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Bile acids receptor FXR agonists repress HBV replication in HepaRG cell

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Introduction

Treatment of hepatitis B relies on pegylated interferon 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 and persistence of the cccDNA pool.

cccDNA forms a circular double stranded DNA 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 at least 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 FXRa in cells of non-hepatic origin is sufficient to allow the transcription and expression of the viral RNAs upon activation by bile acids or FXRa agonists. Moreover, PGC-1a and Sirt1, two key metabolic factors, form a network of proteins regulating the transcriptional activity of the cccDNA in an FXRa 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 binding to NTCP limits its function, inducing a down regulation of FXRa activity with subsequent modifications of the expression of FXRa regulated genes including its own over-expression (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 as a therapeutic approach.

We thus tested the effects of various FXRa ligands on HBV replication in differenciated HepaRG cell.

Material and Methods

Cells and virus

The HepaRG line derived from a human cellular hepato carcinoma can differentiate and 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.

Chemicals

The reference antagonist 052EDL133 described in patent WO 2007052843; Takeda Pharmaceutical Co. Ltd., Osaka, Japan was provided by Edelris Medicinal Keymistry, Lyon, France. The synthetic, non-sterol, reference agonist GW4064 such as disclosed in PCT Publication No. WO 00/37077 or in US2007/0015796 was purchased from Sigma Aldricht. The bile acid derivative FXRa agonist 6a-ethylchenodeoxycholic acid (6-ECDCA) was synthesized by MetaBrain Research (Chilly Mazarin, France) (6). Ursodeoxycholic acid (UDCA), a non FXR-ligand bile acid was bought from Sigma Aldricht.

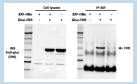
Experimental design

Differentiated HepaRG cells were infected with HBV (100 geq/cell for 24 hr), then treated 3 successive times (days 4, 7 and 11 post infection) with FXRa modulators at indicated concentrations (mM). Cell supernatants were collected 14 days post infection for testings. .

Results

1- HBx directly targets FXRa and its network.

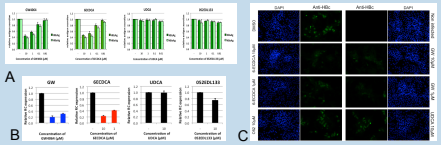
PGC-1a and Sirt1 effects on the transcriptional activity of HBV EnhII/core promoter region depend on FXRa activation. HBx directly binds to Sirt1 and modifies its activity (7). We then wondered if HBx also binds directly to FXRa by a co-immunoprecipitation assay (Fig. 1). Our results strongly suggest an interaction between HBx and FXRa.



HEK293T cells were co-transfected with fusion proteins 3XF-HBx and Gluc-FXR encoding plasmids. 48h post-transfection, cells were lysed and co-immunoprecipitation was performed with Dynabeads®Protein G beforehand coupled with anti-3XF antibody. Cells lysates and co-immunoprecipitation products were analyzed by western blot. FXR expression was similar in control and test conditions (left blot). After immunoprecipitation with anti-3XF antibody, FXR fusion protein was detected in the test condition and not in the control (right blot).

2- FXRa agonists repress HBV replication in HepaRG

The effects of the agonists 6ECDCA and GW4064 and of the antagonist 052EDL133 as well as the FXRa non-ligand bile salt UDCA were tested on a complete virus replication cycle in HepaRG (Fig 2).

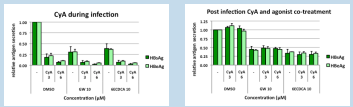


After infection, cells were treated for 10 days with the indicated concentrations of FXRa modulators. Cell supernatants were collected 14 days post infection for, A, quantification of HBsAg, HBeAg (Architec Abbott) and, B, HBV DNA by quantitative PCR using rcDNA primers (n=3 ± SEM).
C, differentiated HepaRG cells grown on coverslips were infected and treated as above (n=3 ± SEM). Cells were fixed on day 14 post infection and immunocytochemistry using anti-HBc antibody was carried out.

Only FXRa agonists, bile salt derivative or synthetic, decrease HBV proteins expression while UDCA and antagonists do not.

3- FXRa agonists repress viral replication at a post-entry step

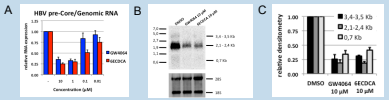
As HBV and bile salts compete for NTCP, FXRa agonists might repress HBV expression by entry inhibition. To test this hypothesis, cyclosporin A, which inhibits HBV binding to NTCP was added during or after infection, in combination with FXRa agonists (Fig. 3)



In addition to the usual protocol, cells were treated with cyclosporin A (CyA) either during HBV infection (i.e. for 24 hr), left panel, or during the treatment with FXRα agonists (i.e. for 72 hr ; from day 4 to 7 post infection), right panel. Cell supernatants were collected 14 days post infection for quantification of HBsAg and HBeAg (n=3 ± SEM).

CyA treatment during HBV infection inhibits viral entry in a dose-dependent manner and does not impair the decrease in HBsAg and HBeAg secretions following treatment with FXR agonists. CyA treatment post infection has no effect on HBV antigen secretion whatever the presence or not of FXRa agonists. These data indicate that action of FXRa agonsits on HBV replication occur at post- entry steps.

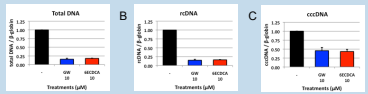
4- FXRa agonists decrease the expression of the viral RNAs



Differentiated HepaRG cells were infected and treated as described. Cells were lysed and RNA was extracted, then reverse transcribed into cDNA for quantitative PCR (qRT-PCR). The expression levels of HBV precore/pregenomic RNAs were quantified, as well as 3 housekeeping genes for normalization (n=3 ± SEM). Expression of all mRNAs was also analysed by Northern Blot (**B**) and quantified by densitometry (**C**).

Treatment with FXRa agonists decrease the expression of all HBV RNAs.

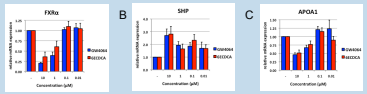
5- FXRa agonists repress the pool of cellular HBV DNA



Differentiated HepaRG cells were infected and treated as described and DNA was extracted. Following plasmid-safe DNase treatment, total HBV DNA (A) and cccDNA (C) expression was quantified by qPCR experiment using specific primers and TaqMan probe (n=3 ± SEM). DNA quantification was normalized by the number of βglobin gene. Quantification of rcDNA was obtained by substracting cccDNA to total DNA (**B**).

Treatment by FXRa dramatically reduce all intra-cellular HBV DNA forms including the cccDNA pool by more than 50%.

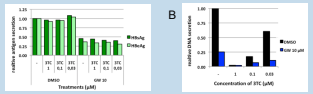
6- Effect of treatment by FXRa on the expression of cellular genes



Differentiated HepaRG cells were infected and treated as described. Cells were lysed and RNA was extracted, then reverse transcribed into cDNA for qPCR. The expression levels of 3 genes of interest were quantified : FXRα, SHP and APOA1, as well as 3 housekeeping genes for normalization (n=3 ± SEM).

FXRa agonists inhibit the expression of FXRa mRNA in a dose-dependent manner. SHP and APOA1 are two genes under the regulation of FXRa : SHP is induced by FXRa while APOA1 is repressed. Here, SHP mRNA expression increases with GW4064 and 6ECDCA treatments, while APOA1 mRNA expression decreases. This suggests an activation of FXRa despite its reduced expression.

7- Effect of RT inhibitor addition to FXRa agonist on HBV replication

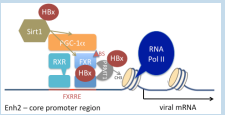


Differentiated HepaRG cells were infected and treated as described plus the indicated lamivudine (3TC) concentrations. Cell supernatants were collected 14 days post infection. (**A**) Quantification of HBsAg and HBeAg secretion. (**B**) Quantification of secreted infectious particles by DNA extraction and quantification by qPCR.

Conclusions

Treatment with BA derived 6-ethyl-chenodeoxycholic acid (6-ECDCA) or synthetic non-steroidal GW4064 agonists, but not with antagonists or ursodeoxycholic acid, strongly inhibited the secretion of HBV DNA, HBsAg, HBeAg and of HBcAg synthesis in a dose dependent manner (70 to 80 % inhibition at 1 or 10 microMol) as well as the viral pregenomic RNA synthesis, cccDNA copies number and total cellular HBV DNA. Cyclosporine A, an NTCP ligand and HBV entry inhibitor, did not modify the effect of agonists suggesting that the effect did not depend on entry inhibition. Treatment consistently increased FXRa activity as indicated by the increase of the small heterodimer partner (SHP) and decrease of the apolipoprotein-A1 mRNAs expression, two FXRa dependent genes, despite reduced FXR mRNA levels. Interestingly FXRa activation neutralizes the effects of HBV infection on the bile acid metabolism at the cellular level.

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