotent concentrations (silvestrol:RBV 1:250–1:32,000) and analyzed using CompuSyn software (Fig. 4). According to the method of Chou-Talalay (Chou and Talalay, 1984), combination indices (CI) were calculated. CIs for IC_{50}, IC_{75} and IC_{90} after 24 h treatment range between 0.70 and 1.39. After 42 h of treatment, indices ranged from 0.76 to 1.42 indicating additive antiviral effects when combining silvestrol and ribavirin. This is also reflected by the positioning of the experimental data points in close proximity to the line of additivity in the isobologram (Fig. 4A, C) for all fractions. In addition, data were analyzed according to the Bliss independence model (Prichard Shipman 1990) and three-dimensional differential surface plots are depicted in Fig. 4B for the 24 h treatment and in Fig. 4D for the 42 h treatment. Here, we observed a minimal elevation above the plane after 24 h for the low silvestrol concentrations and a minor decrease below the plane after 42 h for the low silvestrol concentrations.

Taken together, these results suggest minimal changes in the mode of additivity for the combination of silvestrol and RBV at the different ratios employed. In this assay setup, both drugs showed additive effects in the inhibition of HEV replicon replication.

4. Discussion

Most of cellular and viral mRNAs rely on cap-dependent mRNA translation. The canonical mechanism of initiation commences with recognition of 5′ end m7GpppN cap structure by the eIF4F complex formed by the DEAD-box helicase eIF4A, the scaffolding protein eIF4G

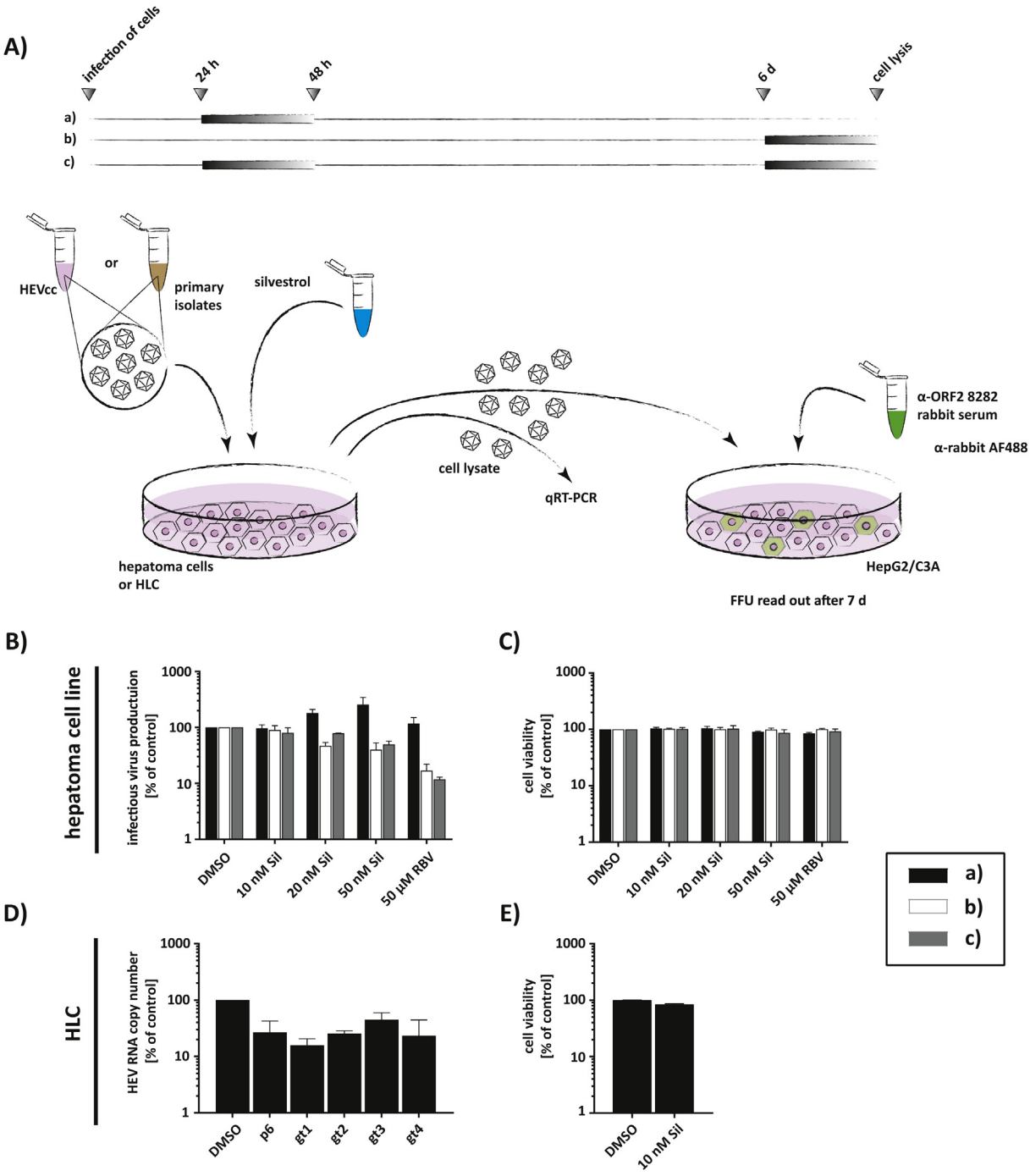


Fig. 2. Antiviral effect of silvestrol on cell culture derived infectious viral particles (HEV_{CC}) and primary Hepatitis E virus (HEV) isolates. A) Assay setup: HEV_{CC} based on HEVp6 or primary isolates of all genotypes were used to infect HepG2/C3A cells or hepatocyte-like cells (HLC). Twenty-four hours after infection (h p.i.), cells were treated in triplicates as indicated: **a)** 24 h–48 h (black bars in **B-E**); **b)** 6 d–7 d (white bars in **B-C**); **c)** 24 h–48 h and 6 d–7 d (gray bars in **B-C**) and lysed after 7 d p.i. Intracellular viruses obtained from freeze-thaw lysis were used to infect HepG2/C3A on 96 well plates and focus forming units (FFU) were counted after 7 d. **B)** Amount of infectious viral particles produced after treatment with different doses silvestrol (Sil) or 50 µM ribavirin (RBV), normalized to DMSO treated control. **C)** Cell viability was monitored via MTT assay and normalized to DMSO control. **D)** Reduction of HEV RNA copy numbers in HLCs infected with primary isolates of HEV genotypes 1–4 and HEV_{CC} p6 after treatment with 10 nM silvestrol for 24 h 1 d p.i. (protocol a)), normalized to DMSO treated control. **E)** Cell viability was monitored via WST assay and normalized to DMSO control.

and the cap recognition factor eIF4E (Sonenberg and Hinnebusch, 2009). The natural compound silvestrol, which has been identified in this study as a novel HEV inhibitor, has been reported to have the ability of modulating translation by preventing ribosome loading onto mRNA templates by targeting this eIF4A eukaryotic initiation factor (Bordeleau et al., 2008; Cencic et al., 2009). Interestingly, Zhou et al. previously investigated the role of the eIF4F complex in HEV replication and found in line with the activity of silvestrol described here, that efficient replication of HEV requires the mRNA translation machinery eIF4A, eIF4G and eIF4E (Zhou et al., 2015). Programmed cell death 4 (PDCD4) and eIF4E-binding protein 1 (4E-BP1), the negative regulatory factors of this complex, exerted anti-HEV activities, which further illustrated the requirement for eIF4A and eIF4E in supporting HEV replication (Zhou et al., 2015). Therefore, molecules like

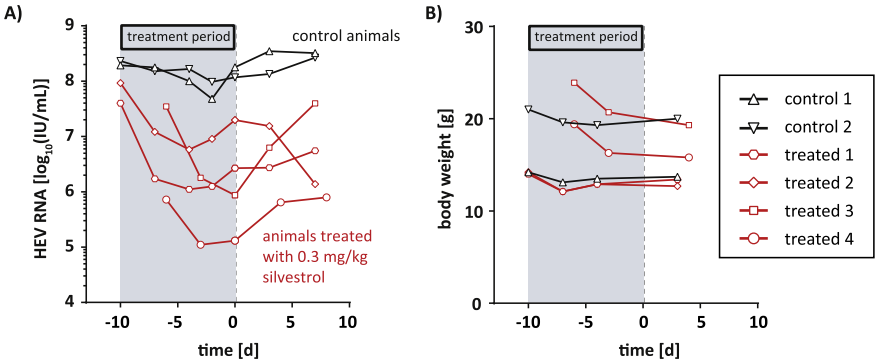


Fig. 3. Antiviral effect of silvestrol in Hepatitis E virus (HEV) infected human liver chimeric mice. A) HEV genotype 1 inoculated mice were treated with a daily intraperitoneal dose of 0.3 mg/kg silvestrol for ten (hexagon and diamond) or six days (circle and square) (red lines, two mice each) or vehicle only (triangles, black lines, two mice). Stool samples were collected on a daily basis and viral RNA was detected and quantified via RT-qPCR. **B)** The body weight of all animals was monitored over the time of the assay (coloring and shapes according to A)).

silvestrol targeting these mRNA translation host factors open new perspectives for future antiviral strategies. Host-targeting agents (HTAs) are likely to exhibit a higher barrier of viral resistance development compared to specific viral targets. An antiviral activity for silvestrol has been described for Ebola virus replication (Biedenkopf et al., 2017). Inhibition of viral propagation by treatment with silvestrol occurred more rapidly than any effects on cellular functions and an IC_{50} lower than 1 nM was observed (Biedenkopf et al., 2017). Very recently, potent antiviral activities were also reported for coronaviruses including MERS-CoV and picornaviruses revealing broad-spectrum activities (Muller et al., 2018). For HEV, we observed also an antiviral activity at low nanomolar concentrations for the human gts 1 and 3, a gt3 swine isolate as well as for a viral variant, which was identified in HEV-infected patients and confers enhanced replication fitness (Fig. 1) (Debing et al., 2014; Todt et al., 2016a). HCV as cap-independent replication control was less susceptible to silvestrol, however, also the

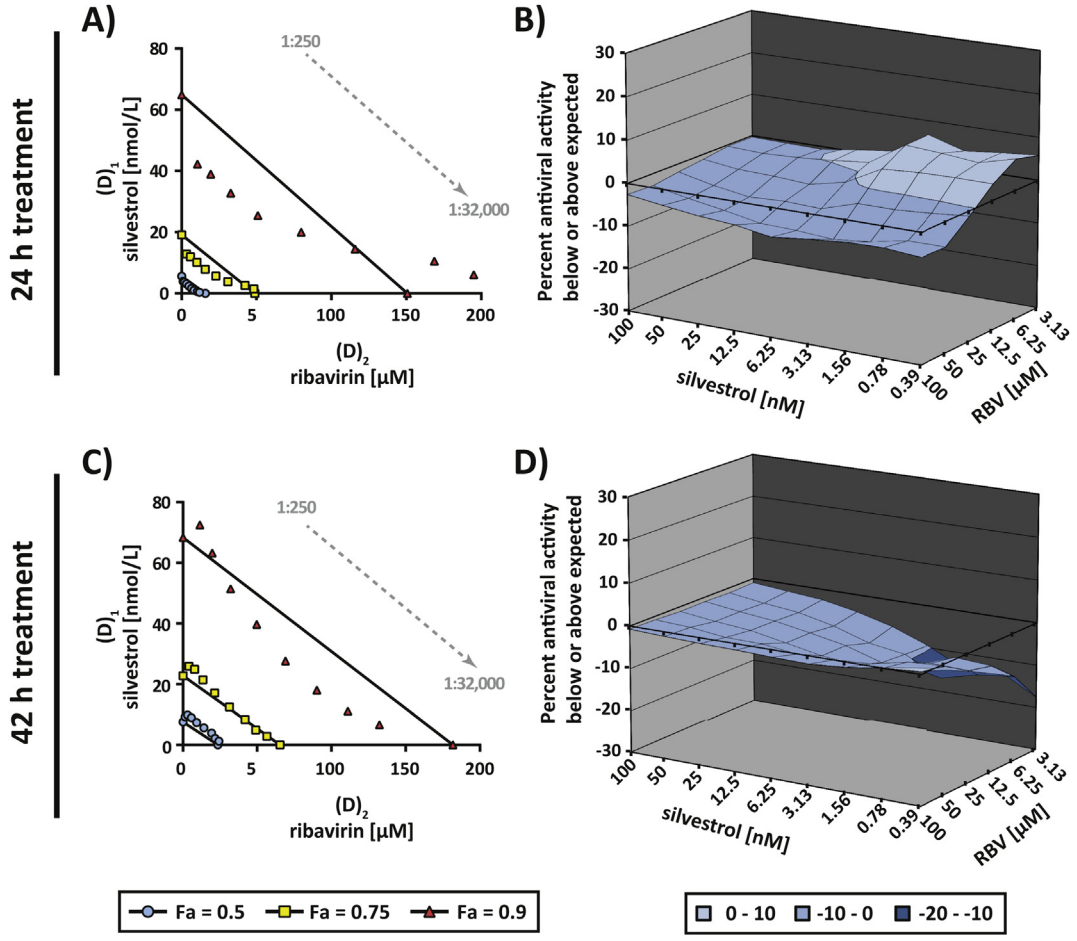


Fig. 4. Silvestrol has an additive antiviral effect when combined with ribavirin. Depicted is the dose-dependent inhibition of transfected HEV replicons after silvestrol and ribavirin combination treatment for 24 h (**A-B**) and 42 h (**C-D**). Reduction in replication was determined via reporter luciferase read-out and normalized to the respective DMSO treated control. **A, C)** Isobolograms with lines denoting the expected additive IC_{50} (left line), IC_{75} (middle line) and IC_{90} (right line) values for the drug combination as calculated from the monotherapies. The experimental IC_{50} (blue circles), IC_{75} (yellow squares) and IC_{90} (red triangles) are shown for the different drug ratios (dotted gray arrows). Symbols far left of the line of additivity indicate synergism, while symbols to its far right indicate antagonism. **B, D)** Three-dimensional surface plots representing potential differences between the actual experimental effects and the theoretical additive effects at various concentrations of the two compounds. Shading indicates different ranges above or below the expected value. An elevation above the zero planes indicates a synergistic effect, a decrease below the plane an antagonistic effect.

IRES-dependent translation machinery might require the help of additional translation initiation factors from the infected host cell (Thompson, 2012). The anti-HEV activity of silvestrol was subsequently confirmed in viral infection assays by determination of production of infectious particles after compound incubation. However, the overall antiviral activity of silvestrol was reduced in this assay compared to the replicon system in Huh7.5, as in HepG2/C3A cells higher concentration of the drug were needed (suppl. Fig. 1). In addition, a pangenotypic activity of silvestrol was observed using primary isolates from four different gts in HLCs derived from human embryonic and induced pluripotent stem cells (Fig. 2). HLCs offer an improved, physiologically relevant traceable system for studying the replication and antiviral response of primary HEV isolates (Wu et al., 2018).

Previously, silvestrol has been mainly characterized in antitumor models such as chronic lymphocytic leukemia, acute lymphocytic leukemia, mantle cell lymphoma and also hepatocellular carcinoma (Cencic et al., 2009; Lucas et al., 2009; Alinari et al., 2012; Kogure et al., 2013). Noteworthy, clinical efforts are underway to target specific components of the translation apparatus or unique mRNA translation elements in cancer therapeutics (Silvera et al., 2010; Bhat et al., 2015). Based on these *in vivo* studies, the inhibitory effect of silvestrol was additionally investigated in humanized mice infected with gt1 HEV, which showed a reduction of viral loads in the first few days of treatment (Fig. 3). This proof of concept validation *in vivo* should guide further silvestrol-based antiviral strategies, which could also involve combination treatments with RBV. Combining two drugs with distinct mechanisms of action involves the advantage of possible synergistic antiviral effects and decreased danger of the emergence of resistances, which is of exceptional importance in the therapeutic intervention for chronically HEV infected immunosuppressed patients (Debing et al., 2016; Todt et al., 2016a). In addition, synergistic antiviral effects imply the possibility to reduce the dose or dosing frequency of any of the two compounds, or even both, minimizing potential toxicity and adverse effects. Here, we employed two models to analyse interactions between silvestrol and RBV in combination according to the recommendations by Zhao and colleagues (Zhao et al., 2010). Both, combination indices and isobolograms calculated based on the Chou-Talalay method, as well as the three-dimensional surface plot determined using the Bliss independence model, suggest additive antiviral effects of the drugs (Fig. 4). As also slight deviations from the line of additivity and the planar plot were observed at extreme doses of antivirals (low and high effect levels), careful titration of the compounds *in vitro* and *in vivo* is recommended to identify the optimal ratio of the drugs for increased antiviral activity along with a reduced danger of the emergence of resistances and improved tolerability in clinical settings.

In summary, the natural compound silvestrol, which specifically targets the host factor eIF4A during cap-dependent translation initiation, could be identified as novel pangenotypic anti-HEV agent. Viral replication was inhibited by silvestrol in a dose-dependent manner at low nanomolar concentrations in different HEV experimental model systems including HEV primary isolates and HEV-infected humanized mice. Thus, silvestrol may be considered in future treatment strategies of chronic hepatitis E in immunocompromised patients.

Disclosure statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.antiviral.2018.07.010.

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