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Effect of a novel synthetic FXR agonist EYP001 on hepatitis B virus replication in HepaRG and primary human hépatocytes

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Introduction

Treatment of hepatitis B relies on pegylated interferon (pegIFN) and/or on polymerase inhibitors. Only a minority of patients respond to pegIFN with HBs seroconversion. Polymerase inhibitors suppress viral replication at the cost of a life-long treatment. Indeed such therapies poorly modify the size of the cccDNA reservoir and treatment interruption is followed by viral replication reactivation with often-deleterious consequences. There is thus an unmeet need for new therapy aiming at controlling the expression of the cccDNA.

cccDNA forms a minichromosme that associates viral proteins HBc and HBx as well as cellular factors including histones, histone acetylases and deacetylases, nuclear receptors and co-activators or repressors. These factors control both the persistence and expression of the minichromosome.

Among the liver restricted nuclear receptors we showed that two response elements for the farnesoid X receptor alpha (the bile acids nuclear receptor FXRa) are located within the enhancer II/ core promoter region (EnhII/Cp) (1). Expression of FXR in cells of non-hepatic origin is sufficient to allow the transcription and expression of the viral RNAs upon activation by bile acids or FXR agonists. Moreover, PGC-1a and Sirt1, two key metabolic factors, form a network of proteins regulating the transcriptional activity of the cccDNA in an FXR dependant manner (2). Interestingly bile acids and HBV compete for the sodium taurocholate cotransporting polypeptide (NTCP), expressed at the hepatocyte baso-lateral plasma membrane (3). HBV infection interferes with FXR signaling with subsequent modifications of the expression of FXR regulated genes including its own over-expression, either by binding and limiting NTCP function, inducing a down regulation of FXR activity or through other mechanisms that may involve binding of viral proteins to FXR (4). HBV infection is thus characterized by important modifications of the bile acids metabolism pathway. This intimate link between bile acids metabolism and HBV replication suggests that manipulating this pathway might be exploited to control cccDNA expression.

We investigated the effect of FXR activity modulation on HBV replication by a novel synthetic non-steroidal FXR agonist EYP001 and compare its activity to the BA derived 6-ethyl-chenodeoxycholic acid (6-ECDCA) and the synthetic FXR agonist GW4064.

Materials and Methods

Cells and virus

The HepaRG line derived from a human cellular hepato carcinoma can regain many phenotypic traits of hepatocytes after 4 weeks of culture under defined conditions (5). After differentiation, these cells are susceptible to infection at high MOI of HBV virions produced by HepG2.2.15 line. Under these conditions viral production can be observed in the second week post infection.

Freshly plated primary human hepatocytes (PHH) were obtained from Human HepCell (Paris, France). Upon arrival, cells were placed in a 5% CO2 atmosphere at 37°C, in Williams medium supplemented with 2% v/v HyClone FetalClone II serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1% v/v insulin-transferrin-selenium, 20 µg/ml gentamicin (Life Technologies), 50 µM hydrocortisone hemisuccinate and 1.8% v/v DMSO (Sigma-Aldrich).

HEK293T line was maintained in standard conditions.

Chemicals

The synthetic, non-sterol, reference FXR agonist GW4064 was purchased from Sigma Aldricht. The bile acid derivative FXR agonist 6a-ethylchenodeoxycholic acid (6-ECDCA) was synthesized by MetaBrain Research (Chilly Mazarin, France) (6). The non-steroid FXR agonist EYP001 was provided by Enyo Pharma, Lyon, France.

Results

1- Effect of EYP001 on GpBAR1 and FXR activity

We first tested the effects of EYP001 on the bile acids plasma membrane receptor GPBAR1 and the nuclear receptor FXR. GPBAR1 was transiently expressed in HEK293T and cells were treated with lithocholic acid, their natural ligand, 6ECDCA, a bile salt derivative agonist for FXR and GPBAR1, GW4064, a synthetic non-steroidal specific FXR agonist, and EYP001. GPBAR1 dependant induction of cAMP synthesis was only observed with 6ECDCA or lithocholic acid and not with GW4064 or EYP001 indicating that this later compound is FXR specific.

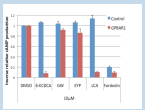


Figure 1: HEK293T cells were transfected with pGPBAR1-EGFP-N1 or pEGFP-N1 empty vector and treated with Forskolin, lithocholic acid, GW4064, 6-ECDCA 10µM for 15 min. cAMP was quantified using cAMP-Screen® System kit (Applied Biosystems).

We next tested the effect of EYP001 on the expression of FXR, SHP and APOA1, three genes under the control of FXR in differentiated HepaRG and PHH and for the latter in HBV or mock infected cells.

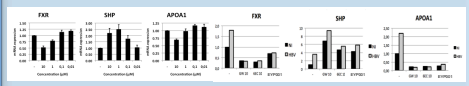


Figure 2: A, dHepaRG were treated for 12 days with EYP001 (10 mM). B, after seeding, PHH were infected or not by HBV for 6h and subsequently treated with EYP001, GW4064 and 6ECDCA (all at 10 mM) for 10 days. The indicated mRNA were quantified by qRT-PCR.

As expected for a FXR agonist, treatment with EYP001 repressed the expression of APOA1 and FXR mRNA and increased that of SHP in both cell culture systems in a dose dependent manner. HBV infection increased the expression of all three mRNA and treatment with any FXR agonists reversed the effect of the HBV induced FXR and APOA1 over expression while still increasing that of SHP.

2- EYP001 represses HBV replication in dHepaRG

EYP001 represses the secretion of HBs and HBe antigens as well as that of viral DNA in a dose dependent manner after 10 days of treatment.

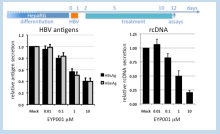


Figure 3 : After infection, dHepaRG cells were treated for 10 days with the indicated concentrations of EYP001. Cell supernatants were collected 14 days post infection for quantification of HBsAg, HBeAg (Architec Abbott), **A** and HBV rcDNA by qPCR, **B** (n=3 ± SEM).

Treatment with the FXR agonist EYP001 inhibited in a dose dependent manner the transcription of the preg and preCore mRNA. The intracellular pool of rcDNA and of cccDNA was also significantly reduced.

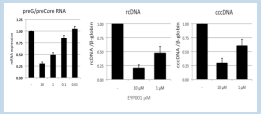


Figure 4: Cells were treated as in figure 3. pg/preCmRNA were quantified from cell lysates by qRT-PCR. Total DNA from HepaRG cells was extracted and treated with Plasmid-Safe ATP-dependent DNase before qPCR experiments were carried out. β-globin gene quantification was used for normalization. (n=3 ± SEM)

3 - Effects of FXR agonists and EYP001 on HBV replication in PHH

➢Effects of FXR agonists and infection on PHH

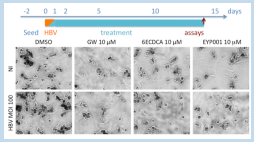


Figure 5: After seeding PHH were infected or mock infected and treated for 2 weeks with FXR agonists GW4064, 6ECDCA and EYP001 at 10mM or vehicle only. No toxicity was detected by monitoring LDH activity and quantification of total cell RNA and DNA contents (not shown). HBV infection induced a mild cytopathic effect.

Treatment with FXR agonists preserved the cell morphology during the 2 weeks treatment and reduced the HBV-induced cell alteration.

➢The FXR agonists repress the viral proteins and rcDNA secretion

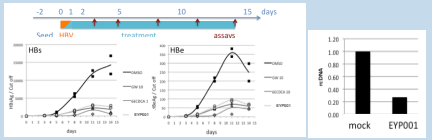


Figure 6: After seeding PHH were treated with 10 µM of the FXR agonists GW4064, 6ECDCA, EYP001 or with vehicle only for 2 weeks. Secretion of HBs (A) and HBe (B) in the supernatant was monitored using the HBsAgII and HBeAg kits (Architect, Abbott Diagnostics). rcDNA secreted in the supernatant was quantified by qPCR at day 14 (C).

Treatment with FXR agonists induced a sustained inhibition of HBs and HBe secretion into the cell culture supernatant. Inhibition was similar for the three FXR agonists. rcDNA secretion was reduced by EYP001 treatment.

➢ FXR agonists repress viral mRNA synthesis and the pool of cellular HBV DNA

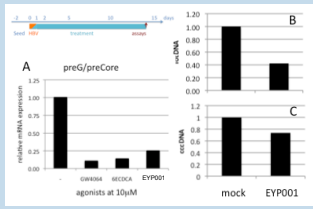


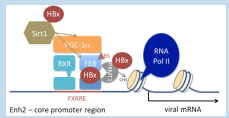
Figure 7 : After seeding PHH were treated with 10mM of the FXR agonists GW4064, 6ECDCA, EYP001 or with vehicle only. Expression of preG/preCore mRNA was quantified by qRT-PCR (A). Total cellular HBV DNA (B) or cccDNA (C) were quantified by qPCR or cccDNA qPCR. Relative fold change variations were normalized with b-globine gene quantification.

Treatment by FXR agonists at 10mM for 2 weeks dramatically reduced the synthesis of the pre-genomic and pre-Core mRNA with some small efficiency differences. EYP001 reduced the pool of cellular total viral DNA and to a lower extent the cccDNA pool. A

Conclusions

Here we showed that EYP001 does not activate GPBAR1, the plasma membrane bile acid receptor but indeed activates the transcription of genes under the control of FXR. HBV infection of PHH modifies the FXR dependent gene profile and EYP001, as well as two other FXR agonists, can reverse some of the HBV-induced modification of gene expression. EYP001 induces a sustained repression of HBV replication in the HepaRG and PHH cell culture systems. The decreased expression of the viral mRNA suggests that the inhibition of the viral replication mostly results from the modulation of FXR activation that could perturb the complex FXR network of transcription factors, which is highly targeted and controlled by HBx. These data stress out the importance to exploit drug regulation of metabolism pathways in controlling HBV replication.

We hypothesize that FXR binds to the cccDNA in an inactivated state under the control of PGC-1α and Sirt1. HBx participates to this network of cellular proteins by binding to FXRα (unpublished data) and regulating the function of Sirt1. This complex might stabilize the cccDNA and contribute to its persistence. Upon activation by its ligands, FXR recruits the transmethylase PRMT1, a transcription inhibitor, the activity of which is repressed by HBx (8,9). In addition FXR activation leads to a negative retro-control of its expression. Therefore, the effects of HBV, which increases FXR expression while decreasing its activity might favor the FXR stabilizing effect on cccDNA without inhibiting the transcription. The opposite effects of FXR agonists on FXR expression and activity might thus decrease the cccDNA stability and repress its transcription leading to a global inhibition of the virus replication.



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Acknowledgments and Contact

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P. André and V. Lotteau are co-founders and consultants at Enyo Pharma, (www.envopharma.com)