Original article

Strong antiviral activity of the new L-hydroxycytidine derivative, L-Hyd4FC, in HBV-infected human chimeric uPA/SCID mice

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Background: Suppression of viral replication with nucleoside/nucleotide inhibitors has been shown to greatly improve the outcome of chronic HBV infection. β-L-nucleoside analogues, especially β-L-deoxycytidine derivatives represent one of the most efficient groups of antiretroviral compounds. We recently described that hydroxylation of the amino group of these β-L-deoxycytidine derivatives preserved their strong HBV inhibitory activity in vitro, but strongly reduced their cytotoxicity. From this new group of compounds we selected β-L-2′,3′-didehydro-2′,3′-dideoxy-N′-hydroxy-5-fluorocytidine (L-Hyd4FC) for a first in vivo investigation. The aim of this study was to determine the antiviral activity of L-Hyd4FC in HBV-infected human liver chimeric urokinase plasminogen activator (uPA)/SCID mice. Methods: Stably infected animals (median 6×10⁷ HBV DNA/ml) were injected daily with either L-Hyd4FC (50 mg/kg) or saline as controls. Mice treated with lamivudine served to compare the in vivo antiviral potency of L-Hyd4FC. Virological changes were determined by quantitative PCR.

Results: Treatment with L-Hyd4FC for 4 weeks induced a 2-log reduction of viraemia, while a median 1.5-log decline was achieved with lamivudine. Intrahepatically, L-Hyd4FC induced a median eightfold decline of viral activity (relaxed circular DNA/covalently closed circular DNA), and threefold reduction of pregenomic RNA/GAPDH levels. No significant decline of subgenomic HBV transcripts, as well as of circulating hepatitis B e antigen and hepatitis B surface antigen was detected. Maintenance of human serum albumin concentrations throughout the study, negative TUNEL staining and occurrence of viral rebound after drug withdrawal indicated that L-Hyd4FC was not toxic in human hepatocytes.

Conclusions: Administration of L-Hyd4FC in uPA/SCID mice harbouring HBV-infected human hepatocytes demonstrated the high antiviral potency of this drug in vivo. Such characteristics make L-Hyd4FC a good candidate for further investigations as a potential HBV therapeutic agent.

Introduction

Chronic infection with HBV is a global problem with >350 million people affected worldwide and an estimated 0.5–1.2 million related deaths per year due to the development of liver cirrhosis and hepatocellular carcinoma [1]. Treatment with pegylated interferon-α is only effective in a minority of the patients and is associated with significant side effects. Treatment with nucleoside/nucleotide analogues (NUCs) that inhibit the viral polymerase activity can effectively lower viraemia, reduce intrahepatic inflammation, improve liver fibrosis and reduce the risk of hepatic decompensation in patients with severe liver damage [2]. Nevertheless, administration of NUCs does not prevent formation or inhibit the activity of the HBV transcription template, the covalently closed circular viral minichromosome (cccDNA), which guarantees
leads to liver damage [8] and after crossing uPA mice. The liver-specific expression of the uPA transgene urokinase plasminogen activator (uPA)/SCID mice. The urokinase plasminogen activator (uPA) compound able pharmacokinetic properties was selected. Recently we described the hydroxylation of the amino group of some of these l-deoxycytidine analogue-produced compounds, which maintained their strong anti-HBV activity but exhibited markedly reduced cytotoxicity [6]. The most effective compound was β-L-2′,3′-didehydro-2′,3′-dideoxy-N4-hydroxyctydine (L-Hyd4C; 50% effective dose in HepG2.2.15 cells 0.03 µM and 50% cytotoxic dose in HepG2 cells 2,500 µM) [6,7]. For the first in vivo study of this new group of compounds, a 5-fluoromodified derivative of the L-Hyd4C, the β-L-2′,3′-didehydro-2′,3′-dideoxy-N4′,5′-fluorocytidine (L-Hyd4FC) predicting the most favourable pharmacokinetic properties was selected.

To investigate the antiviral potency of this new compound in vivo we employed human liver chimeric urokinase plasminogen activator (uPA)/SCID mice. The liver-specific expression of the uPA transgene leads to liver damage [8] and after crossing uPA mice with immunodeficient SCID mice, this system allows the transplanted human hepatocytes to engraft and repopulate the diseased murine liver. The human liver cells reconstituting the livers of uPA/SCID mice remain highly differentiated, maintain typical metabolic and detoxification functions of the human hepatocytes [9–11] and remain susceptible to HBV and HCV infections [11–17].

In this study, humanized uPA/SCID mice stably infected with HBV were used as a preclinical model to access the efficacy of l-Hyd4FC in reducing the levels of circulating virions, while treatment with lamivudine, which is a well characterized cytosine derivative, additional mice received oral administration of lamivudine for 4 weeks (30 mg/kg/day; Zefix Solution; GlaxoSmithKline, Munich, Germany). Serum samples were collected from all mice shortly before treatment, after 2 and 4 weeks. Mice were sacrificed 24 h after the last compound injection either after 2 or 4 weeks of treatment. Liver specimens were removed and snap-frozen in liquid nitrogen for further histological and molecular analyses.

Virological measurements
Viral DNA was extracted from serum samples (10 µl) using the QIAmp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). Quantitative virological measurements were performed by real-time PCR using the LightCycler (Roche Applied Science, Mannheim, Germany) and HBV-specific primers in combination with hybridization FRET probes as previously reported [20,21]. Defined amounts of cloned HBV DNA were dissolved in sterile physiological saline and stored at -20°C until used.

Generation of human chimeric mice
Animals were housed and maintained under specific pathogen free conditions in accordance with institutional guidelines under approved protocols. Breeding and genotyping of the UPA+/+; SCID+/+ transgenic mice and liver cell transplantation was performed as reported [13,18]. Briefly, 3–4-week-old homozygous uPA/SCID mice were anesthetized with isoflurana and injected intrasplenically with 1×10⁶ viable thawed human hepatocytes [19,20]. All animal experiments were conducted in accordance with the European Communities Council Directive (86/EC) and were approved by the City of Hamburg, Germany. Human hepatocyte repopulation levels were determined by measuring human serum albumin (HSA) concentrations in mouse serum by using the Human Albumin ELISA Quantitation Set (Bethyl Laboratories, Biomol GmbH, Hamburg, Germany), as recommended by the manufacturer. Human chimeric animals displaying HSA concentrations of ≥1 mg/ml were used for the study.

HBV infection and antiviral treatment
To establish HBV infection in mice, human chimeric mice received a single intraperitoneal injection of mouse-derived HBV-positive serum (2×10⁷ HBV DNA copies, genotype D, hepatitis B e antigen [HBeAg]-positive). Only mice that had been infected with HBV for ≥10 weeks and hence displayed stable viraemia levels of >10⁷ HBV DNA copies/ml were used for the study. Chimeric mice received 100 µl of either a daily intraperitoneal injection of 50 mg/kg body weight L-Hyd4FC or physiological saline (control group). To compare the antiviral efficacy of this new compound with another cytosine derivative, additional mice received oral administration of lamivudine for 4 weeks (30 mg/kg/day; Zefix Solution; GlaxoSmithKline, Munich, Germany). Serum samples were collected from all mice shortly before treatment, after 2 and 4 weeks. Mice were sacrificed 24 h after the last compound injection either after 2 or 4 weeks of treatment. Liver specimens were removed and snap-frozen in liquid nitrogen for further histological and molecular analyses.

Methods
Drugs
L-Hyd4FC was synthesized and purified by Vibhut K Dabral, (BioDeTek, Griesheim, Germany) and Sönke Petersen (Chemische Laboratorien, Würms, Germany). The purity of the compound was estimated to be >98% by high pressure liquid chromatography. L-Hyd4FC was dissolved in sterile physiological saline and stored at -20°C until used.

Persistence of infection in hepatocytes, despite the long-term suppression of viral replication achieved during antiviral therapy with NUCs [3–5]. Furthermore, several years of such treatment regimens are complicated by both the emergence of drug-resistant viral strains and the appearance of side effects. Therefore, the challenge will be to develop new efficient drugs that could be employed either for shorter treatment regimens or in combination therapy in order to reach synergistic or at least additive drug action.

L-nucleoside analogues, especially L-deoxycytidine derivatives, represent one of the most efficient groups of antiretroviral compounds available, which display high activity against both HBV and HIV replication. During antiviral therapy with NUCs [3–5]. Further-long-term suppression of viral replication achieved persistence of infection in hepatocytes, despite the emergence of drug-resistant viral strains and the appearance of side effects. Therefore, the challenge will be to develop new efficient drugs that could be employed either for shorter treatment regimens or in combination therapy in order to reach synergistic or at least additive drug action.
amplified in parallel to establish a standard curve for quantification. The Master Pure DNA purification kit (Epicentre®, Biozym, Hessisch Oldendorf, Germany) and RNeasy RNA purification kit (Qiagen) were used to extract the DNA and RNA from mouse liver samples [21]. Intrahepatic HBV DNA values were normalized for cellular DNA contents using the β-globin gene kit (Roche DNA Control Kit; Roche Diagnostics, Mannheim, Germany), which specifically recognize sequences of human origin [13,18]. Purified DNA was then treated with plasmide-safe DNAase-I (Epicentre®, Biozym) to enrich the cccDNA fraction, and quantitative cccDNA measurements were performed using cccDNA-specific primers and FRET probes [21]. Levels of relaxed circular DNA (rcDNA) were estimated by subtracting cccDNA amounts from the total HBV DNA. Viral RNA was reverse transcribed from 1 µg total RNA using oligo-dT primers and the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) and quantified by using primers specific for pregenomic RNA (pgRNA) [21]. Steady-state levels of intrahepatic pgRNA and preS/S RNA amounts were normalized to human-specific GAPDH RNA contents (QuantiTect Primer Assay, Qiagen) [20]. Hepatitis B surface antigen (HBsAg) quantification was performed using the Abbott Architect I8000 platform (quantitative HBsAg kit; Abbott, Wiesbaden, Germany), after diluting the mouse serum samples (1:50–1:500) in Abbott human control serum according to the manufacturer’s recommendations. The HBsAg Abbott test (HBsAg kit; Abbott) was used to determine HBsAg levels in mice after diluting the serum samples (1:40) in dilution medium as suggested by the manufacturer.

Immunohistochemistry and cell death assay
Cryostat sections of transplanted mouse livers were immunostained with Monoclonal Mouse Anti-Human Cytokeratin 18 (hu-CK18; Dako, Hamburg, Germany) recognizing human CK18 and not cross-reacting with mouse proteins and rabbit antibody against hepatitis B core antigen (HBcAg) [13,20]. Specific signals were then visualized with Alexa546-labelled secondary antibodies or TSA amplification (Perkin Elmer, Waldham, MA, USA) [20]. Stained sections were then mounted with fluorescence mounting media (Dako) and analysed by fluorescence microscopy. Frozen liver sections obtained from uPA recipients were analysed for the presence of apoptotic and/or necrotic human hepatocytes in mouse liver sections. Briefly, cryosections were fixed in a 4% Paraformaldehyde before TUNEL labelling was performed following the manufacturer’s instructions (In situ Cell Death Detection Kit; Roche Applied Science). As positive controls, serial liver sections were incubated for 15 min at 37°C with 100 IU DNase I (Epicentre®, Biozym), prior to TUNEL labelling. To identify human hepatocytes in the mouse liver, the sections were stained with hu-CK18 and visualized by Alexa546-labelled secondary antibody as describe above.

Statistics
GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) was used for creating the graphs and statistical analysis of the data. The non-parametric Kruskal–Wallis Test was used for non-parametric group-wise comparisons and Dunns post test for comparison between groups with known differences. P-values <0.05 were considered significant.

Results
l-Hyd4FC treatment induces strong reduction of HBV viraemia in chimeric mice
To investigate the efficacy of the new compound l-Hyd4FC (Figure 1A) to reduce HBV viraemia in vivo, human chimeric uPA mice with stable viraemia (median 4×10^7 HBV DNA copies/ml; n=7) received daily injections of 50 mg/kg l-Hyd4FC. As control, chimeric mice displaying similar viraemia levels were either injected with saline (n=4) or received 4 weeks of oral administration of 30 mg/kg/day lamivudine (n=4). As shown in Figure 1B, both drugs efficiently lowered viraemia levels, although the antiviral effect of l-Hyd4FC versus lamivudine appeared slightly superior both after 2 weeks (1.7-log versus 1.4-log reduction, respectively) and 4 weeks (2-log versus 1.5-log reduction, respectively) of treatment. Figure 2A shows in detail viraemia changes determined over time in individual mice treated with l-Hyd4FC. Treatment of 2 weeks induced a rapid and highly significant reduction of HBV viraemia (to a median of 6×10^5 copies/ml; P=0.006), whereas viraemia levels in untreated control mice remained unchanged (median 5×10^5 copies/ml; P=0.8). Notably, treatment prolongation to 4 weeks did not induced further significant reduction of viraemia levels (n=3; median 8×10^5 copies/ml; P=0.7). Exemplary reversibility of the antiviral effect was demonstrated in one mouse. After 2 weeks of treatment with l-Hyd4FC the drug was withdrawn and the viral titres returned to baseline within the next 2 weeks (Figure 2A). Furthermore, HSA levels remained stable during the whole experiment, both in the l-Hyd4FC treated and untreated control mice (Figure 2B). Altogether these serological analyses indicated that in vivo treatment with l-Hyd4FC did not affect the viability of the human hepatocytes.

Subviral particles are generally produced and secreted in large excess during infection and represents the major source of HBsAg determined in serum of HBV-infected individuals. To investigate whether l-Hyd4FC administration affected the production...
and secretion of this very sensitive HBV serological marker, HBsAg levels were quantitatively measured in individual mice both at baseline (mean 3,670 IU/ml) and after 4 weeks of therapy (mean 4,060 IU/ml; \( n = 3 \)). As expected, 4 weeks of treatment did not induce significant changes in HBsAg levels (\( P = 0.9 \)). Similarly, as shown in Figure 2C, the levels of circulating HBsAg did not decrease significantly in mice treated with lamivudine (mean 3,400 versus 2,430 IU/ml at baseline and 4 weeks, respectively). The levels of circulating HBeAg also remained similar both in mice receiving \( l \)-Hyd4FC or lamivudine for 4 weeks (Figure 2D).

Intrahepatic analysis of humanized mice treated with \( l \)-Hyd4FC

Mice were sacrificed after 2 (\( n = 3 \)) or 4 (\( n = 3 \)) weeks of \( l \)-Hyd4FC treatment to analyse the intrahepatic viral activity, as well as possible signs of drug-induced toxicity in human hepatocytes. Intrahepatic virological measurements revealed that stably HBV-infected control mice displayed a median of 445 rcDNA copies per cccDNA (\( n = 4 \)), whereas in mice that received either 2 or 4 weeks of \( l \)-Hyd4FC treatment, virion productivity was significantly reduced to 180 and 59 rcDNA copies per cccDNA molecule, respectively (Figure 3A; \( P = 0.004 \)). Thus, intrahepatic measurements showed that 4 weeks of in vivo treatment with \( l \)-Hyd4FC reduced the amount of newly produced HBV DNA by 87% (ratio of rcDNA/cccDNA). Quantitative analysis of intrahepatic cccDNA contents also revealed that the average cccDNA copy number determined per human hepatocyte reconstituting the liver of the uPA/SCID mice did not differ significantly between antiviral treated and untreated control mice (\( P = 0.1 \); Figure 3B), confirming that the antiviral effect of \( l \)-Hyd4FC was due to the inhibition of the HBV DNA polymerase and not to cccDNA reduction.

Quantitative analysis of the steady-state levels of HBV RNA transcripts performed by real-time PCR revealed that pgRNA levels remained unchanged during the first 2 weeks of treatment (Figure 3C; from a median 0.07 pgRNA/GAPDH at baseline versus median 0.06 pgRNA/GAPDH at week 2). However, after 4 weeks of treatment, the levels of pgRNA appeared significantly lower (median 0.02/GAPDH; \( P = 0.04 \)).

By contrast, the amount of subgenomic viral transcripts (preS/S RNAs) did not vary significantly between \( l \)-Hyd4FC treated (median 1.1 preS/S RNA/GAPDH at week 2 and median 0.7 preS/S RNA/GAPDH at week 4) and untreated controls (median 0.7 preS/S RNA/GAPDH; Figure 3D; \( P = 0.09 \)), indicating that the transcription of HBV-specific proteins was not significantly affected by \( l \)-Hyd4FC administration. This was also in line with the lack of significant reduction of serologic HBsAg levels determined at the end of treatment.

The comparative analysis of liver sections obtained from untreated and treated mice failed to detect a clear reduction of core protein levels by immunohistochemistry, both in mice treated for 4 weeks with \( l \)-Hyd4FC or with lamivudine (Figure 4), since most of the human hepatocytes that were identified in mouse liver sections by using hu-CK18 antibody stained positive for HBcAg and showed a predominant nuclear distribution. Furthermore, the TUNEL staining, which was performed to detect possible signs of cell death or apoptosis in liver tissues, was negative in all mice, indicating that 4 weeks of treatment with the new compound \( l \)-Hyd4FC did not induce detectable damage of the human hepatocytes under the antiviral conditions employed (Figure 5).
Suppression of HBV replication by \( \text{l-Hyd4FC} \) in humanized uPA mice

**Discussion**

Small animal models amenable to infection with human hepatotropic viruses are in great need for the preclinical validation of new compounds and novel therapeutic approaches before going into clinical trials [17,22]. In the chimeric uPA mouse model the engrafted hepatocytes undergo several rounds of cell division within the first 2 months after transplantation to replace the diseased mouse hepatocytes, but remain highly differentiated throughout the lifespan of the recipient, creating a unique *in vivo* environment stably supporting the full life-cycle of HBV and HCV. Therefore, the model is suitable to evaluate the potency of new antiviral compounds interfering both with viral entry [13,23] and replication [15,17,20,24], as well as to investigate the possible cytotoxicity of new antiviral compounds in human hepatocytes, the real target cells [9,10].

Figure 2. Suppression of HBV viraemia induced by the novel antiviral substance \( \text{l-Hyd4FC} \) in human chimeric mice

A total of 11 HBV-infected mice displaying median viraemia of \( 6 \times 10^7 \) HBV copies/ml were treated either with 2',3'-didehydro-2',3'-dideoxy-\( N^4 \)-hydroxy-5-fluorocytidine (\( \text{l-Hyd4FC; n=7} \)) or saline (\( n=4 \)). Serum was taken after 2 and 4 weeks of treatment to determine changes of (A) viraemia levels and (B) human serum albumin (HSA) concentrations. (A) Treatment with \( \text{l-Hyd4FC} \) induced a 2-log decrease of circulating virions compared to baseline (\( P=0.006 \)), whereas viraemia levels remained stable in the control group receiving saline. Viraemia rebound after drug discontinuation was analysed in one mouse. (B) HSA levels were determined at baseline and after 4 weeks of \( \text{l-Hyd4FC} \) administration in serum samples obtained both from treated and untreated control mice by ELISA. Levels of circulating (C) hepatitis B surface antigen (HBsAg) and (D) hepatitis B e antigen (HBeAg) were determined in individual mice shortly before starting therapy and after 4 weeks of treatment with \( \text{l-Hyd4FC; n=3} \) or lamivudine (\( n=3 \)).
In this study we first showed that daily application of l-Hyd4FC was able to reduce viraemia by 2-logs during a treatment period of 4 weeks. By comparison, treatment of chimeric mice with lamivudine, which is the best known cytosine derivative already used in the clinic, indicated that this new β-l-deoxycytidine compound was slightly more effective in lowering viraemia levels, although lamivudine was administered to the mice at high dosage [15]. Additional serological analyses also revealed that 4 weeks of l-Hyd4FC or lamivudine administration did not induce significant changes in the levels of circulating HBsAg and HBeAg, supporting the notion that administration of these drugs does not affect the transcription of HBV-specific proteins.

To further analyse the antiviral potency and rule out the occurrence of drug-induced toxicity on the human hepatocytes, l-Hyd4FC-treated mice were sacrificed either after 2 or 4 weeks of treatment. We found that intrahepatic virion productivity (relaxed circular DNA [rcDNA] copies/covalently closed circular DNA [cccDNA]), intrahepatic rcDNA copies per human hepatocyte, steady-state levels of pregenomic RNA (pgRNA) and of subgenomic HBV RNA (preS/S RNA) were normalized to human GAPDH. All measurements were compared to levels determined in the HBV-infected untreated control group of mice. l-Hyd4FC administration for 4 weeks induced significant reduction of (A) rcDNA/cccDNA (P=0.004) and of (C) pgRNA (P=0.04).

Stable HBV-infected human chimeric mice were treated either with 50 mg/kg 2',3'-didehydro-2',3'-dideoxy-N4-hydroxy-5-fluorocytidine (l-Hyd4FC) or saline. After 2 weeks (n=3) or 4 weeks (n=3) of antiviral treatment, mice were sacrificed 24 h after the last injection to determine intrahepatic viral changes. (A) Levels of virion productivity (relaxed circular DNA [rcDNA] copies/covalently closed circular DNA [cccDNA]), (B) Intrahepatic rcDNA copies per human hepatocyte, (C) Steady-state levels of pregenomic RNA (pgRNA) and of (D) subgenomic HBV RNA (preS/S RNA) were normalized to human GAPDH. All measurements were compared to levels determined in the HBV-infected untreated control group of mice. l-Hyd4FC administration for 4 weeks induced significant reduction of (A) rcDNA/cccDNA (P=0.004) and of (C) pgRNA (P=0.04).
Suppression of HBV replication by L-Hyd4FC in humanized uPA mice

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Figure 4. Expression levels of HbcAg in antiviral treated and untreated mice

Hepatitis B core antigen (HbcAg) staining (green, upper panels) of liver tissues obtained from HBV-infected chimeric mice receiving either 2,3'-didehydro-2,3'-dideoxy-N4-hydroxy-5-fluorocytidine (L-Hyd4FC; central panels), lamivudine (right panels) or saline (left panels) for 4 weeks indicated that antiviral treatment did not induce significant reduction of intracellular HbcAg levels. Double staining with Monoclonal Mouse Anti-Human Cytokeratin 18 (hu-CK18; Dako, Hamburg, Germany) antibodies identifies human cells in urokinase plasminogen activator/SCID mouse livers (red, lower panels).

In conclusion, this study provides further evidence that humanized uPA/SCID mice represent a very effective small animal system for preclinical studies, enabling us to investigate, both at serological and intrahepatic level, the antiviral potency of a new compound in vivo and, at the same time, its possible cytotoxicity. Our analysis indicates that L-Hyd4FC is a good candidate for further investigations. In particular, it will be important to determine the resistance profile of this new potential HBV therapeutic agent.

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TV, ML and LA contributed to the acquisition and analysis of data. TV, LA and MW contributed to mouse transplantation. ML, JMP and JB contributed to human hepatocyte preparation. MD, ML, EM and JP contributed to study concept, design and manuscript preparation.

Disclosure statement

The authors declare no competing interests.
**References**

7. Matthes E, Bunger H. Cellular pharmacology of the anti-hepatitis B virus agent beta-t-2',3'-didehydro-2',3'-dideoxy-N4-hydroxy-5-fluorocytidine (l-Hyd4FC; right). Serial sections incubated with DNAase were used as positive controls as recommended by manufactures (left). Staining of the same sections with Monoclonal Mouse Anti-Human Cytokeratin 18 (hu-CK18; Dako, Hamburg, Germany; red) antibodies allows the identification of human hepatocytes that have reconstituted the mouse livers. DAPI staining (blue) was utilized to visualize cell nuclei of human and mouse origin present on the same liver sections.