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Characterization of the antiviral properties and mode of action of FXR ligands in Hepatitis Delta Virus (HDV) infection

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Université Claude Bernard  Lyon 1

**THESE de DOCTORAT DE
L'UNIVERSITE CLAUDE BERNARD LYON 1**

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Biologie Moléculaire Intégrative et Cellulaire (BMIC)**

Discipline : Virologie

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**Characterization of the antiviral
properties and mode of action of FXR
ligands in Hepatitis Delta Virus (HDV)
infection**

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« Il n'y a pas de plus belle aventure que celle qui se termine ; mais il est quand même très difficile de dire au revoir. Longtemps Archibald avait souhaité connaître la fin de son livre préféré, mais à présent que Rousseau le lui avait prêté et qu'il le tenait entre ses pattes, il n'était pas vraiment sûr de vouloir le lire... A partir de cette histoire inachevée, il avait imaginé des conclusions personnelles, des retournements mystérieux, des trahisons insupportables et des vengeances assouvies : là où le roman lui avait fait défaut, son imagination avait pris le relais pour lui conter ce que ses yeux ne pouvaient lire. Ce second volume serait-il à la hauteur de ses projections imaginaires ? mais le lire c'était cesser de l'espérer. Quand enfin il découvrirait, l'histoire prendrait fin, et il faudrait dire au revoir aux personnages qu'il avait appris à aimer et qui l'avaient accompagné toutes ces années. Oui, c'est quand même très difficile de dire au revoir, pensa le libraire. »

Mémoires de la forêt, Mickaël Brun-Arnaud

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Résumé

L'infection chronique par le virus de l'hépatite B (VHB) touche environ 250 personnes dans le monde. Parmi ces patients infectés par le VHB, on estime que 5 à 10 % sont également co-infectés par le virus de l'hépatite Delta (VHD), un virus défectueux et satellite du VHB. L'hépatite delta chronique (CHD) est considérée comme la forme la plus agressive des hépatites virales chroniques et est associée à une progression rapide vers la fibrose et la cirrhose ainsi qu'à une augmentation du risque de carcinome hépatocellulaire et de décès. Le VHD ne code pas pour des protéines enzymatiques et n'utilise que des polymérases cellulaires pour sa réplication, compliquant le développement de traitements antiviraux spécifiques. Pour le traitement de la CHD, les directives internationales recommandent l'utilisation de l'IFN alpha pégylé (PEG-IFN α) en combinaison ou non avec des analogues de nucléos(t)ides qui inhibent la transcriptase inverse du VHB. Néanmoins, ces traitements donnent des résultats insatisfaisants sur le VHD et sont associés à des effets secondaires et à des rechutes. Le développement de nouveaux antiviraux est donc nécessaire.

Nous avons montré précédemment que certains ligands du récepteur farnésioïde-X alpha (FXR), le principal récepteur nucléaire des acides biliaires (BA), sont des inhibiteurs de la réplication du VHB. Hormis le rôle de NTCP, le transporteur hépatique des acides biliaires qui est utilisé comme récepteur cellulaire par le VHB et le VHD pour infecter les hépatocytes, le lien entre le métabolisme des BA et le cycle de vie du VHD n'a pas encore été exploré. Dans ce travail, nous avons donc voulu déterminer si le récepteur nucléaire FXR jouait un rôle dans l'infection par le VHD.

Pour ce faire, des mono-infections par le VHD ou des co-infections et surinfections par le VHB et le VHD ont été établies dans deux modèles pertinents, la lignée cellulaire différenciée HepaRG (dHepaRG) et les hépatocytes humains primaires (PHH). L'effet de plusieurs ligands de FXR sur l'infection par le VHD ont été analysés. Nous avons constaté que le GW4064, ligand de FXR, diminuait de manière significative les ARN du VHD dans les cellules dHepaRG et PHH, de 44 % et 60 % respectivement. La quantité d'antigènes delta a également été réduite. La diminution de ces marqueurs intracellulaires de la réplication du VHD a été associée à une réduction de 60 % de la sécrétion des virions du VHD. En outre, l'infectivité spécifique des particules de VHD a été réduite de plus de 95 %, ce qui indique que les propriétés infectieuses des particules sécrétées ont été profondément altérées par le traitement aux ligands de FXR.

L'activité antivirale des ligands du FXR a été confirmée comme étant dépendante du FXR. En conclusion, les ligands du FXR peuvent inhiber la réplication du VHD de manière dépendante du FXR, ainsi que la sécrétion des virions et l'infectivité spécifique. L'effet antiviral était supérieur à celui obtenu avec l'IFN- α *in vitro*, ce qui suggère que le ciblage du FXR est une approche thérapeutique prometteuse pour le traitement des patients infectés par le VHD.

Mots clés : Virus de l'Hépatite Delta, FXR, Métabolisme des acides biliaires, Antiviraux.

Abstract

Chronic hepatitis B virus (HBV) infection affects around 250 million people worldwide. Of these HBV-infected patients, an estimated 5-10% are also co-infected with hepatitis delta virus (HDV), a defective virus that is a satellite of HBV. Chronic hepatitis delta (CHD) is considered the most aggressive form of chronic viral hepatitis and is associated with rapid progression to fibrosis and cirrhosis, as well as an increased risk of hepatocellular carcinoma and death. HDV does not code for enzymatic proteins and uses only cellular polymerases for replication, complicating the development of specific antiviral treatments. For the treatment of CHD, international guidelines recommend the use of pegylated IFN alpha (PEG-IFN α) in combination or not with nucleos(t)ides analogs that inhibit HBV reverse transcriptase. Nevertheless, these treatments give unsatisfactory results on HDV and are associated with side effects and relapses. This calls for the development of new antivirals.

We have previously shown that certain ligands of the farnesoid-X alpha receptor (FXR), the main nuclear bile acid receptor (BA), are inhibitors of HBV replication. Apart from the role of NTCP, the hepatic bile acid transporter that is used as a cellular receptor by HBV and HDV to infect hepatocytes, the link between BA metabolism and the HDV life cycle has not yet been explored. In this work, we set out to determine whether the FXR nuclear receptor plays a role in HDV infection.

To this end, HDV mono-infections or co-infections and superinfections with HBV and HDV were established in two relevant models, the differentiated cell line HepaRG (dHepaRG) and primary human hepatocytes (PHH). The effect of several FXR ligands on HDV infection was analyzed. We found that FXR ligand GW4064 significantly decreased HDV RNAs in HepaRG and PHH cells, by 44% and 60% respectively. The amount of delta antigens was also reduced. The decrease in these intracellular markers of HDV replication was associated with a 60% reduction in the secretion of HDV virions. In addition, the specific infectivity of HDV particles was reduced by over 95%, indicating that the infectious properties of secreted particles were profoundly altered by FXR ligand treatment.

Key words: Hepatitis Delta Virus, FXR, Bile acid metabolism, Antiviral drugs.

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List of abbreviations

ADAR1: Adenosine Deaminase Action on RNA 1

AF: Activation Function

AGL: Antigenic Loop

AIP1: Actin-interacting Protein 1

ALIX: ALG-2-interacting Protein X

ApoB: Apolipoprotein B

ApoCII: Apolipoprotein CII

ApoCIII : Apolipoprotein CIII

AP-2: Adaptor Protein 2

ASBT: Apical Sodium-dependent Bile Salt Transporter

ASO: Antisense Oligonucleotide

BACS: Bile Acid CoA Synthase

BAs: Bile acids

BSEP: Bile Salt Export Pump

CA: Cholic Acid

CAR: Chimeric Antigen Receptor

cccDNA: Closed Covalent Circular DNA

CDCA: Chenodeoxycholic Acid

CETP: Cholesterol-ester Transfer Protein

CHB: Chronic Hepatitis B

CHC: Clathrin Heavy Chain

CH25H: Cholesterol 25-hydroxylase

COX-2: Cyclooxygenase-2

CpAM: Capsid Assembly Modulator

CYP7A1: Cholesterol 7 α -hydroxylase

CYP8B1: Sterol 12 α -hydroxylase

CYP27A1: Sterol 24-hydroxylase

CYP46A1: Cholesterol 24-hydroxylase

DBD: DNA Binding Domain

DCA: Deoxycholic Acid

DDB1: Damage DNA Binding Protein 1

ddPCR: Digital Droplet PCR

DHBV: Duck Hepatitis B Virus

dHepaRG: Differentiated HepaRG

DR: Direct Repeat

EGFR: Epidermal Growth Factor Receptor

EMSA: Electrophoretic Mobility Shift Assay

Enh: Enhancer

ER: Endoplasmic Reticulum

ERK: Extracellular Signal-regulated Kinase

ERR α : Estrogen Receptor-related Receptor alpha

FBP1: Fructose 1,6-bis Phosphatase

FEN1: Flap Structure Specific Endonuclease 1

FGF15/19: Fibroblast Growth Factor 15/19

FGFR4: Fibroblast Growth Factor Receptor 4

FOXm1b: Forkhead Box m1b

FTI: Farnesyl Transferase Inhibitor

FXR: Farnesoid X Receptor

FXRE: FXR Response Element

GLUT2: Glucose Transporter Type 2

GLUT4: Glucose Transporter Type 4

GR : Glucocorticoid Receptor

GSK3 β : Glycogen Synthase Kinase-3 β

G6P: Glucose-6-Phosphate

HBV: Hepatitis B Virus
 HBcAg : HBV Core Antigen
 HBeAg: HBV E Antigen
 HBsAg: HBV Surface Antigen
 HBx: HBV X Protein
 HCC: Hepatocellular Carcinoma
 HCV: Hepatitis C Virus
 HDAg: Hepatitis Delta Antigen
 HDL : High-density Lipoprotein
 HDV: Hepatitis Delta Virus
 HDV-G RNA: HDV Genomic RNA
 HIV: Human Immunodeficiency Virus
 HNF4 α : Hepatocyte Nuclear Factor 4 alpha
 HP1: Heterochromatin Protein 1
 HSD3B7: Hydroxysteroid Dehydrogenase 3B7
 HSPG: Heparan Sulfate Proteoglycan
 IDL: Intermediate-density Lipoprotein
 IRS-1: Insulin Receptor Substrate 1
 IFN α : Interferon alpha
 IFN γ : Interferon gamma
 IFN λ : Interferon lambda
 IL-1 β : Interleukine 1 beta
 iNOS: Nitric Oxide Synthase
 LBL: Ligand Binding Domain
 LCA: Lithocholic Acid
 L-HDAg: Large Delta Antigen
 LDL: Low-density Lipoprotein
 LDLR: Low-density Lipoprotein Receptor
 LPK: L-type Pyruvate Kinase
 LPL: Lipoprotein Lipase
 MDR3: Multidrug-resistance 3 P-glycoprotein
 mRNA: Messenger RNA
 MRP2: Multidrug-Resistance-associated Protein 2
 MTP: Microsomal triglycerides Transfer Protein
 MVBs: Multivesicular Bodies
 NAFL: Nonalcoholic Fatty Liver
 NAFLD: Nonalcoholic Fatty Liver Disease
 NAP: Nucleic Acid Polymer
 NASH: Nonalcoholic Steatohepatitis
 NCOR1: Nuclear Receptor Corepressor 1
 NES: Nuclear Export Signal
 NF- κ B: Nuclear Factor Kappa B
 NHEJ: Nonhomologous DNA End Joining
 NLS: Nuclear Localization Signal
 NPC: Nuclear Pore Complex
 NR: Nuclear Receptor
 NTCP: Sodium Taurocholate Cotransporter Polypeptide
 NUC: Nucleos(t)ide Analog
 OATP: Organic Anion Transporting Polypeptide
 OCA: Obeticholic Acid
 ORF: Open Reading Frame
 OST α/β : Organic Solute Transporter α/β
 PBC: Primary Biliary Cholangitis
 PD-L1: Programmed Death-ligand 1
 PEPCK: Phosphoenolpyruvate-Carboxylase
 PEG-IFN: Pegylated Interferon
 pgRNA: Pre-genomic RNA
 PHH: Primary Human Hepatocyte
 PKC: Protein Kinase C
 POL II: Polymerase II

PPAR α : Peroxisome Proliferator-activated Receptor alpha
PLTP: Phospholipid Transfer Protein
PRR: Pattern Recognition Receptor
PSC: Primary Sclerosing Cholangitis
PXR: Pregnane X Receptor
RIP: RNA Immunoprecipitation
RIP14: RXR-interacting Protein 14
RIP15: RXR-interacting Protein 15
RISC: RNA-induced silencing complex
RNP: Ribonucleoprotein
rcDNA: Relaxed Circular DNA
RXR α : Retinoid X Receptor alpha
siRNA: Small Interfering RNA
S-HDAg: Small Delta Antigen
SHP: Small Heterodimer Partner
SMC4/5: Structural Maintenance of Chromosome 5/6

SMRT: Silencing Mediator Retinoic Acid and Thyroid Hormone Receptor
SR-B1: Scavenger Receptor Class B Member 1
SVP: Subviral Particle
SWI/SNF: Switch/sucrose Non-fermentable
TCR: T Cell Receptor
TDP2: Tyrosyl-DNA phosphodiesterase 2
TGF β : Transforming Growth Factor beta
TNF α : Tumor Necrosis Factor
UDCA: Ursodeoxycholic Acid
VLDL: Very Low-density Lipoprotein
VLDL: Very Low-density Lipoprotein Receptor
VPS4B: Vacuolar Protein Sorting 4 Homolog B
WHV: Woodchuck Hepatitis Virus
WHO: World Health Organization

Part 1: The hepatitis B virus

The hepatitis B virus (HBV) is a virus that specifically targets the liver and is responsible for hepatitis B. In cases of chronic infection, it can cause progressive destruction of liver cells, leading to the formation of scar tissue and eventually cirrhosis, liver failure, or hepatocellular carcinoma. Despite the existence of a safe and effective vaccine, HBV still affects around two billion people worldwide, of whom almost 296 million are chronically infected. Unfortunately, no curative treatment can completely eliminate the virus, making it a major global public health problem.

I. The discovery of the hepatitis B virus

The first descriptions of transmissible liver diseases, and more specifically epidemics of jaundice, date back to Antiquity, as witnessed by the “First Treatise on Medicine” written by Hippocrate¹. Over centuries, several explanations have been attributed to these epidemic cases of jaundice, but it was not until the end of the 19th century and the beginning of the 20th that the involvement of a transmissible agent was mentioned. Epidemics of jaundice were recorded after two smallpox vaccination campaigns in 1885, as well as following mass vaccinations against measles, mumps, and yellow fever in the 1930s and 1940s. The source of these epidemics was the use of human lymph or plasma administered with the vaccines. Based on these observations, Mac Callum proposed a viral origin as an explanation for these cases of transmissible jaundice². He made the first historical distinction between infections resulting from fecal-oral transmission, to which the name hepatitis A was attributed, and infections resulting from transmission via the blood, to which he gave the name serum hepatitis or hepatitis B.

In 1965, Baruch Blumberg and his team identified the « Australia » antigen in Aboriginal patient during a systematic study of sera from poly transfused patients. Today, The Australia antigen is known as the HBV surface antigen (HBsAg)³. This Australia antigen was subsequently identified as the causative agent of hepatitis B⁴. Blumberg was awarded the Nobel Prize for Medicine in 1976 for this discovery. In 1970, the work of David Dane and his team enabled the particles of HBV to be visualized for the first time using electron microscopy⁵. In 1979, Pierre Thiollais and his team sequenced and published the HBV genome⁶.

HBV belongs to the *Hepadnaviridae* family (group VII of the Baltimore classification) a group of small, spherical, enveloped viruses with mainly hepatotropic, partially double-stranded DNA. The *Hepadnaviridae* family is divided into two genera *Orthohepadnavirus* and *Avihepadnavirus*. The *Orthohepadnavirus* genus includes viruses that mainly affect mammals, such as HBV and WHV (Woodchuck Hepatitis Virus). The *Avihepadnavirus* genus includes viruses that infect birds, such as DHBV (Duck Hepatitis B Virus).

II. The epidemiology of HBV

Approximately two billion worldwide are infected with HBV in their lifetime, and there are around 1.5 million new infections every year. According to the World Health Organization (WHO), around 296 million people were living with chronic hepatitis B in 2019. HBV is responsible for around 820,000 deaths from cirrhosis or hepatocellular carcinoma (HCC).

HCC is one of the most common cancers in the world, and HBV is responsible for over 50% of cases, rising to 80% in regions where it is highly endemic. The prevalence of HBV varies considerably around the world, from 1% in low-endemicity regions to 30% in high-endemicity regions, with an overall prevalence of 3.61% worldwide (**Figure 1**). The two areas most affected are Asia Pacific (prevalence of 5.26%) and Africa (prevalence of 8.83%) with even higher rates in countries such as Sudan⁷. In contrast, HBsAg prevalence is low (below 2%)

in countries in Europe or the Americas, the Middle East, and South-East Asia. However, local variations and disparities in prevalence can be observed.

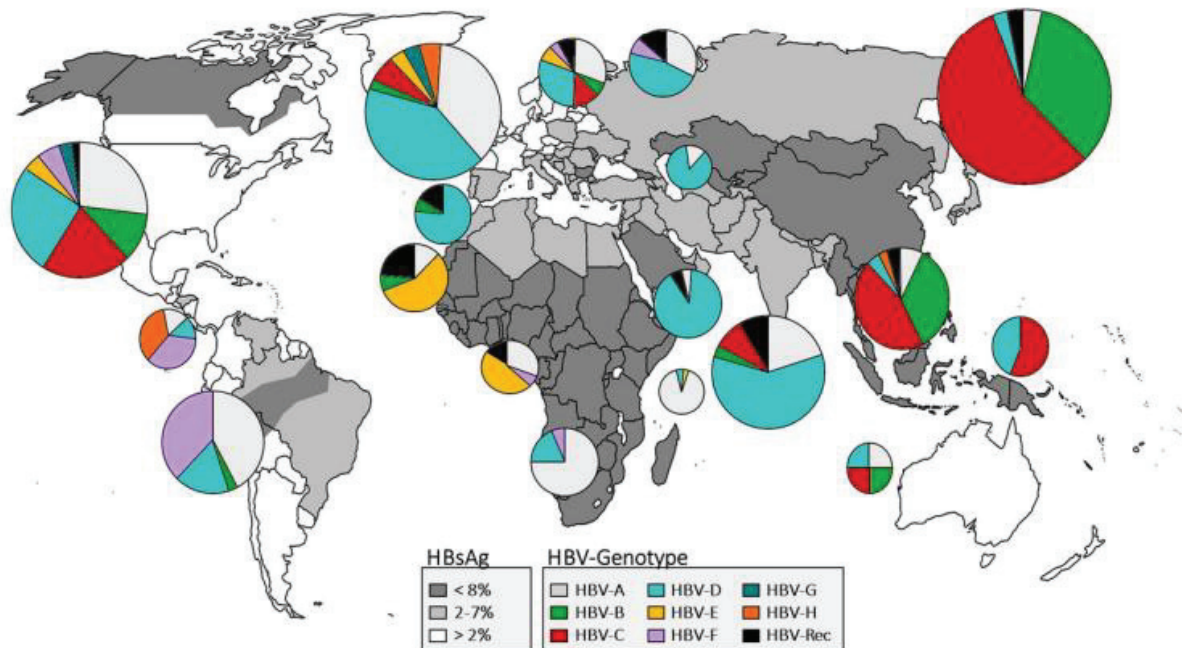


Figure 1: The anti-HBsAg prevalence and geographic distribution of HBV genotypes worldwide. From ⁸.

III. The modes of transmission of HBV

HBV can be transmitted in two main ways, by vertical and horizontal transmission. Vertical transmission occurs from mother to child and is particularly important in areas where the infection is highly endemic. This transmission generally occurs during the perinatal period or in the first few months after birth through breastfeeding. Horizontal transmission, on the other hand, is more frequent in areas where the endemicity of the HBV infection is low. It can occur through parenteral exposure to infected blood, including intravenous drug use, occupational exposure, tattoos, blood transfusions, or other medical procedures. Horizontal transmission can also occur through the exchange of infected body fluids during sexual intercourse.

IV. The HBV genotypes

HBV is classified into different genotypes, of which there are 10, designated by the alphabetical letters A to J. These genotypes represent distinct genetic variations of the virus, with a divergence of around 8% between them⁹. Each genotype can also be subdivided into subgenotypes, with an observed genetic divergence of around 4 to 7.5%. To date, more than 35 subgenotypes have been described. These HBV subgenotypes are distinguished by characteristics such as the length of the viral genome, the size of the open reading frame, and the proteins translated. Their geographical distribution can vary, and some regions of the world have a higher prevalence of certain genotypes than others (*Figure 1*)^{10,11}. Genotypes A and D are widespread and found throughout the world. Genotypes B and C are mainly found in Asia, while genotype E is more common in sub-Saharan Africa. Genotypes F, G, and H are more common in the Americas. Genotype I is present in Asia and genotype J is specific to Japan.

It has been observed a link between HBV genotypes and the progression of liver disease and the development of hepatocellular carcinoma¹². Among the different genotypes, HBV genotype C is considered to have a higher oncogenic potential compared to other genotypes A, B and D. Moreover, variations in treatment response are observed especially with interferon alpha (IFN α) treatment¹². Patients infected with HBV genotype A and to a lesser extent genotype B, tend to respond better to IFN α treatment, showing improved HBeAg and HBsAg seroclearance rates, while patients infected with HBV genotype C and D exhibit poorer response rates.

V. HBV nucleic acids and proteins

1- The structure of the virion and sub-viral particles

HBV can form and secrete both infectious and non-infectious particles (*Figure 2A*). First described in 1970 by electron microscopy, the infectious particle, known as the Dane particle,

is a particle approximately 42 nm in diameter⁵. It consists of an envelope made up of lipids of cellular origin acquired during budding at the endoplasmic reticulum, where the HBV surface envelope proteins are anchored in three forms: small (S-HBsAg), medium (M-HBsAg), and long (L-HBsAg) (**Figure 2B**). S-, M- and L-HBsAg make up the envelope with a respective ratio of 4:1:1¹³. The lipoprotein envelope encloses an icosahedral nucleocapsid characterized by the assembly of 90 or 120 dimers of the core protein (HBcAg). This nucleocapsid itself contains a copy of the HBV viral genome covalently associated with the viral polymerase, as well as cell proteins such as type C protein kinase (PKC) and chaperone proteins (Hps70, Hsp90, and p23).^{14,15} In some HBV-infected patients, it has been observed that Dane particles can be secreted up to approximately 10^9 - 10^{10} particles/mL¹⁶. Different types of non-infectious particles are secreted by HBV. These include sub-viral particles (SVPs), which were identified in 1977¹⁷. SVPs can be secreted as spheres 20-22 nm in diameter or as filaments of varying lengths and diameters. SVPs are secreted in large excess compared with Dane particles, up to 10^{14} particles/mL. SVPs are thought to play a role in the persistence of HBV infection by saturating the host immune system, for example by acting as a decoy for neutralizing antibodies¹⁸.

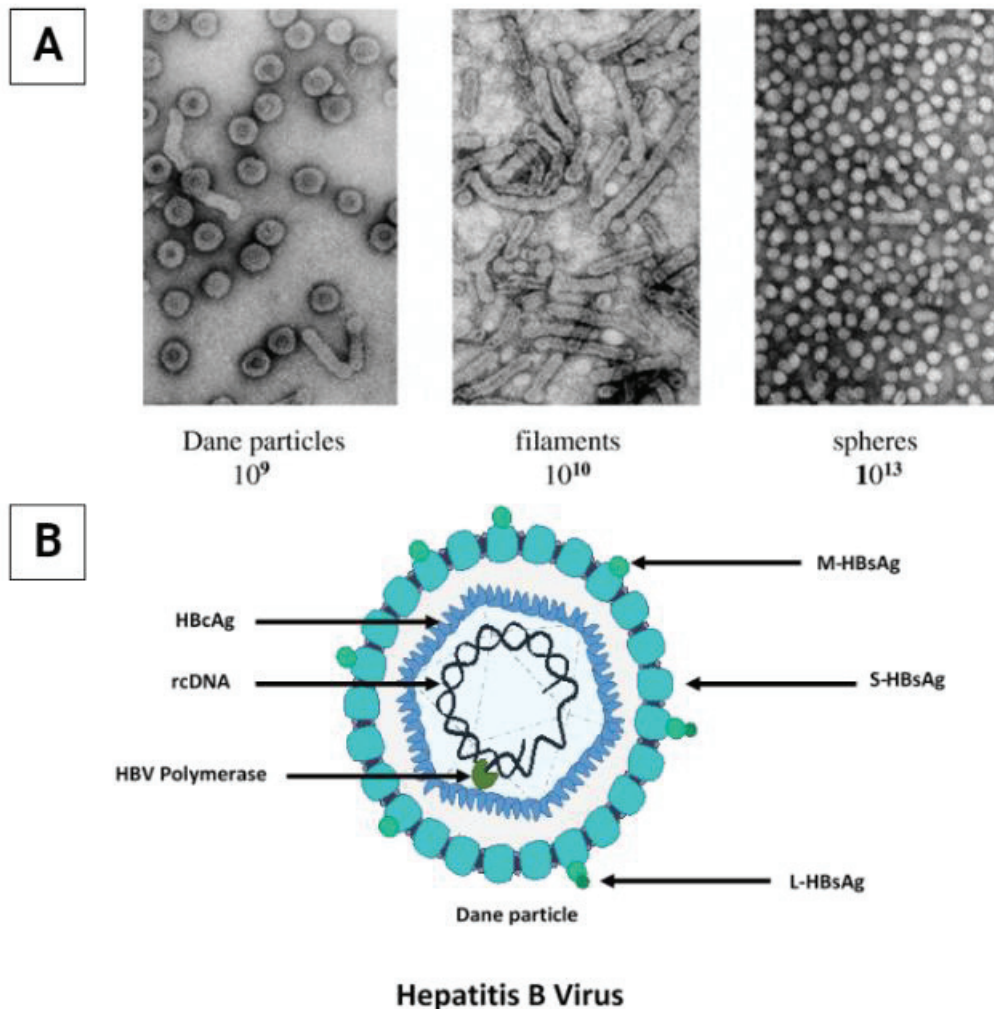


Figure 2: *The structure of HBV infectious and non-infectious particles. The electron microscopy images (negative staining) of different HBV particles and approximate numbers of HBV-associated particles in 1mL of the serum from a highly viremic chronically infected HBV carrier (A). From ¹⁹. The schematic representation of Dane particle (B). Adapted from²⁰.*

2- The HBV viral genome

The HBV genome contained in the Dane particle is characterized by a circular, relaxed, partially double-stranded DNA called rcDNA, measuring approximately 3.2kb (**Figure 3**)²¹. The (-) strand is the transcribed strand that runs the length of the HBV genome, *i.e.* around 3200 nucleotides. It is bound at its 5' end to the viral polymerase and is therefore not closed. It also

has repeated parts at its 3' end, allowing a change of matrix necessary for the synthesis of the (+) strand. Conversely, the (+) strand is not transcribed and is incomplete. It extends over only two-thirds of the HBV genome. It is linked at its 5' end to a short RNA oligomer derived from the end of the pre-genomic RNA (pgRNA) that served as a template for its synthesis²². A cohesive region of 200 nucleotides is present in 5' of each strand to ensure the circularization of the genome during replication. This region contains redundant sequences called direct repeat 1 (DR1) and direct repeat 2 (DR2), which are essential for viral DNA synthesis²³.

The HBV viral genome has four open reading frames (ORFs), some of which enable the synthesis of structural proteins (envelope proteins and capsid protein) and non-structural proteins (viral polymerase, HBe protein (HBeAg), and HBx protein). The P ORF accounts for over 80% of the HBV genome and codes for the viral polymerase. The ORF PreS1/PreS2/S contains three initiation codons (ATGs) for the synthesis of the three envelope glycoproteins. The ORF PreC/C contains two ATG codons for the translation of HBcAg and the HBeAg. The ORF X, the smallest of the HBV ORFs, codes for the X protein (HBx). The HBV viral genome also contains numerous elements regulating transcription and replication of the virus, such as two enhancers (Enhancer I (EnhI) and Enhancer II (EnhII)), four promoters, and a single polyadenylation signal used to terminate all viral RNAs²⁴. These transcriptional regulatory elements are controlled by both cellular and viral factors.

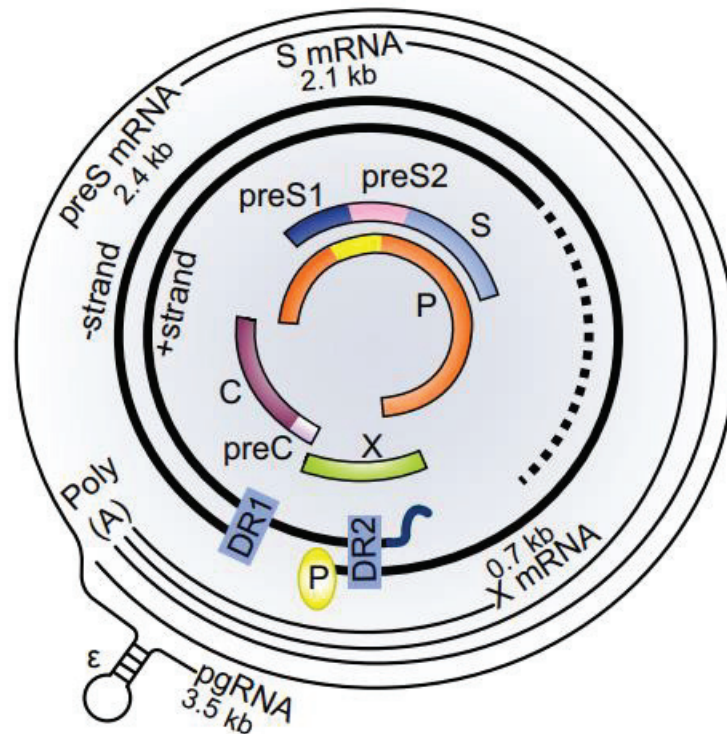


Figure 3: The organization of the HBV genome and four classes of HBV RNAs transcribed from the cccDNA template. P (in yellow): polymerase, DR1: direct-repeat 1, DR2: direct-repeat 2, X: X ORF, preC/C: preC/C ORF, preS1/preS2/S: preS1/preS2/S ORF, P (in orange): polymerase ORF. From ²⁵.

3- The HBV RNAs

During transcription of the HBV genome, 5 viral transcripts are synthesized by the host cell machinery (**Figure 3**)²⁶. Each transcript has its transcription initiation site, but all share the same 3' polyadenylation site. Like cellular transcripts, viral transcripts are capped at 5' and have a polyA tail at 3'.

Two 3.5kb transcripts were identified, pgRNA and, PreCore RNA. The pgRNA is produced from the PreC/C promoter and codes for the viral polymerase and HBcAg. Carrying the entire genetic information of HBV, pgRNA is also an essential intermediary for viral replication by retrotranscription. It consists of a stem-loop structure downstream of the 5' cap, known as the

epsilon (ϵ) loop or encapsidation signal²⁷. PreCore RNA is also transcribed from the PreC/C promoter and encodes the HBeAg protein.

A 2.4kb mRNA is transcribed from the PreS1 promoter and encodes the large envelope protein L-HBsAg. A 2.1kb mRNA is synthesized from the PreS2/S promoter. It codes for the S- and M-HBsAg protein. A final mRNA of 0.7kb, the shortest of the HBV viral transcripts, is transcribed from the X promoter and enables the synthesis of the HBx protein.

4- The HBV proteins

a- The HBV surface antigen (HBsAg)

HBsAg glycoproteins are synthesized from two distinct HBV mRNAs. The 2.4kb mRNA encodes the L-HBsAg form, while the 2.1kb mRNA encodes the S- and M-HBsAg forms via two translation initiation codons. Synthesized from the same ORF, the three forms of HBsAg differ in their N-terminal domains. S-, M-, and L-HBsAg share a common 226 amino acid domain, known as the C-terminal S domain (**Figure 4A**). The S domain is made up of four hydrophobic transmembrane domains (I-IV) and is rich in cysteines that induce the formation of disulfide bridges necessary for the self-assembly of the glycoproteins required for virion assembly. Domains I/II and III/IV are separated by a cytosolic loop, while domains II/III are separated by an antigenic loop (AGL) which is exposed on the outside of the virus particle. The AGL is also known as the a-determinant (**Figure 4B**). The M-HBsAg protein is 55 amino acids longer than S-HBsAg (**Figure 4A**). This extra domain is called preS2. In addition to the S and preS2 domains, the L protein has an extra sequence of 108 to 119 amino acids called preS1 (**Figure 4A**). Two distinct conformations have been observed for the anchoring of L-HBsAg to the endoplasmic reticulum (ER) membrane, depending on whether or not the transmembrane domain I is translocated into the ER cytosol (**Figure 4B**)²⁸. If the transmembrane domain I is translocated into the cytosol of the ER, the preS1 and preS2 domains are also exposed on the

cytosolic side. This is the i-preS form, with i for internal. This configuration allows interaction between the preS1 and preS2 domains and the newly formed nucleocapsids. Conversely, if the transmembrane domain I is not translocated and remains intramembrane, the preS1 and preS2 domains are exposed on the luminal side of the ER. This is the e-preS form, with e for external. Exposure of preS1 and preS2 on the surface of virions facilitates attachment and entry into the host cell.

S-, M-, and L-HBsAg are synthesized in the ER and then matured in the Golgi apparatus, where they may or not undergo post-translational modification. The three glycoproteins share the same N-glycosylation site in the S domain at the asparagine 146 residue of the AGL²⁹. It is estimated that the N-glycosylation site is functional for half of the envelope proteins resulting in similar levels of glycosylated and non-glycosylated S, M, and L forms. A second potential N-glycosylation site is contained in the preS2 domain at asparagine 4. Unlike glycoprotein M, the L protein is only N-glycosylated at the S domain. L-HBsAg also has a site, at glycine 2 in the preS1 domain, which can covalently bind a myristic acid by amide bonding, a process known as myristylation¹¹. This modification enables the protein to be anchored in the membrane and is essential for viral entry^{30,31}.

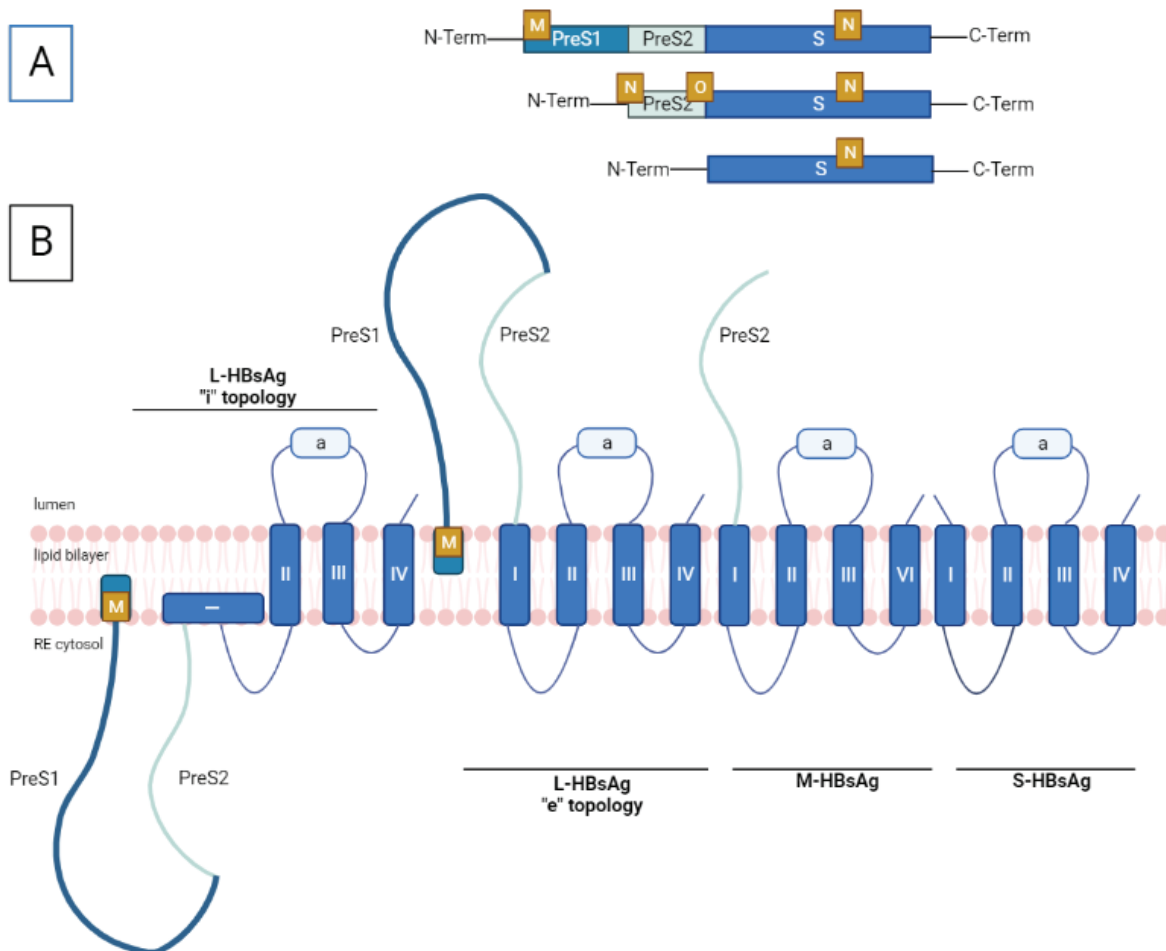


Figure 4: The three forms of HBV envelope proteins. (A): Schematic representations of HBV envelope proteins, N: N-glycosylation, O: O-glycosylation, M: myristic acid. (B): The arrangement of HBV envelope proteins in the endoplasmic reticulum after processing, e: external, i: internal, a: a determinant.

b- The core protein (HBcAg) and HBeAg

The HBcAg is a protein of 183 to 185 amino acids (21.5 kDa), depending on the HBV genome under consideration. It is the structural unit of the icosahedral capsid of HBV. HBcAg consists of two distinct domains separated by a short hinge region. The N-terminal domain, extending from amino acids 1 to 140, is an assembly domain that is essential for the self-assembly of Core proteins into dimers, then into multimers, and finally into the capsid. This N-terminal domain is also thought to have an interaction site with L-HBsAg, which plays a role in capsid envelopment. The C-terminal part, from amino acids 151 to 183 or 185, is rich in

arginines and serines and contains various regulatory regions³². This C-terminal domain is thought to be involved in the encapsidation of pgRNA and DNA replication. The C-terminal domain also contains nuclear localization sequences (NLS) and nuclear export sequences (NES), which allow HBcAg to be transferred between the nucleus of the cell and the cytoplasm, where the capsids are assembled³³⁻³⁵.

The HBe protein is produced from the cleavage of a precursor protein called the Pre-Core protein. HBeAg is secreted in soluble form without being associated with viral or sub-viral particles. It is neither necessary for the infectivity of the virus nor its replication. Like the SVPs, HBeAg is characterized as being highly immunogenic and is also thought to act as a decoy to saturate the immune system³⁶.

c- The HBV viral polymerase

HBV polymerase is an 832 amino acid (90kDa) protein that replicates the viral genome (*Figure 5*). It is composed of four distinct domains. The N-terminal domain of the polymerase, known as the Terminal Protein (TP), recognizes the epsilon (ϵ) loop of the pgRNA and attaches it to initiate retrotranscription of HBV. The spacer domain separates the TP from the retrotranscriptase domain in the central part. The retrotranscriptase (RT) domain is responsible for RNA-dependent DNA polymerase and DNA-dependent DNA polymerase activity, ensuring reverse transcription of pgRNA into the (-) strand of DNA and synthesis of the (+) strand from the (-) strand respectively. Finally, the polymerase contains a C-terminal RNase H domain that is responsible for degrading the pgRNA used as a template during synthesis of the (-) DNA strand using its ribonuclease activity.



Figure 5: *The schematic representation of viral polymerase of HBV. N-term: N-terminal domain, TP: terminal protein, RT: retrotranscriptase domain, C-term: C-terminal domain.*

d- The X protein (HBx)

The X protein consists of 146 to 154 amino acids (17kDa) depending on the genotype, making it the smallest of the HBV proteins. HBx consists of N-terminal helical domains and a C-terminal coiled-coil motif. Various studies have shown that HBx is essential for viral transcription^{37,38}. The precise role of HBx in viral transcription has been a subject of debate, but recent researches have shed more light on this matter. HBx is essential for initiating and sustaining the transcription of HBV RNAs driven by cccDNA^{37,39,40}. It possesses the ability to degrade the host repressor factor, the structural maintenance of chromosome 5/6 (Smc5/6) complex which represses RNA transcription through the recruitment of the damage DNA binding protein 1 (DDB1) thereby promoting HBV transcription^{41,42}. In the absence of HBx, a decrease of cccDNA-bound histone acetylation was observed, along with impaired recruitment of the transcriptional coactivator p300 and histone deacetylases and heterochromatin protein factor 1 (HP1)⁴³.

HBx is also thought to be involved in the process of hepatocarcinogenesis through its interaction with numerous host proteins which deregulate numerous cellular pathways⁴⁴.

VI. The life cycle of HBV

The different stages of the HBV replication are summarized in **Figure 6**.

1- The viral entry

As they share common features, the viral entry of HBV is discussed in the **Part 2 : The hepatitis Delta virus.**

2- The particle disassembly and nuclear import of the viral genome

Once inside the host cell, the Dane particle interacts with endosomal compartments via Rab5 and 7 proteases, inducing proteolytic cleavage of envelope components and releasing the capsid into the cytoplasm⁴⁵. The capsid, containing the viral genome, is transported to the nucleus via the microtubule network and an NLS carried by the Core protein⁴⁶. At the nuclear pore complex (NPC), the capsid is disassembled and the viral genome is released and migrates into the nucleus, potentially via the NLS carried by the viral polymerase^{47,48}.

3- The formation and transcription of the cccDNA

Once in the nucleus, rcDNA is converted into a closed covalent circular DNA called cccDNA. The formation of cccDNA involves several steps that have yet to be fully elucidated. Firstly, the viral polymerase must be detached from the (-) strand of the cccDNA with which it is covalently linked. The detachment of the viral polymerase is thought to be mediated by tyrosyl-DNA phosphodiesterase 2 (TDP2)⁴⁹. The oligoribonucleotide, derived from the pgRNA attached 5' to the DNA strand (+), and the repeated sequences at the ends of the DNA strand (-), are then eliminated by cellular endonucleases or exonucleases such as FEN1 (Flap structure-specific Endonuclease 1)⁵⁰. Next, the incomplete (+) DNA strand is synthesized. This involves cellular polymerases such as polymerase κ and DNA topoisomerase I and II^{49,51}. Finally, the two ends of the (+) and (-) strands are bound together by a cellular ligase. Once formed, the cccDNA is compacted into nucleosomes forming a minichromosome in the nucleus via the binding of histone (H1, H2A, H3, and H4) and non-histone proteins^{52,53}. cccDNA is responsible for the persistence of HBV in the cell and viral rebound.

Transcription of cccDNA requires the use of the host transcription machinery, in particular DNA-dependent RNA Polymerase II (Pol II). All HBV RNAs are produced from cccDNA and all carry a 5' cap and a polyA tail. HBV RNAs are then transported to the cytoplasm and translated like cellular RNAs by the translational machinery, enabling viral proteins to be synthesized.

The regulation of cccDNA transcription is ensured by several regulatory sequences present in the HBV genome, as previously mentioned. The viral protein HBx, as well as cellular proteins such as the transcription factor C-EBP, are involved in this transcriptional regulation process⁵⁴. cccDNA transcription is also regulated by epigenetic modifications such as the acetylation of histones H3 and H4⁵⁵.

4- The encapsidation and retrotranscription of pgRNA

Of the 5 mRNAs transcribed from cccDNA, only pgRNA is supposed to be encapsidated. Initiation of encapsidation is mediated by the interaction between pgRNA and viral polymerase at the ϵ -loop of pgRNA, allowing interaction between pgRNA and HBcAg^{56,57}.

The pgRNA serves as a template for the synthesis of a new copy of viral DNA by retrotranscription. Retrotranscription of pgRNA takes place in several stages, beginning with the synthesis of the (-) strand of the rcDNA. Retrotranscription begins with the synthesis of a short complementary DNA primer at the 5' ϵ -loop by the viral polymerase itself via its primase activity. The complex formed between the viral polymerase and the primer is then translocated to the 3' DR1 region of the pgRNA. Using this primer as a template, the viral polymerase synthesizes the (-) DNA strand while simultaneously degrading the pgRNA via the RNase activity of the viral polymerase. A small 20-nucleotide oligomer of the lncRNA, located at its 5' end, escapes degradation and serves as a primer for the synthesis of the (+) DNA strand. The (+) strand of rcDNA is then synthesized using the (-) strand as a template. The cause of

incomplete synthesis has not yet been elucidated, but steric hindrance imposed by the capsid or depletion of the nucleotides available in the capsid may be responsible.

Capsid maturation is closely linked to the retrotranscription of pgRNA. During the reverse transcription process, HBcAg is progressively dephosphorylated. After maturation, a change in the conformation of the nucleocapsids is described and they can interact with surface proteins at the level of the endoplasmic reticulum to lead to the formation and secretion of viral particles. Alternatively, they can be recycled to the nucleus to amplify the number of cccDNA copies⁵⁸.

5- The assembly and secretion of HBV particles

The envelope proteins S-, M-, and L-HBsAg anchor themselves in the membrane of the ER where they are synthesized and accumulate. On the one hand, they allow the budding of infectious particles resulting from the envelopment of the mature nucleocapsid. The S- and L-HBsAg proteins are strictly required for virion formation, unlike the M-HBsAg protein. However, the S-HBsAg protein alone cannot support nucleocapsid translocation. It has been shown that the assembly and secretion of the nucleocapsid are dependent on the presence of the L-HBsAg protein and, more specifically, its PreS1 domain, which interacts specifically with the Core^{59,60}. It has also been documented that the short cytosolic loop between domains I and II of HBsAg proteins may interact with the nucleocapsid^{61,62}. Introducing a mutation into this loop inhibited virion formation without affecting the secretion of sub-viral particles.

The secretion of infectious HBV particles takes place via multivesicular bodies (MVBs), *i.e.* late endosomes⁶³. The inhibition of the actin-interacting protein 1 (AIP1)/ ALG-2-interacting protein X (ALIX) and vacuolar protein sorting 4 homolog B (VPS4B) proteins resulted in virions not budding.

On the other hand, viral envelope proteins are capable of budding into SVPs. The mechanisms of morphogenesis of these empty particles remain poorly understood. It has been

shown that the S-HBsAg protein is essential and sufficient for SVP budding, unlike the M protein, which is dispensable for this phenomenon. Coexpression of the S- and L-HBsAg forms tend to form filamentous SVPs. Confocal microscopy has shown that S-HBsAg proteins self-assemble in the lumen of the ER before being directed to the intermediate compartment between the endoplasmic reticulum and the Golgi (ERGIC) and being secreted⁶⁴. Spheres are secreted by the host's constitutive secretory pathway, while filaments are secreted by the multivesicular body pathway and the endosomal sorting complex required for transport (ESCRT) complex^{63,65,66}.

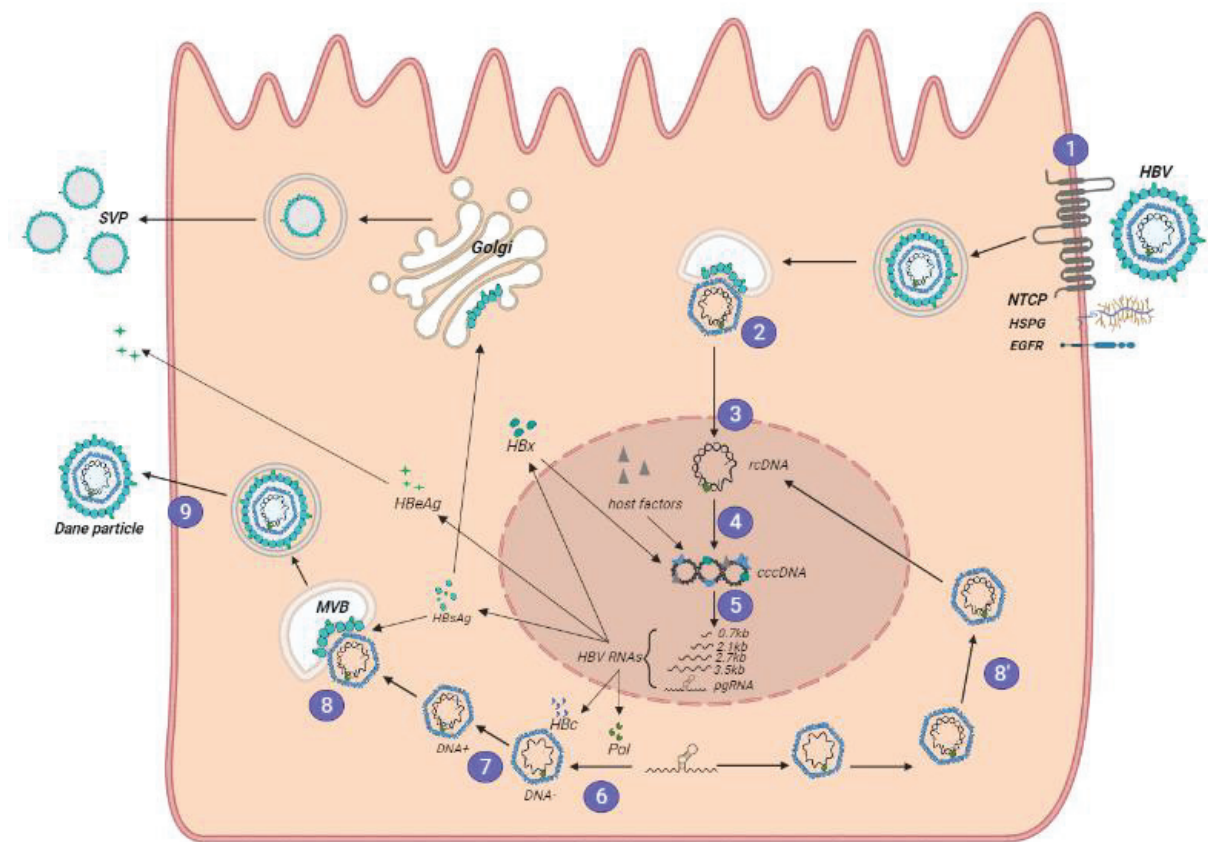


Figure 6: The HBV life cycle. The entry of HBV into hepatocytes involves NTCP, in collaboration with EGFR and HSPGs (1). The nucleocapsid is internalized in clathrin endosomes and subsequently released into the cytoplasm (2). Upon reaching the nuclear pore, the capsid disassembles, releasing the relaxed circular DNA (rcDNA)(3). rcDNA is then converted into covalently closed circular DNA (cccDNA)(4), which serves as the template for viral transcription (5). Viral transcription produces various RNA, an intermediate RNA named pregenomic RNA (pgRNA), which undergoes encapsidation and reverse transcription to form negative-sense-DNA (DNA-)(6). The synthesis of the positive-sense DNA (DNA+) results in the formation of rcDNA(7). The DNA-containing nucleocapsids can be enveloped (8) and secreted (9) to form new infectious particles, or they can be recycled in the nucleus to replenish the cccDNA pool (8'). Adapted from²⁰.

VII. The natural history of HBV infection

After being infected with HBV, there is an incubation period of between 1 and 6 months, depending on the individual. Incubation is followed by acute hepatitis B, which is asymptomatic in most cases. In some cases, however, it may manifest as symptoms such as jaundice, malaise, nausea, anorexia, or fever. In less than 1% of cases, acute hepatitis can develop into a severe form known as fulminant hepatitis, which can be fatal, with an overall survival rate of only 20% in the absence of a liver transplant. This serious complication is thought to be due to an excessive immune response resulting in massive destruction of infected hepatocytes.

After the acute phase, HBV infection can take one of two courses: spontaneous recovery or progression to chronic hepatitis. Chronic hepatitis B (CHB) is characterized by the persistence of viral DNA and HBsAg for more than six months. Progression to CHB appears to be influenced by several factors, including the immune response of the infected individual and age at infection. For example, it has been observed that the risk of developing chronic infection was 90% in newborns infected at birth, while it was only 5% in adults⁶⁷.

CHB can be subdivided into 5 phases of variable duration and not necessarily sequential: phase 1 of HBeAg-positive chronic infection (immunotolerance phase), phase 2 of HBeAg-positive chronic hepatitis (immune reaction or immune clearance phase), phase 3 of HBeAg-negative chronic infection (immune control or inactive carriage phase), phase 4 of HBeAg-negative chronic hepatitis and phase 5 or HBsAg-negative phase (*Figure 6*)⁶⁸. The impact on the liver varies from one individual to another, ranging from the simple asymptomatic carriage of the virus (characterized by low viral replication and the absence of liver lesions) to various stages of fibrosis that can progress to cirrhosis or severe liver failure, and even to the development of hepatocellular carcinoma. The outcome of chronic B infection varies from one individual to another. It is determined by numerous factors linked to the virus (genotype, HBV DNA level), the infected individual (sex, age, and genetic history), and the environment (co-

infection with other viruses such as hepatitis c virus (HCV), HDV, or human immunodeficiency virus (HIV).

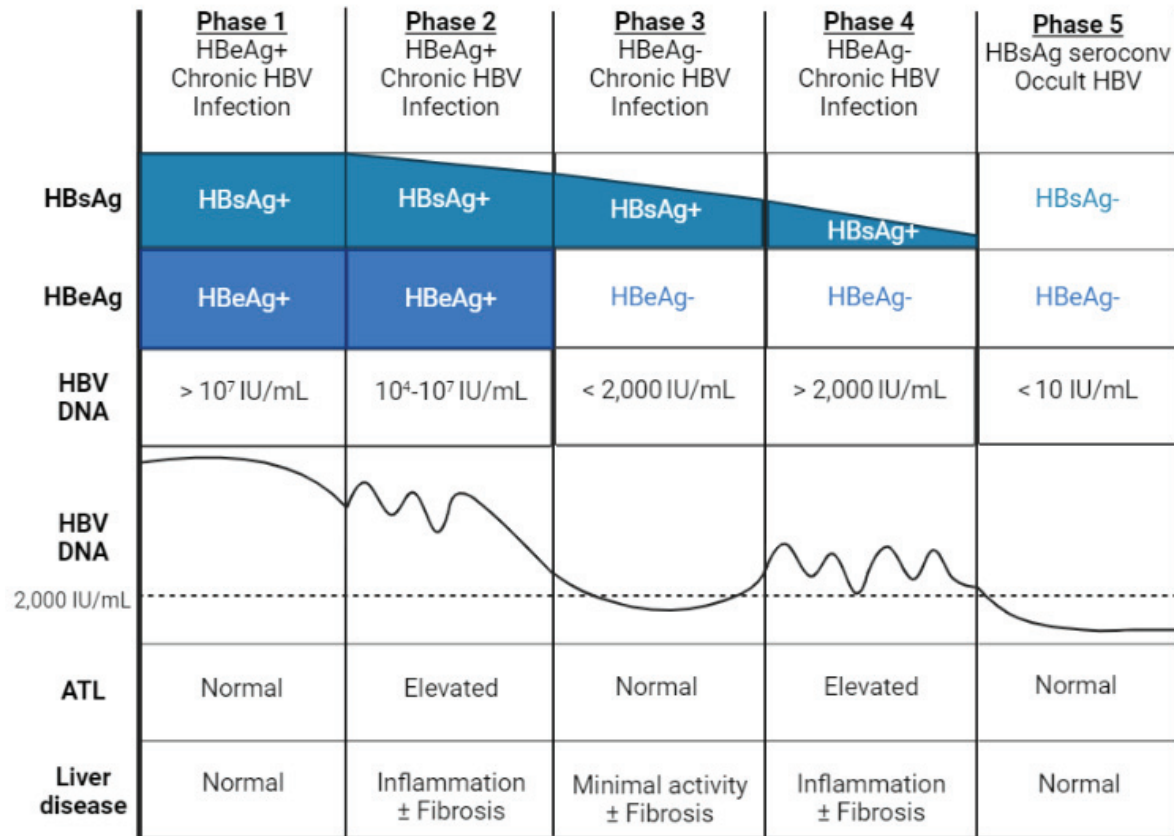


Figure 7: The different phases of hepatitis B infection natural course. ATL: Alanine aminotransferase, IU: International units, seroconv: seroconversion. Adapted from ⁶⁸.

VIII. The progression of chronic hepatitis B to hepatocellular carcinoma

Soon after the discovery of HBV, numerous studies showed an association between HBV infection and the development of HCC⁶⁹. The risk of developing HCC is multiplied by 10 to 25 in a person chronically infected with HBV compared with an uninfected person⁷⁰. This risk increases with the progression of liver disease and co-infection with the hepatitis Delta virus (discussed in [Part 2: The hepatitis Delta virus](#)) or HIV. The development of HCC from chronic HBV infection is a multifactorial process combining both viral factors and cellular

factors. The primary mechanisms leading to the development of HCC is the immune response against HBV infection, resulting in chronic necroinflammation of the liver which can alter the host cell genome, causing genetic instability, mutations and integrations, among other things. One of the viral factors contributing to HCC is the integration of HBV DNA into the host genome. This integration occurs early in HBV infection through non-homologous end joining (NHEJ) and has been observed in about 80% of patients infected with HBV⁷¹. Although this integration appears to be random, it can occur in transcriptionally active regions close to key cellular genes in the process of carcinogenesis, leading to the activation of oncogenes or the inactivation of tumor suppressor genes⁷²⁻⁷⁴. Another viral factor implicated in HCC is the HBx protein. The role of HBx in oncogenesis is subject to debate, but various studies have reported its interaction with numerous cellular pathways modulating epigenetic modifications, affecting cell proliferation and influencing cellular senescence and apoptosis⁷⁵.

IX. The treatments for HBV infection

There is no specific treatment for acute hepatitis B. The general recommendations are to rest and limit the consumption of fatty foods, medicines, and alcohol to prevent further damage to the liver.

Three definitions have been proposed to describe the cure of CHB, complete cure, functional cure, and partial cure. Complete cure is characterized by the absence of detectable HBsAg and viral load in the serum, along with the complete elimination of cccDNA and/or integrated forms. Functional cure is defined as the sustained loss of HBsAg with an undetectable viral load in the serum, with or without the appearance of antibodies against HBsAg (anti-HBs) after the completion of treatment. Partial cure refers to the persistence of detectable serum HBsAg levels but an undetectable viral load after successful treatment.

The persistence of cccDNA in the nucleus of infected hepatocytes and the integration of HBV into the host genome are difficult targets to achieve a complete cure of HBV infection. As a result, the management of patients chronically infected with HBV is primarily aimed at controlling the progression of the infection to limit serious liver damage that could lead to the patient's death, which can be achieved by prolonged HBV viral suppression over time. Ideally, this would result in loss of HBsAg with HBs seroconversion, although this is rarely achieved with currently available treatments.

Currently, two therapeutic options are available in the management of hepatitis B, IFN α and nucleos(t)ide analogs (NUCs), either in monotherapy or combined. The indication for treatment depends on viral load, transaminase levels, and the severity of liver damage.

The different therapeutic strategies presented in this part are summarized in *Figure 8*.

1- The interferon-alpha (IFN α)

The first treatment available for patients infected with HBV was IFN α . IFN α plays a major role in the immune response and is widely used in the treatment of viral infections. It can both induce antiviral factors at the cellular level and rapidly stimulate the innate immune response.

The antiviral effects of IFN α on HBV replication were discovered in 1976 and its use was approved in the 1990s⁷⁶. Initially, IFN α was administered to patients in its classic form, but numerous side effects have been reported. The most common include flu-like symptoms, fatigue, anorexia, and weight loss. However, IFN α can also be associated with serious psychiatric side effects, ranging from anxiety to depression and even suicidal tendencies⁷⁷.

Subsequently, IFN α was modified by adding a polyethylene glycol molecule (PEG-IFN α), which prolongs its half-life and allows less frequent administration (from three times a week to once a week). It has been observed that PEG-IFN α has a more sustained antiviral effect than conventional IFN α but that the associated side effects are similar.

The use of IFN is generally limited in time, with a treatment duration of 48 weeks administered subcutaneously once a week. However, its efficacy is modest. After 48 weeks of treatment, loss of HBsAg is observed in only 3 to 7% of cases, and this proportion rises slightly to 3 to 8% three years after treatment is stopped^{78,79}. The use of IFN α is rarely recommended but can be used in selected patients (such as HBeAg-positive patients with low viral load and persistent liver necroinflammatory activity, or HBV/HDV coinfecting patients).

2- The nucleos(t)ides analogs (NUCs)

NUCs act as inhibitors of HBV viral polymerase, blocking the retrotranscription of pgRNA into DNA. By competitively mimicking natural nucleos(t)ides, they incorporate into newly synthesized DNA, leading to premature termination of the DNA chain. As a result, the formation of new infectious particles is inhibited, limiting the infection of new, uninfected hepatocytes. NUCs reduce the risk of cirrhosis and the development of HCC^{80,81}.

Although NUCs do not directly target cccDNA, they can nevertheless inhibit cccDNA amplification and progressively contribute to its elimination. However, although NUCs are effective in suppressing the virus in the majority of cases (around 95%), with a better tolerance profile than IFN, loss of HBs antigen expression remains a rare event (only 1% each year)⁸². In addition, stopping treatment leads to relapses and must therefore be taken for life by most patients⁸⁰.

The use of NUCs exposes patients to the risk of selecting resistance mutations, which can occur and affect several molecules, creating a source of virological escape. The risk of resistance mutations appearing varies according to the genetic barrier of resistance of each compound. For first-generation NUCs (lamivudine, adenofovir, and telbivudine), resistance rates ranging from 0 to 27% after one year of treatment and from 0 to 80% after five years of treatment have been reported⁸³. They have been replaced by the second-generation NUCs

(entecavir, tenofovir disoproxil fumarate, and tenofovir alafenamide). Indeed, their rate of resistance is lower, ranging from 0 to 1.2% after 5 years of treatment.

NUCs are commonly prescribed for patients with high HBV activity and signs of liver inflammation.

3- The development of new treatments against HBV

The treatments currently available for chronic hepatitis B do not provide a complete cure and generally require lifelong therapy to control the infection. The treatments currently available do not target the viral reservoir constituted by cccDNA and do not sufficiently stimulate the host's immune responses. No new treatment for HBV has been approved in the last 25 years, despite the efforts of the scientific community to develop new molecules.

a- The entry inhibitors: the example of Myrcludex (see [Part 2 : The hepatitis Delta virus](#))

To date, there are no clear recommendations for the introduction of this treatment in people infected solely with HBV.

b- The secretory inhibitors : the nucleic acid polymers (NAPs) (see [Part 2 : The hepatitis Delta virus](#))

c- The post-transcriptional inhibitors : siRNA and antisense oligonucleotides (ASOs)

Short interfering RNA (siRNA) molecules target HBV transcripts by inducing their degradation, thereby preventing gene expression. This approach is commonly used *in vitro* and can be applied in humans by subcutaneous injections of metabolically stable siRNA. It is based on the use of a non-coding double-stranded RNA, comprising a passenger strand (sense) and a guide strand (antisense) complementary to the target mRNA. The double-stranded RNA is recognized by the RNA-induced silencing complex (RISC) protein complex, which releases the

sense strand and causes it to bind to the target mRNA. Once bound, the Argonaute protein cleaves the target mRNA, leading to its degradation. An example of a siRNA currently undergoing clinical evaluation is ARC-520, which was developed to target all HBV transcripts. It has been shown to reduce the amount of HBV DNA in the serum of patients with chronic hepatitis B, whether they are positive or negative for the HBeAg marker⁸⁴. Treatment with ARC-520 was well tolerated and a long-term virological response was observed after cessation of treatment⁸⁵. However, only a moderate reduction in HBsAg levels was observed mainly in HBeAg-positive patients, probably due to the integration of HBV DNA which allows HBsAg expression independently of the cccDNA. As a result, other siRNAs were subsequently developed to target both mRNAs transcribed from cccDNA and those produced from the HBV genome integrated into the host genome, such as the JNJ-3989 (or ARO-HBV) siRNA⁸⁶. Promising results were obtained in a phase IIa study, showing a significant reduction in HBsAg, HBV RNA, HBeAg, and HBcAg in HBeAg-positive and -negative patients.

Using the same principle of mRNA degradation, antisense oligonucleotides (ASOs), which are nucleic acids of approximately 8 to 50 nucleotides complementary to their target mRNA, have also been evaluated on HBV. Thanks to their complementarity with the target mRNA, these ASOs induce degradation of this mRNA by the action of RNase H⁸⁷. *In vitro* and *in vivo* studies have demonstrated the antiviral effects of ASOs against HBV⁸⁸. Currently, three compounds are being evaluated in phase I clinical trials, including RO7062931 (Roche) and ALG-020572-40 (Aligos Therapeutics), and Bepirovirsen (GSK), which is in phase II trials. In this trial, patients were treated for 24 weeks with different doses of Bepirovirsen (150 mg and 300 mg), placebo or Bepirovirsen in combinations with NUCS. The primary endpoint, which was defined as achieving an undetectable HBsAg level (<0.05UI/mL) and an HBV RNA level below 20IU/mL 24 weeks after discontinuing treatment, was reached by 9% of patients who

received 300mg of Bepirovirsen plus NUCs and by 10% of patients who received Bepirovirsen alone⁸⁹.

d- The modulators of capsid assembly

Directly targeting the HBc protein offers a promising prospect as a therapeutic strategy. It could potentially interfere with the encapsidation of the HBV viral genome and reverse transcription, blocking the formation of infectious virions and the reintegration of the viral minichromosome into the nucleus of infected cells⁹⁰. Capsid assembly modulators (CpAMs) interact with HBc in different ways depending on their structure. They can induce the formation of unstable nucleocapsids which are then degraded (class I CpAMs), or promote the assembly of empty capsids by accelerating their formation (class II CpAMs)⁹¹⁻⁹⁴.

Several clinical trials are currently underway, both as monotherapy and in combination, in phases I and II. A phase Ib trial of the compound JNJ-56136379 showed that, as a single agent after 28 days of treatment, a rapid reduction in HBV DNA was observed in chronically infected patients⁹⁵. However, there was no impact on HBsAg or HBeAg levels. Another phase I trial of NVR3-778 reported that after 4 weeks of treatment in combination with PEG-IFN α , a decrease in HBV DNA and RNA levels as well as HBeAg in HBeAg-positive patients was observed⁹⁶.

e- The inhibitors of the cccDNA

Directly targeting the cccDNA of HBV appears to be a promising therapeutic strategy. However, a complete understanding of the mechanisms involved in its formation and persistence within cells remains unclear. Many of the crucial steps in cccDNA formation require cellular factors, such as TDP2, FEN1, and DNA polymerase kappa (POLK), as well as histones and host chromatin components^{49,97}. Consequently, therapies targeting these host factors can potentially lead to more side effects, as they do not directly target viral factors. More needs to be known about this area to develop new therapies.

The use of nucleases to target established cccDNA has shown promising results *in vitro* in cleaving cccDNA⁹⁸⁻¹⁰⁰. CRISPR/Cas9 technology is also a potential strategy for specifically inactivating cccDNA. *In vitro* studies have shown that this approach has led to the appearance of mutations and deletions rendering cccDNA transcriptionally inactive, with an efficiency of up to 90% of cccDNA cleavages¹⁰¹⁻¹⁰³.

f- The immunomodulators

A further approach to achieving a functional cure for HBV is to restore and boost the antiviral immune response, both innate and adaptive, which is weakened over time during chronic infection.

i. The utilization of direct immune pathways activators

The first strategy is to exploit the antiviral properties of cytokines to target innate immunity. *In vitro*, studies have shown that treatment with IFN gamma (IFN γ) and lambda (IFN λ) interferons, interleukin-1beta (IL-1 β), or tumor necrosis factor (TNF α) can inhibit HBV replication¹⁰⁴⁻¹⁰⁷. Cytokines not only reduce HBV replication but may also eliminate cccDNA in some cases. IFN γ and TNF α have been shown to reduce cccDNA in hepatocytes by inducing deamination of cccDNA and hence its degradation¹⁰⁸.

Another strategy is to target molecular pattern recognition receptors (PRRs) and more specifically by using agonists of toll-like receptors (TLRs) (TLR7 and TLR8) or RIG-I, which are expressed in hepatocytes and/or adjacent immune cells. A well-studied example currently in clinical trials is GS-9620, a TLR-7 agonist, which induces the production of IFN α ¹⁰⁹. Although it has no side effects, an initial clinical trial reported that its antiviral effect was weak, probably due to the use of low doses. Higher doses showed a more pronounced antiviral response in chimpanzees¹¹⁰. GS-9620 induces the expression of interferon-stimulated genes (ISGs) and promotes the activation of NK cells and the HBV-specific response of T

lymphocytes¹¹¹. TLR-8 agonists are currently being developed. They induce the expression of interleukins 15 and 18, which in turn trigger the production of IFN γ by NK cells, making it possible to restore the HBV-specific T cell response¹¹². Another promising candidate is inarigivir, an agonist of RIG-I, which has a direct antiviral effect on HBV or via the IFN pathway. It has been shown to reduce HBV DNA and RNA levels, particularly in HBeAg-negative patients. In addition, it reduced HBsAg levels in 26% of patients¹¹³.

ii. The immune checkpoint inhibitors

Patients with chronic hepatitis B generally have impaired specific immunity to HBV, partly due to the abundance of sub-viral particles contributing to the depletion of the immune response, particularly of T lymphocytes. Inhibitors of immune checkpoints are capable to reinvigorate the pre-existing antiviral immune response by blocking the action of signaling pathways that restrict the duration and strength of immune responses. These negative regulatory mechanisms are normally triggered to minimize tissue damage. For example, the use of anti-programmed cell death 1 ligand 1 (PD-L1) antibodies, to prevent the inhibitory signals generated from the interaction between PD-L1 and its receptor PD1, has restored the function of HBV-specific T and B lymphocytes in patients chronically infected with HBV¹¹⁴⁻¹¹⁷.

iii. The immune cell transfer

Another approach to restore immunity is the adoptive transfer of engineered HBV-specific T cells. Various methods have been developed and tested for engineering HBV-specific T cells, including the use of chimeric antigen receptors (CARs) containing an anti-HBsAg specific antibody or canonical HBV-specific T cell receptors (TCRs). *In vitro* and *in vivo* studies of these engineered HBV-specific T cells have shown promising outcomes in significantly reducing HBV infection¹¹⁸⁻¹²¹. Similar findings were observed in clinical settings, where gene-modified T cells exhibited survival, expansion, and the ability to reduce HBsAg levels without

exacerbating liver inflammation or causing other toxicities¹²². However, the strict regulation and requirement of highly skilled personnel pose challenges to the implementation of these approaches. An alternative strategy involves the use of antibodies with TCR-like specificities or soluble TCRs that can redirect endogenous T cells to infected hepatocytes¹²³. These strategies do not require the manipulation of T cells outside the patient's body.

iv. The therapeutic vaccination

Therapeutic vaccines have the potential to enhance the response of CD4⁺ and CD8⁺ T lymphocytes against HBV by priming new antiviral immune responses¹²⁴. Unlike preventive vaccines that solely aim to prevent infection, therapeutic vaccines have a different mode of action and administration. Various types of therapeutic vaccines have been developed, such as vaccines based on HBV recombinant HBsAg, naked DNA, possibly combined viral vectors, and/or those containing T-cell peptides epitopes derived from HBV proteins, and tested.

For example, HepTcell (FP-02.2) is a peptide-based immunotherapeutic that has completed a phase I trial, demonstrating a robust T cell response when combined with an adjuvant¹²⁵. Although it was found to be safe, there was no observed decline in HBsAg levels after three administrations. Nevertheless, it has been approved to proceed to phase II trials. TG-1050/T101, a replication-defective adenovirus serotype 5 expressing multiple HBV-specific antigens, has shown promising results in reducing circulating viral parameters in a mouse model. Phase I trials confirmed its safety profile, and it resulted only in a mean 0.45 log decrease in HBsAg levels after 197 days¹²⁶. It is currently being evaluated in phase II trials. Another vaccine, GS-4774, contains epitopes derived from HBsAg, HBx and HBcAg. It has demonstrated the ability to induce IFN- γ and IL-2 production by CD8⁺ T cells¹²⁷. However, it has not shown a significant association with a decrease in HBsAg levels.

The strong immune stimulatory effects of therapeutic vaccines could be beneficial when used in combination with other antiviral or immune modulator agents to enhance the response against HBV.

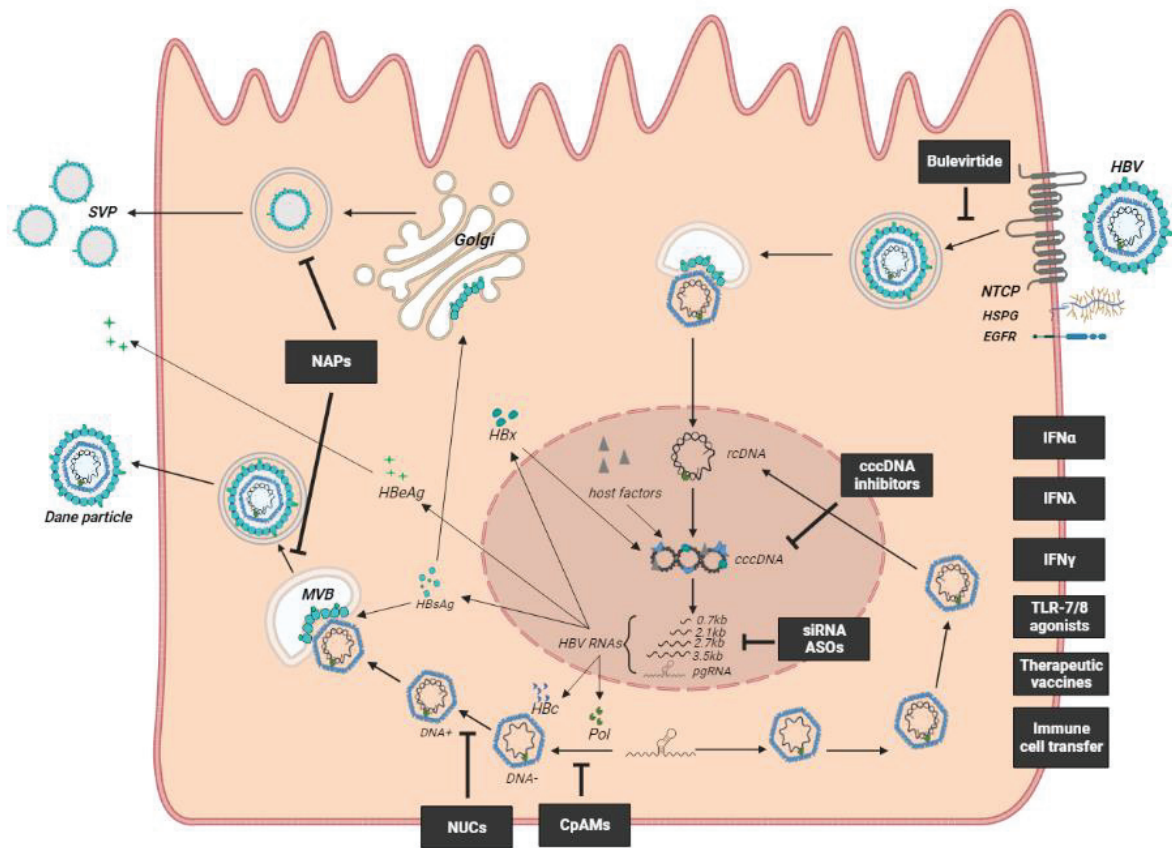


Figure 8: *The therapeutic strategies deployed against HBV. Several therapeutic approaches have been developed to target the HBV life cycle, the entry inhibitors (Bulevirtide), RNAs metabolism modulators (siRNA and ASOs), the cccDNA inhibitors, the capsid assembly modulators (CpAMs), the secretory inhibitors (NAPs), and the immunomodulators (IFN α , IFN λ , IFN γ , TLR-7/8 agonists and therapeutic vaccines). Adapted from ²⁰.*

Part 2 : The hepatitis Delta virus (HDV)

Among HBV-infected patients, around 5-10% are also coinfecting with the hepatitis Delta virus (HDV), a satellite virus of HBV. HDV dissemination depends on HBV and especially on the expression of HBV surface antigen. The use of three forms of HBV surface proteins confers the same tropism to both viruses. The coinfection by both viruses is considered as the most aggressive form of viral chronic hepatitis and is associated with a progression towards fibrosis and cirrhosis as well as an increase of hepatocellular carcinoma risk and death. Currently, there are no effective antiviral treatments against HDV.

I- The discovery of hepatitis Delta virus

The hepatitis Delta virus was first identified in 1977 in Italy by Mario Rizzeto, a gastroenterologist, and his colleagues in a cohort of patients infected with HBV and suffering from severe chronic hepatitis. The study of liver biopsies using direct immunofluorescence revealed the presence of a new antigen-antibody system, called delta antigen and anti-delta antibody respectively, in the nuclei of liver cells¹²⁸. Although this system appeared to be uniquely associated with HBV infection, the delta antigen was immunologically different from HBsAg, HBcAg, and HBeAg. Initially described as a new marker of HBV infection, transmission experiments in chimpanzees demonstrated that the delta antigen was not a component of HBV but was associated with a defective transmissible agent requiring the presence of HBV for infection and/or replication¹²⁹. The inoculation of serum from patients with delta-positive hepatitis into chimpanzees already infected with HBV or not showed that the delta antigen could infect these animals. Furthermore, it was demonstrated in the serum of these same chimpanzees that the delta antigen was an internal component of virus-like HBsAg particles associated with a low molecular weight RNA molecule smaller than the genomes of

known RNA viruses. This transmissible pathogen is now known as HDV. It is a unique human pathogen classified in the *Deltavirus* genus, of which it is the only representative.

II- The epidemiology of HDV

Among HBV-infected patients, an estimated 5-10% are also co-infected with HDV, according to the WHO. However, recent reports suggest that these figures are underestimated due to non-standardized and heterogeneous screening practices, as well as the inaccessibility of HDV screening tests in regions where the virus is endemic. According to two meta-analyses, between 60 and 70 million people could be infected with HDV in 2018 and 2019, reinforcing the idea that HDV is a major global health problem^{130,131}. In 2020, one study estimated the prevalence of HDV at 12 million people¹³².

HDV infection is distributed worldwide and overlaps with that of HBV. However, this distribution is not uniform, highlighting geographical hotspots of high prevalence (**Figure 9**). Central and West Africa, the Mediterranean basin, the Middle East, Eastern Europe, North Asia, certain regions of South-East Asia, and the Amazon basin of South America have all been described as areas of high HDV prevalence. In low-prevalence areas, such as Western Europe and North America, HDV infection is sporadic and largely confined to high-risk groups such as injecting drug users or people with high-risk sexual behavior.

At the end of the 1980s, the implementation of vaccination programs against HBV, combined with improvements in hygiene and living conditions, as well as the effects of measures to control the spread of HIV, helped to restrict the circulation of HBV. As a result, a significant reduction in the prevalence of HBV infection was observed at the end of the 1990s. Deprived of HBsAg, the prevalence of HDV has also been reduced by around 5-10% in Europe¹³³. For example, the prevalence of HDV among injecting drug users has fallen from 30% in the 1990s to 4.2% in 2018¹³⁴. However, no further decline has been described since. This may be partly

explained by the presence of residual reservoirs of HDV, such as elderly people who were infected during the epidemic of the 1980s. In Italy, for example, 513 HBsAg carriers were identified in 2014, 61 of whom were also positive for delta antigen¹³⁵. Only 3% were aged under 30, while 80% were aged 50 or over. In addition, there are migratory flows of people from highly endemic areas to low-endemic areas.

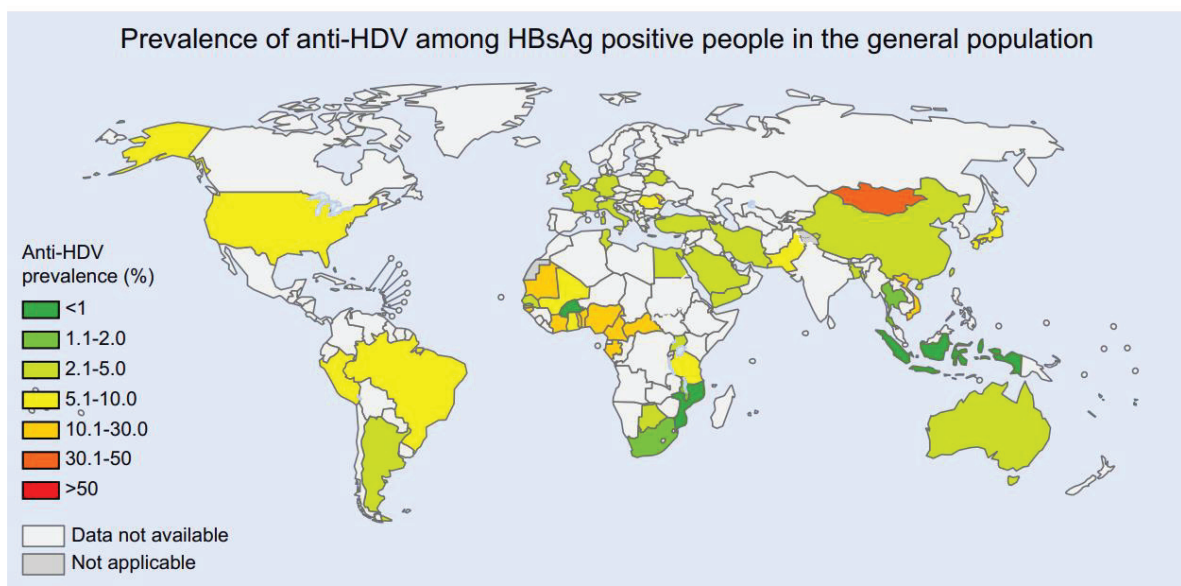


Figure 9: The country-level estimates of anti-HDV prevalence among HBsAg-positive people. From ¹³²

III- The HDV acquisition and transmission modes

As a satellite virus, HDV requires the presence of its helper virus, HBV, to spread. Clinically, HDV can be acquired in two possible ways: either by simultaneous infection with HBV, corresponding to co-infection, or by infection of patients already chronically infected with HBV, corresponding to superinfection. A third rare mode of HDV acquisition, known as "helper-independent latent infection", has been reported and may occur after liver transplantation. HDV infection of the new liver may occur without apparent help from HBV and remain asymptomatic unless reactivated by HBV¹³⁶.

The routes of transmission of HDV are the same as for HBV. Although HDV is mainly transmitted parenterally, the route of transmission can change between areas of low and high prevalence. In low-endemic areas, the transmission of HDV occurs mainly through exposure to infected blood, particularly among injecting drug users who share needles, syringes, or drug-preparation equipment. Vertical transmission of HDV, *i.e.* from mother to child, has been described in previous case reports but appears to be a rare occurrence.

IV- HDV nucleic acids and proteins

HDV is characterized by a spherical particle 35 to 37 nm in diameter containing a viral ribonucleoprotein (RNP) composed of a circular single-stranded RNA of negative polarity (HDV-G) associated with the two forms of the viral protein known as the delta antigen (HDAg), which are surrounded by HBV envelope proteins (*Figure 10*).

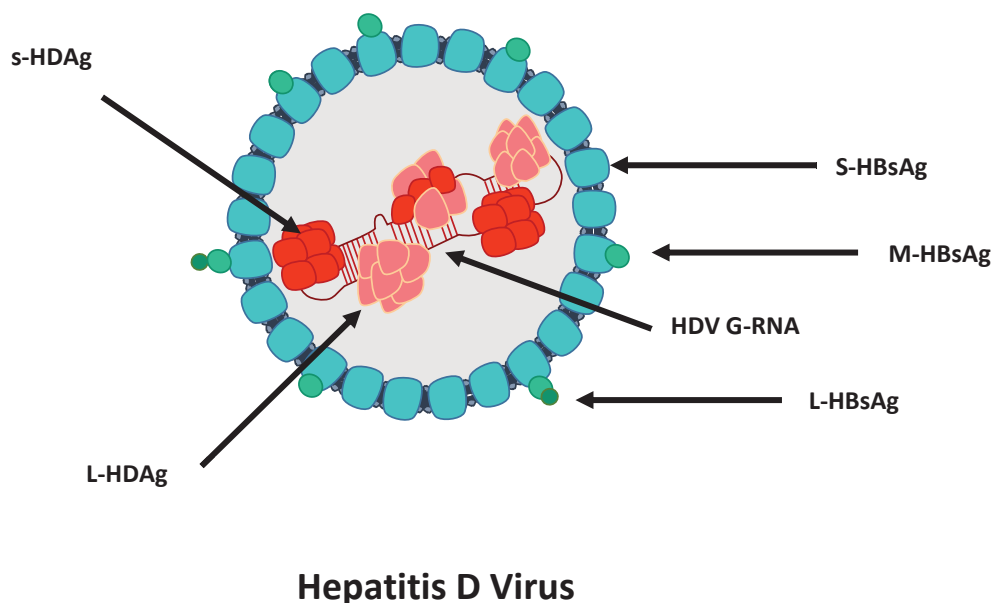


Figure 10: The structure of HDV. Adapted from²⁰.

1- The HDV envelope

Being a defective virus, the dissemination of HDV depends on HBV, and more particularly on the expression of the HBsAg (See [Part 1: The hepatitis B virus](#)). HDV does not code for its own envelope proteins. In the absence of HBV, *in vitro*, replication of HDV results only in the formation of RNP and the accumulation of HDV viral RNA in the host cell. The use of HBsAg from HBV confers the same tropism on both viruses, allowing them to exit and re-enter hepatocytes.

The composition of the viral envelope of HDV is similar to that of HBV, in particular to that of the SVPs of HDV¹³⁷. The HDV envelope consists mainly of membrane lipids in which the three forms of HBV surface antigen are anchored: the small (S-HBsAg, 24-27 kDa), medium (M-HBsAg, 31 kDa), and large (L-HBsAg, 42 kDa) forms, with 95% of the S-HBsAg form, 5% of the M-HBsAg form and 1% of the L-HBsAg form¹³⁷.

2- The viral ribonucleoprotein (RNP) of HDV

The HDV RNP consists of the association of a molecule of HDV genomic RNA with the two isoforms of HDAg. This RNP is present both in infected cells and in viral particles. Depending on the method used, between 30 and 200 HDAg are thought to be bound to a HDV RNA molecule¹³⁸⁻¹⁴⁰. More precisely, the HDAg form dimers via an antiparallel 'helical coil' and then interact to form homomultimeric S-HDAg or L-HDAg structures or heteromultimeric S-HDAg/L-HDAg structures that bind to the HDV genome. The interaction between HDAg and the HDV genome results in the formation of a nuclease-resistant complex.

3- The HDV genome

The HDV genome consists of a single-stranded circular RNA of negative polarity with 1675-1697 nucleotides, depending on the genotype studied (*Figure 11*). It is currently the smallest genome of all known mammalian viruses. The HDV genome is characterized by an almost double-stranded conformation containing numerous stem-and-loop structures resulting from 74% base pairing and 60% guanine-cytosine. This specific conformation gives the HDV genome a flexible configuration essential for both genome replication and viral RNP assembly. During replication, an infected cell can contain around 300,000 HDV genome molecules distributed between the cytoplasm and the nucleus¹³⁹. In addition to genomic RNA, two other types of viral RNA can be detected in infected cells: antigenomic RNA and HDV messenger RNA (mRNA) (*Figure 11*). Antigenomic RNA is the strict antiparallel and circular complement of genomic RNA. It is located only in the nucleus of infected cells and is not packaged in virions. It mediates HDV replication and also possesses the open reading frame (ORF) encoding the two isoforms of the delta antigen. HDV mRNA, around 800 nucleotides long, is produced following the transcription of genomic RNA. It is linear and has a cap complex and a polyadenylation tail^{141,142}.

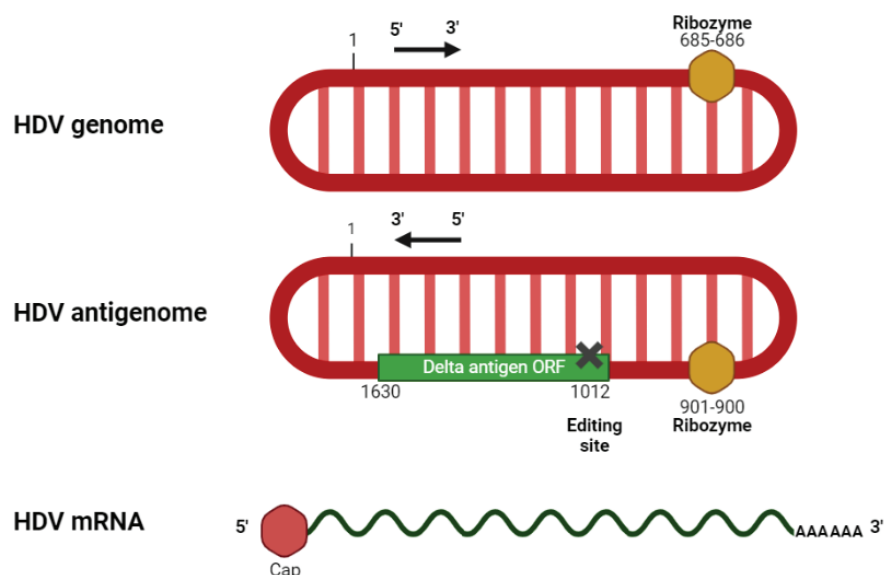


Figure 11: The structure of HDV RNAs.

4- The ribozyme of HDV

Both genomic and antigenomic RNA contain a self-cleavage sequence of approximately 85 contiguous nucleotides, called ribozyme¹⁴³. This ribozyme is essential for HDV replication and is highly conserved among the different HDV genotypes. Ribozyme catalyzes the self-cleavage of multimeric RNA molecules neo-synthesized during virus replication. Specifically, self-cleavage involves transesterification, modulated by divalent metal ions, converting a 3'-5' phosphodiester bond into a 2'-3' cyclic monophosphate group and a 5' hydroxyl group. Self-cleavage is the only enzymatic activity reported for HDV to date.

5- The HDV viral proteins and editing

The HDV genome contains several ORFs in the genomic RNA and antigenomic RNA sequences. However, only one of these ORFs appears to be actively transcribed during HDV replication, leading to the synthesis of the unique viral protein called delta antigen (HDAg)¹⁴⁴. The translation of HDAg takes place from HDV mRNA. Two isoforms of HDAg are translated from the same ORF: the small (S-HDAg) (24kDa) and large (L-HDAg) (27kDa) forms. The S-HDAg form is translated early in the HDV viral cycle, *i.e.* as early as the first transcription cycle, from an unedited mRNA transcribed from the HDV genome (**Figure 12**). Conversely, the L-HDAg form is produced later in the cycle following editing of the antigenome, *i.e.* around the third day after infection of HepaRG cells with HDV¹⁴⁵. Editing does not occur directly on the mRNA but on the specific Ambre/W site present on the antigenome at position 1012. Editing of the Ambre/W site is catalyzed by the cellular protein ADAR1 (Adenosine Deaminase Action on RNA), which leads to the conversion of the Ambre/W stop codon (UAG) into a tryptophan codon (UGG), allowing translation to continue at the next stop codon, extending the ORF and giving rise to a protein 19 amino acids longer¹⁴⁶. ADAR-1 exists in two distinct forms: the small (ADAR1-S, 110kDa), constitutively expressed in the nucleus, and the large (ADAR1-L, 150kDa) form of ADAR1, localized mainly in the cytoplasm and whose expression is induced by IFN. Editing of the antigenome, which takes place in the nucleus, is thought to be

mediated solely by the ADAR1-S form¹⁴⁷. However, other studies have demonstrated the involvement of the ADAR1-L form in the production of HDV^{148,149}.

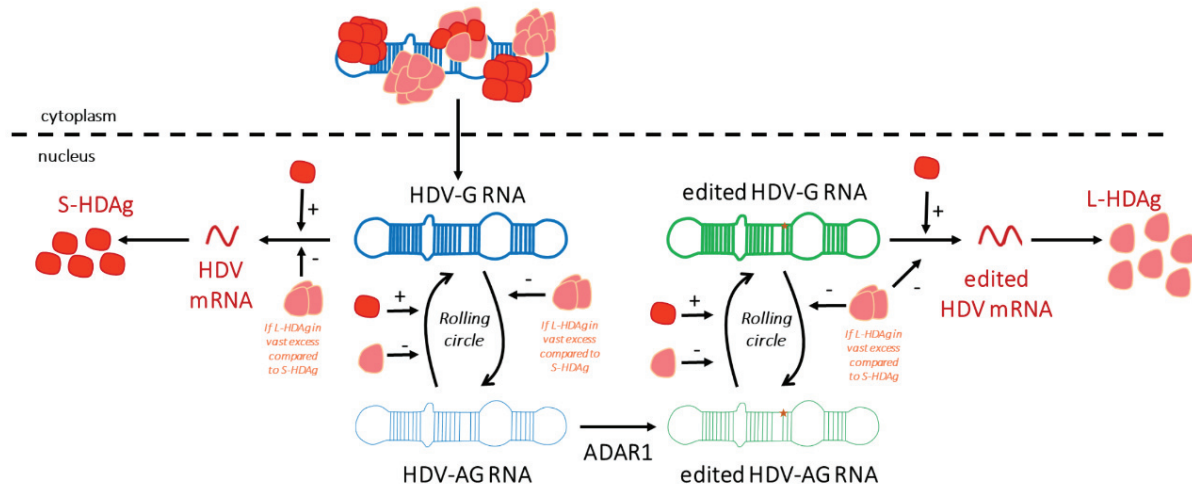


Figure 12: The schematic representation of the production of both delta antigen forms. The incoming genome of HDV (HDV-G RNA) acts as a template for synthesizing unedited HDV mRNAs, which code for the small delta antigen (S-HDAg). Additionally, HDV-G RNA is produced through a rolling circle mechanism. S-HDAg plays a role in enhancing the replication and transcription of HDV. The antigenome of HDV (HDV-AG RNA) undergoes editing by ADAR1, an RNA deaminase, followed by a second round of rolling circle replication, resulting in the production of edited mRNA and the synthesis of the large delta antigen (L-HDAg). Produced in large amounts compared to S-HDAg, L-HDAg inhibits HDV replication. Adapted from²⁰.

The S- and L-HDAg isoforms share a 195 amino acid sequence and similar functional domains (**Figure 13**). These domains include a helix-loop-helix domain that is involved in the three-dimensional structure of S- and L-HDAg, as well as a NLS that facilitates their transport into the cell nucleus. In addition, both isoforms possess an RNA-binding domain, enabling them to interact with viral RNA and perform their functions in the HDV viral cycle. The additional 19 amino acid sequence of L-HDAg contains a nuclear export signal (NES), a viral assembly signal, and a prenylation site^{150,151}.

The S- and L-HDAg isoforms undergo post-translational modifications that influence their activity in the HDV viral cycle. S-HDAg undergoes phosphorylation at the serine and threonine residues, whereas only the serine residues are phosphorylated in the L-HDAg form¹⁵²⁻¹⁵⁴. Phosphorylation of serine 177 of S-HDAg plays an essential role in its interaction with RNA Pol II, and phosphorylation of serines 177 and 2 increases HDV replication^{154,155}. It has also been shown that both forms of HDAg can be acetylated^{156,157}. The S-HDAg form is acetylated at lysine 72 by the cellular acetyltransferase p300, ensuring nuclear retention of S-HDAg and thus enabling HDV transcription and replication. In the absence of this post-translational modification, early synthesis of the L-HDAg form is observed. The S-HDAg protein can also be methylated, via the protein arginine methyltransferase PRMT1, which adds a methyl group to arginine 13 located in the RGGR motif of the RNA-binding domain. Methylation of S-HDAg is essential for HDV replication, as a mutation at arginine 13 inhibits replication, preventing retention of the protein in the nucleus¹⁵⁸. Furthermore, it has been observed that the S-HDAg protein can be sumoylated at lysine residues by the creation of a covalent bond between the latter and a SUMO¹⁵⁹. Studies have shown that sumoylation of the S-HDAg protein increases HDV genomic RNA and mRNA synthesis, but does not affect antigenomic RNA synthesis. Isoprenylation of the L-HDAg protein is a major post-translational modification of the HDV viral cycle. It results from the farnesylation of cysteine 211 contained in the CRPQ isoprenylation motif located in the C-terminal part of the protein¹⁵¹. Farnesylation of L-HDAg plays an essential role in the HDV viral cycle, since farnesylated L-HDAg inhibits HDV transcription and replication, and promotes viral particle assembly and secretion^{160,161}.

In the HDV viral cycle, the S-HDAg and L-HDAg proteins play distinct roles. While S-HDAg is involved in the initiation of HDV transcription and replication, L-HDAg acts as an inhibitor of replication and plays an essential role in viral particle assembly and release^{160–162}.

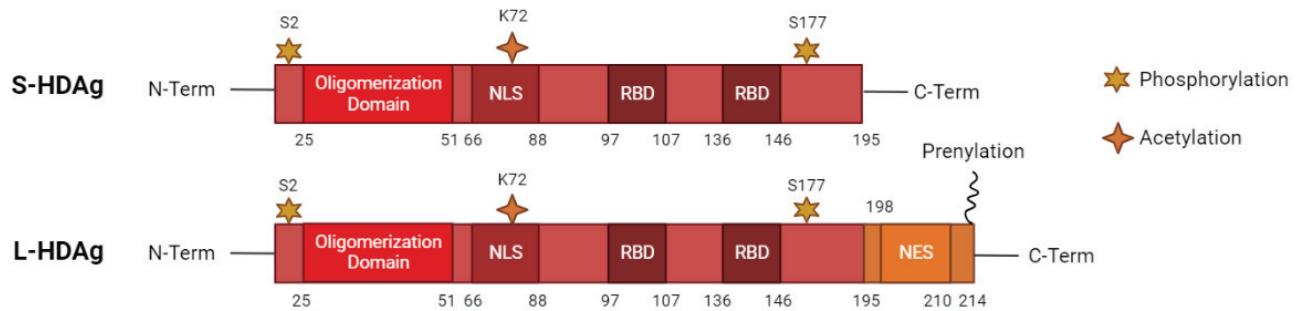


Figure 13: Schematic representation of delta antigen structure. NLS: nuclear localization signal, RBD: RNA-binding domain, NES: nuclear export signal, K: lysine, S: serine.

V- The viral life cycle of HDV

The different stages of HDV replication are summarized in **Figure 14**.

1- The attachment of HDV particles and viral entry into cells

Using the envelope proteins of HBV, HDV is expected to share the same entry mechanisms and cell receptors with HBV. Infection of both viruses is based on a multi-stage process. Firstly, the initial step in this entry process is the attachment of viral particles to the cell surface, involving cell surface molecules such as heparan sulfate proteoglycans (HSPGs). The attachment step is carried out by HSPG side chains, which enable non-specific, low-affinity, and reversible binding. The involvement of HSPGs in the entry process has been demonstrated by incubating virions in the presence of heparin, or by pre-treating target cells with heparin, resulting in inhibition of HDV and HBV infection^{163,164}. Recently, the Glypican 5 protein (GPC5), highly expressed in the liver, has been identified as a key HSPG in the attachment of HDV and HBV¹⁶⁵. The interaction of both viruses and HSPGs is mediated by the antigenic loop of HBV envelope proteins^{166,167}. It results from an electrostatic interaction between negatively

charged HSPGs and the positively charged Arg122 and Lys141 residues of the antigenic loop¹⁶⁷. Low-affinity attachment to HSPGs is necessary to stabilize viruses on the cell surface, but this interaction is not sufficient for viral entry. HDV and HBV particles must interact with their specific receptor(s) with higher affinity to trigger the entry process. The sodium taurochlorate cotransporter polypeptide (NTCP) has been identified as the functional and specific entry receptor for HDV and HBV^{168,169}. NTCP, encoded by the *SLC10A1* gene, is a bile acid transporter involved in the enterohepatic circulation of bile acids and is expressed on the basolateral surface of hepatocytes. The interaction of HDV and HBV with NTCP is mediated by the pre-S1 domain of L-HBsAg^{168,169}. Recently, the epidermal growth factor receptor (EGFR) has been identified as a potential cofactor for HBV entry¹⁷⁰. Thanks to a direct interaction between EGFR and NTCP, HBV internalization could be mediated by clathrin-dependent endocytosis¹⁷¹. Inhibition of clathrin heavy chain (CHC) and adaptor protein 2 (AP-2) significantly reduced HBV infection. It has also been reported that the preS1 domain of HBV envelope proteins interacts with clathrin and AP-2 upon entry into immortalized primary human hepatocytes¹⁷².

2- The uncoating and nuclear transport of HDV

After entering the host cell, the virus is uncoated in the cytoplasm, and the HDV RNP is transported to the nucleus. Our understanding of virus uncoating and RNP transport remains to be clarified. HBV appears to be internalized by clathrin-dependent endocytosis, which occurs across early and late endosomal compartments independently of acidification and protease activity^{45,172}. Although this has not been demonstrated, this pathway may be also used by HDV. Indeed, the L-HDAg protein is a clathrin adaptor protein¹⁷³. RNP import into the nucleus is mediated by HDAg and more specifically by the NLS nuclear-addressing signal and the RNA-binding motif¹⁷⁴. Indeed, the deletion of these determinants impaired the nuclear import of HDV RNA. Moreover, RNP translocation appears to be mediated by importins.

3- The replication and transcription of HDV

Once in the cell nucleus, genomic RNA serves as a template for mRNA synthesis. This mRNA is then exported from the nucleus and translated, resulting in the production of S-HDAg. S-HDAg migrates to the nucleus of the cell, where it becomes part of the HDV replication process. HDV genomic RNA is replicated by a rolling circle mechanism similar to that proposed for plant viroids, but modified to include HDV mRNA synthesis. Rolling circle replication relies on two reverse-polarity circular RNA models, namely the genome and antigenome, as well as the generation of multimeric linear transcripts. Firstly, the incoming circular genomic RNA serves as a template for the very first round of HDV RNA replication to generate multimeric antigenomic RNAs. The antigenome is self-cleaved via its intrinsic ribozyme activity and each antigenomic monomer is circularized probably by self-ligation or by a cellular ligase. Secondly, this antigenomic monomer serves as a template in turn for the second similar round of rolling circle replication to generate circular genomic RNAs.

HDV does not possess its own RNA polymerase, nor does it use the polymerase of its helper virus, unlike other satellite viruses. Instead, it uses the host's transcriptional machinery for replication and transcription. This requires the recruitment of a cellular DNA-dependent RNA polymerase to synthesize oligomeric strands from circular RNA templates. RNA polymerase II (Pol II) is thought to be involved in HDV replication and transcription, as shown by several studies. Indeed, like cellular RNAs, HDV mRNA has a 5' cap and a 3' poly-A tail, as previously mentioned. Furthermore, it has been shown that HDV transcription can be inhibited by low doses of α -amanitine, a well-known inhibitor of RNA Pol II^{175,176}. On the other hand, RNA Pol II has been shown to interact with HDV genomic and antigenomic RNA^{176,177}. Other studies have suggested the potential involvement of RNA Pol I and III in HDV replication. Indeed, it was shown that antigenome synthesis appeared to be resistant to high doses of α -amanitine but was inhibited by actinomycin D, suggesting a probable involvement of RNA Pol I^{175,178}. This

is supported by the fact that RNA Pol I leads to the transcription of non-polyadenylated transcripts and that the polyadenylation signal of the antigenome remains silent during its synthesis and no mRNA is synthesized from the antigenome. Furthermore, another study reported that HDV RNAs interact with the RNA components Pol I and Pol III¹⁷⁹.

Taken together, these data suggest that DNA-dependent RNA polymerase(s) is (are) hijacked by HDV to use RNA as a template for replication and/or transcription instead of the usual DNA. On the one hand, HDV quasi-double-stranded conformation seems to facilitate the detour of RNA polymerase from its initial substrate. Indeed, it has been shown that RNA Pol II, TBP (TATA-binding protein), and the general transcription factors TFII-A, B, E, F, H, and S bind to the stem-loop ends of HDV¹⁸⁰. On the other hand, S-HDAg has been identified as playing a crucial role in HDV transcription and replication, and more specifically in the hijacking of RNA polymerase(s)¹⁶². Firstly, S-HDAg was found to interact with 9 of the 12 subunits of RNA Pol II¹⁸¹. S-HDAg also facilitates the recruitment of cellular factors required for the establishment of the transcription pre-initiation complex (*Figure 15*). Studies have revealed that S-HDAg interacts with several cellular proteins, including the transcription factor YY1, the activation cofactors CBP and p300, B23, BAZ2B (bromodomain-associated to zinc finger protein 2B), which regulates the bromodomain remodeling factor BRF, nucleolin and the Pol I coactivator SL1 (selectivity factor 1)^{156,157,178,182,183}. In addition to its involvement in the recruitment of the transcription pre-initiation complex, S-HDAg contributes to RNA elongation by destabilizing the negative elongation factor (NELF) - DRB sensitivity-inducing factor (DSIF) complex, which inhibits elongation by binding to RNA Pol II¹⁸⁴. S-HDAg appears to compete with the NELF-A subunit.

The activity of S-HDAg as a regulator of HDV transcription and replication is intrinsically linked to the latter's post-translational modifications, as previously mentioned. For example, it has been shown that phosphorylation of S-HDAg at serine 177 is crucial for the interaction between S-HDAg and RNA Pol II and that phosphorylation of serine 177 and serine 2 enhances HDV replication^{155,185}.

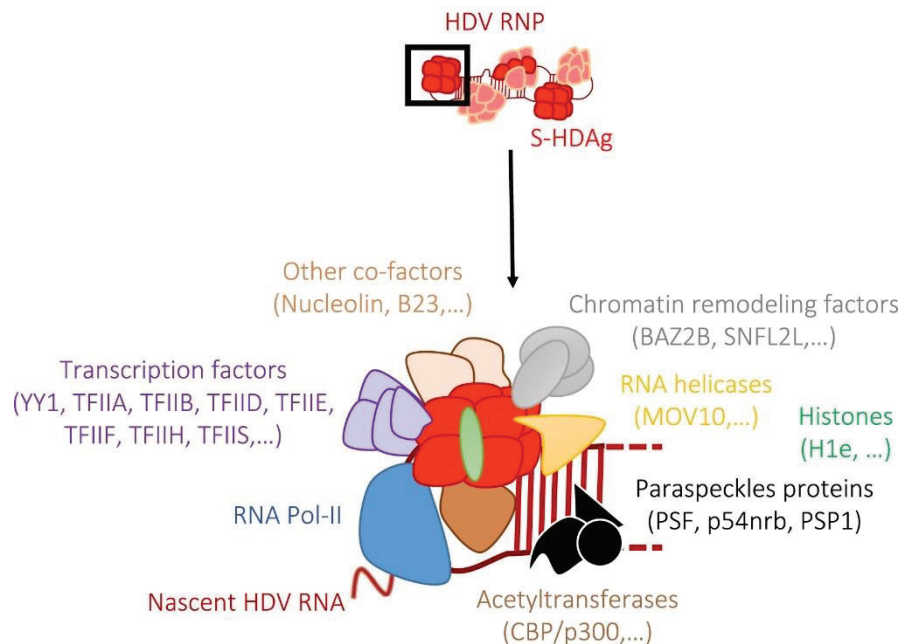


Figure 15 : Schematic representation of HDV replication complex. From²⁰.

4- The assembly and secretion of HDV particles

Although HDV is enveloped by HBV surface proteins, the mechanisms of virion assembly and secretion for the two viruses appear to be different. Before being packaged, HDV genomic RNA associates with HDAg to form the viral RNP, which is then translocated from the nucleus to the cytoplasm. L-HDAg has been identified as a nucleocytoplasmic shuttle protein characterized by an NES nuclear export signal in its C-terminal domain¹⁸⁶. A protein that interacts with this nuclear export signal, called NESI, has been identified as essential for the nuclear export of the HDV RNP¹⁸⁷. The NESI protein seems to facilitate the formation of an RNP export complex by interacting with lamin A/C as well as with nucleoporin complexes, thus ensuring the nuclear export of RNP¹⁸⁸. At the same time, the nuclear factors TAP and Aly

have been identified as essential for the translocation of RNP from the nucleus to the cytoplasm¹⁸⁹. TAP (NXF1) is a cellular export receptor that facilitates the anchoring and translocation of RNPs into the NPC, while Aly is an RNA-binding adaptor protein.

After translocation, the new HDV RNPs are transported to the endoplasmic reticulum. The L-HDAg protein anchors via its farnesyl moiety in the ER membrane, where it associates with HBsAg and assembles to form new HDV particles¹⁸. Unlike infectious HBV virions, which use the multivesicular body, HDV appears, like HBV subviral particles, to use the secretory pathway for assembly and budding. L-HDAg appears to be involved in the clathrin-mediated trafficking of HDV viral particles. Indeed, L-HDAg has been identified as a clathrin adaptor-like protein¹⁷³. Nevertheless, inhibition of CHC expression did not completely suppress HDV particle assembly and secretion, suggesting that other pathways may be involved.

The association between L-HDAg and HBsAg is the driving force behind HDV assembly and is mediated by viral determinants present in L-HDAg and HBsAg. Isoprenylation of L-HDAg is necessary but not sufficient for virion assembly¹⁶¹. An additional signal is required for packaging. The 15 amino acids preceding the isoprenylation motif are essential. The proline-rich C-terminal part of L-HDAg can interact with the S-domain of envelope proteins, and in particular with a motif rich in tryptophan residues¹⁹⁰. Together, the isoprenylation signal and the C-terminus mediate direct interaction between L-HDAg and HBsAg, and thus the assembly of viral particles. HDV particles can be assembled and secreted using only the S-HBsAg form, but the particles are not infectious¹⁹¹. Conversely, the addition of L-HBsAg is essential to make HDV particles infectious, but not sufficient alone for particle assembly. Unlike the S- and L-HBsAg proteins, the M-HBsAg protein does not appear to be necessary for viral particle

assembly and infectivity¹⁹². The amount of HBsAg determines the assembly, release, and infectivity of HDV particles^{193,194}.

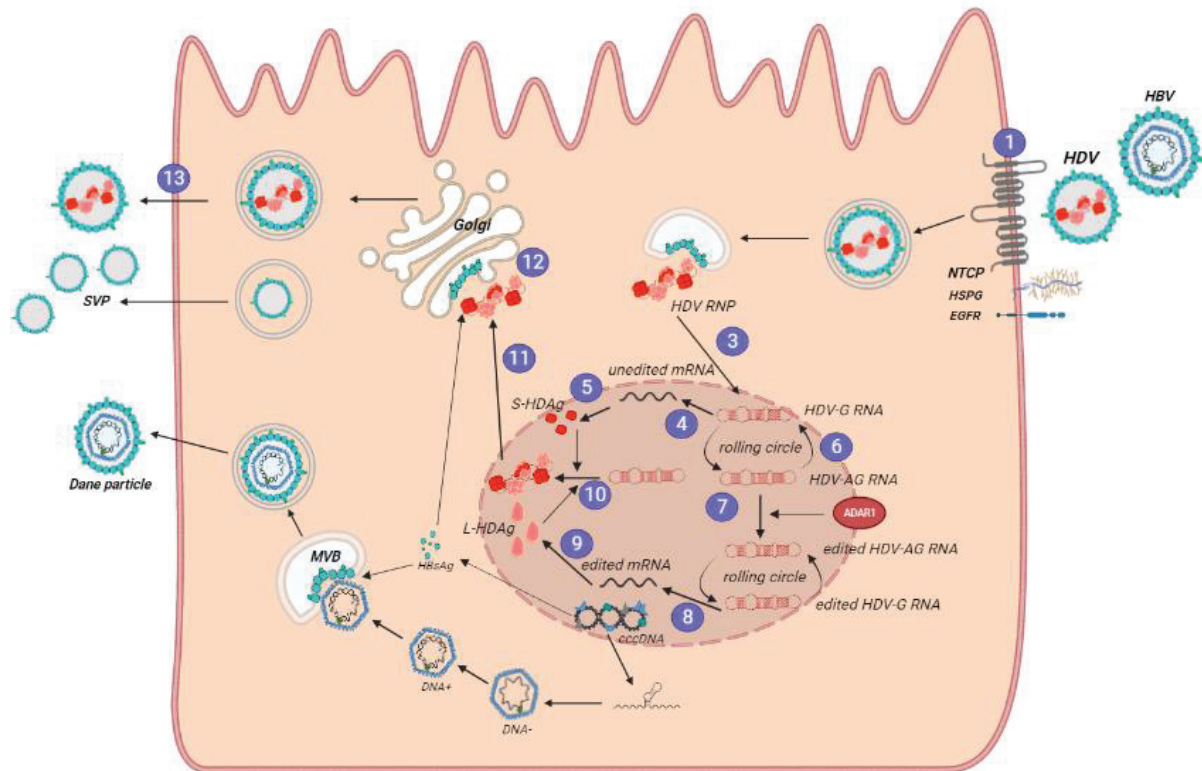


Figure 14: *The HDV life cycle. The entry of HDV into hepatocytes involves NTCP, in collaboration with EGFR and HSPGs (1). The virus is internalized, and subsequently, the viral ribonucleoprotein (HDV RNP) is released into the cytoplasm (2) and transported to the nucleus (3). The incoming HDV genome (HDV-G RNA) serves as a template for the transcription of HDV mRNAs (4), enabling the synthesis of the small delta antigen (S-HDAg)(5). Concurrently, HDV-G RNA and HDV antigenome (HDV-AG RNA) are synthesized through a rolling circle mechanism (6). In the later stages of the HDV life circle, HDV-AG RNA undergoes editing by ADAR1 (7), leading to the synthesis of edited mRNA (8) and the production of the large delta antigen (L-HDAg)(9). L-HDAg and S-HDAg associate with HDV-G RNA to form HDV RNP (10), which is then exported from the nucleus to the endoplasmic reticulum and/or Golgi apparatus (11) to generate new HDV particles (12) for secretion (13). Adapted from²⁰.*

VI- The HDV genotypes

HDV exhibits considerable genetic variability. According to phylogenetic analyses of HDV sequences worldwide, 8 genotypes have been identified (HDV-1, -2, -3, -4, -5, -6, -7, and -8), with subgenotypes within each genotype (**Figure 16**)¹⁹⁵. The classification of HDV genotypes is based on a nucleoside sequence divergence of more than 20% over the entire viral genome, or more than 15% if the R0 region is considered (region encoding the terminal part of HDAg)¹⁹⁶. The sub-genotypes of the genotypes differ from each other by more than 10% in the complete genome.

The different genotypes of HDV are characterized by different geographical distributions. Genotype 1 is found in Europe, North America, Africa, and Asia, while genotypes 2 and 3 are restricted to South and East Asia, Japan, and Siberia. Genotype 3 is the most divergent. It has been observed in South America. Genotypes 5, 6, 7, and 8 appear to be restricted to African patients from the sub-Saharan region. The pathogenicity of HDV has been shown to differ according to the genotype considered.

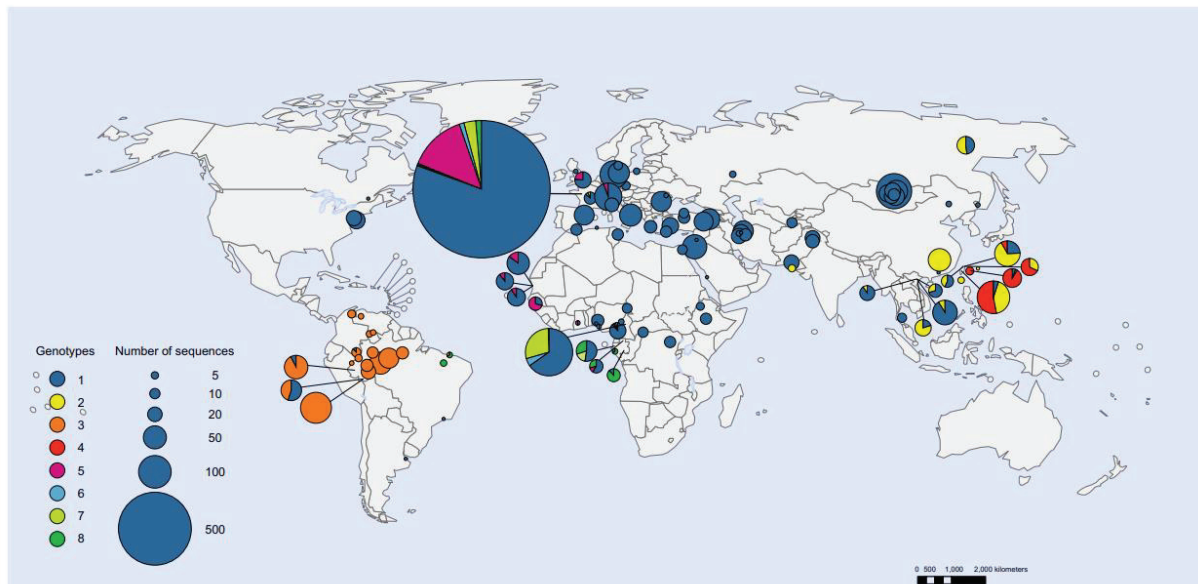


Figure 16 : *The geographic distribution of HDV genotypes worldwide. From*¹³².

VII- The impact of HDV/HBV co-infection on the severity of liver disease

Patients chronically infected with both HDV and HBV have been shown to develop more severe liver disease than patients infected with HBV alone¹⁹⁷. In fact, between 70% and 80% of patients develop cirrhosis within 5 to 10 years of infection. In addition, HCC occurs in 42% of cases after 15 years of infection⁷⁰. The risk of developing HCC is thus multiplied by three in patients with cirrhosis in the context of an HDV/HBV coinfection compared with an HBV mono-infection alone and is accompanied by a mortality rate multiplied by two¹⁹⁸⁻²⁰⁰. The factors behind the severity of hepatitis in co-infection with HDV and HBV appear to be multiple, both virological and immune. However, the molecular mechanisms by which HDV promotes more rapid and severe disease progression are not yet fully understood. Indeed, this progression could be caused by a cumulative effect of the two viruses, an underlying effect of cirrhosis, or a direct oncogenic effect of HDV.

In vitro, studies suggest a potential oncogenic role for HDV, but these data are limited and mostly obtained in hepatoma cell lines. Indeed, HDV could modulate key cellular pathways associated with the development of fibrosis. It has been reported that L-HDAg, and not S-HDAg, can activate the transforming growth factor- β (TGF- β) and c-Jun signaling pathways that regulate numerous cellular processes²⁰¹. Prenylation of L-HDAg appears to mediate this activation. TGF- β is involved in the control of cell proliferation, differentiation, and apoptosis. It plays a major role in hepatic fibrosis by stimulating the expression of fibrosis-associated genes. By activating TGF- β signaling, L-HDAg may promote the development of fibrosis. L-HDAg has also been reported to activate the nuclear factor kappa B (NF- κ B) via induction of TNF α and STAT3 expression in Huh7 cells^{202,203}. The transcription factors NF- κ B and STAT3 play a key role in numerous cellular pathways such as inflammation, apoptosis, cell proliferation, and tumor development. For example, activation of NF- κ B and STAT3 by L-HDAg induces oxidative stress in Huh7 cells via NOX4 expression, promoting the synthesis of

reactive oxygen species (ROS)²⁰⁴. This ROS synthesis was associated with increased apoptosis. In addition, S-HDAg was shown to decrease the expression of glutathione S-transferase Pi 1 (GSTP1), which plays a key role in ROS detoxification²⁰⁵. Finally, HDAg may induce epigenetic modifications, as suggested by a study in which an increase in the expression of DNMT-3b, a DNA methyltransferase, was observed²⁰⁶. An altered methylation profile could therefore play a role in the development of HCC²⁰⁷.

Prolonged stimulation of immunity could also play a role in the pathogenicity of HDV. Indeed, deregulation of the innate and adaptive immune response could lead to the development of liver damage. Strong induction of the IFN (β and λ) pathway, as well as the specific expression of a large number of ISGs and cytokines, was observed during infection with both viruses, compared with HBV monoinfection²⁰⁸. This suggests that HDV increases inflammation. An increase in the number of NK cells has also been reported in patients chronically infected with HDV producing large amounts of IFN α and IFN γ ²⁰⁹. Moreover, a significant increase in the number of cytotoxic CD4⁺ LT in the presence of HDV was observed, which may contribute to disease progression²¹⁰.

VIII- The interplay of HDV on HBV

Despite its dependence on HBV, HDV has been reported to interfere with HBV. HDV has been shown to inhibit HBV replication in animal models such as chimpanzees, woodchucks infected with WHV, a humanized mouse model, and humans^{129,211–213}. A cross-sectional study of a cohort of patients coinfecting with HDV and HBV reported a dominance of HDV over HBV in 75% of cases²¹⁴.

Different mechanisms have been envisaged to explain the origin of HDV interference on HBV. On the one hand, experimental data suggest a direct interaction between HDV and HBV via the delta antigen. Studies have reported that HDAg can reduce the amount of HBV RNA

via an overexpression system or in co-infected hepatocytes^{145,215}. However, cccDNA levels did not appear to be affected. Another study showed that delta antigen could inhibit HBV replication by repressing both HBV enhancers²⁰³. More recently, both forms of delta antigen have been shown to reduce the level and stability of newly synthesized HBV RNAs²¹⁶. However, the mechanisms underlying this interaction remain to be elucidated. No significant changes in the levels of RNA Pol II, its phosphorylation status, HBcAg, or acetylated histone H3 (H3K27) associated with cccDNA were observed, indicating that the inhibition of HBV synthesis is not directly mediated by these factors. Instead, it is possible that the impact of HDV on HBV RNA transcription involves a rapid effect on the stability of newly synthesized HBV RNAs. RNA immunoprecipitation assays revealed that both forms of HDV Ag bind to HBV RNAs, and preliminary data from UV-crosslinking experiments suggest a direct interaction between them²¹⁶. The association of HDV Ag with HBV may contribute to their degradation. Furthermore, it appears that HDV does not affect the m⁶A modifications induced by the 3' epsilon stem loops of HBV transcripts, which are known to play a role in controlling their stability.

On the other hand, an additional interference mechanism is suggested. HDV infection is associated with strong induction of ISGs, such as RSAD2 (Viperin), and IFI78 (MxA), a specific signature of which has recently been identified²¹⁶. Some of these ISGs may be responsible for the interference of HDV on HBV replication. For example, TRIM22, which is up-regulated upon infection with HDV, has shown antiviral activity against HBV by acting as a transcriptional repressor²¹⁷. DDX60, an RNA helicase, promotes the degradation of HBV RNAs²¹⁸. ISG20, an exoribonuclease, induces HBV RNA degradation by recognizing m⁶A modifications on the epsilon stem loop^{219,220}.

IX- The current treatments and therapeutic prospects for HDV

To date, the best way to eradicate HDV remains the prevention, *i.e.* vaccination against HBV. Vaccination is 95% effective in combating HBV transmission, but also in preventing future HDV infection. Around 5% of those vaccinated fail to respond by developing antibodies to the vaccine²²¹. The first vaccine was developed in 1976 by Professor Philippe Maupas and his team by purifying HBsAg from the sera and plasmas of chronic hepatitis B patients. The vaccine received its marketing authorization in 1981 but is gradually being replaced by recombinant vaccines produced by genetic engineering.

The main objective in the search for treatments for HDV is to reduce the replication of the virus to reduce liver inflammation and prevent the progression of fibrosis and its complications, such as the development of cancer. However, therapeutic management of patients with HDV infection remains complex and unsatisfactory. Indeed, HDV does not code for enzymatic proteins and uses only cellular polymerases for replication, complicating the development of specific antiviral treatments. What's more, treatments commonly used for HBV do not affect HDV, such as the NUCs used as first-line therapy in the management of patients with CHB²²². NUCs inhibit HBV reverse transcriptase, thereby reducing replication. However, they do not affect envelope protein synthesis. Envelope proteins are always synthesized, either from cccDNA or from HBV DNA integrated into the host genome.

The different therapeutic strategies presented in this part are summarized in ***Figure 17***.

1- The current therapeutics

a- The interferon alpha (IFN α)

Until recently, the use of IFN α and mostly PEG-IFN α in the management of patients with chronic hepatitis delta was the only treatment regimen recommended by international guidelines to date, in combination or not with NUCs. PEG-IFN α is administered to patients by

subcutaneous injection once a week for 48 consecutive weeks. Treatment showed some efficacy, leading to undetectable HDV viremia by PCR in serum in some cases. The precise mechanisms by which IFN α acts on HDV are not fully understood. However, in addition to its activation of immune cells, IFN α has demonstrated direct effect on various steps of the HDV life cycle, including entry, replication and secretion^{145,223}. In HepaRG cells, IFN α treatment reduced HDV infection by 50 % after early treatment while only a minor effect was observed after late treatment when the infection is already established²²⁴. Furthermore, it has been observed to inhibit the spread of HDV through cell division²²⁵. Nevertheless, only 25% of patients respond after 48 weeks of treatment, and of these, 50% relapse after discontinuation^{222,226}. Treatment prolongation does not improve the antiviral efficacy of PEG-IFN α ²²⁷. Besides, the combination of PEG-IFN α with NUCs does not both improve the virologic response and prevent post-treatment relapses^{228,229}. The HIDIT-II clinical trial demonstrated that HDV became undetectable in 48% of patients who received a combination of 180 μ g weekly PEG-IFN α -2a plus tenofovirs, and in 33% of patients who only received PEG-IFN α -2a after 96 weeks of treatment. 24 weeks later, HDV RNA was undetectable in 31% of patients who received the combination of PEG-IFN α -2a and tenofovir, and in 3% of patients receiving PEG-IFN α -2a alone. In addition to relapses, taking IFN α is also associated with significant side effects^{226,228,230,231}. Indeed, the use of PEG-IFN α is contraindicated in patients with decompensated cirrhosis, psychiatric disorders, and autoimmune diseases.

It is therefore necessary to develop new antiviral treatments directly targeting the HDV viral cycle. New treatments are currently under development, and some of them have recently obtained marketing authorization and/or are in the process of being registered.

b- The entry inhibitors: bulevirtide

One of the new antiviral strategies being considered is to directly target the first stage of the viral cycle, i.e. the entry of HDV and HBV into hepatocytes. Although at first sight, this strategy

does not appear to be directly curative, it could lead to a progressive elimination of the viruses by impeding the establishment of the infection. In the case of chronic infection, it would enable a reduction in the number of infected cells resulting from the inhibition of the infection of new cells coupled with the permanent renewal of hepatocytes.

As previously explained, HDV and HBV entry relies on the specific interaction between HBsAg and the viral receptor NTCP via the PreS1 region of L-HBsAg. Based on this knowledge of the viral entry mechanism, a synthetic myristoylated peptide corresponding to the amino acid sequence of the PreS1 domain of L-HBsAg required for the interaction was developed: Bulevirtide (or MyrcludexB or Hepcludex). Bulevirtide has been shown to inhibit both HDV and HBV entry into hepatocytes by binding competitively to NTCP *in vitro* and *in vivo* in humanized mouse models^{212,232}.

In multicentre phase IIb of Bulevirtide (named MYR202), 120 patients were randomized to different doses of Bulevirtide (2, 5, or 10 mg/day) or tenofovir monotherapy for 24 weeks followed by a period of tenofovir alone for 24 weeks for each arm of the study. This clinical trial showed that Bulevirtide was well tolerated by patients, with no adverse effects or immunogenic response, and excellent bioavailability when administered subcutaneously to patients²³³. A 2 log decline or undetectable HDV RNA was reached by 54%, 50% and 77% of patients treated with 2, 5 or 10mg of Bulevirtide at week 24 but only 3% of those on tenofovir monotherapy. At week 48, a relapse occurred in 60%, 80% and 83% in the 2, 5 and 10mg Bulevirtide treatment arms. However, HBsAg levels did not significantly decline during the Bulevirtide monotherapy. Next, the effect of Bulevirtide monotherapy at different doses (2mg or 5mg/day) or dual therapy with PEG-IFN α -2a or tenofovir on 90 patients chronically infected with HBV and HDV was evaluated over 48 weeks in a Phase IIa clinical trial (named MYR203)²³⁴. The results showed that Bulevirtide alone reduced the amount of HDV and HBV RNAs after 24 weeks of treatment and that its effect was more pronounced when combined (at

2mg/day) with PEG-IFN α . After 48 weeks of dual therapy, a 4.81 log decrease in HDV RNA was observed²³⁴. In contrast to the rebound usually observed with PEG-IFN α , dual therapy with Bulevirtide maintained the decrease in HDV RNA levels at 4.04log 24 weeks after treatment discontinuation. A 1 log IU/mL decline of HBsAg was only observed in 40 %, 13.3%, and 13.3% of patients treated with 2, 5 or 10 mg of Bulevirtide combined with PEG-IFN α respectively²³⁵. The dual therapy with PEG-IFN α appeared to show a strong synergism with respect to HDV RNA decline but off-treatment HDV responses at week 72 were only observed in patients achieving an HBsAg response.

Following on from clinical phase IIa, a second phase IIb study (named MYR204) is ongoing to evaluate the use of Bulevirtide in dual therapy with PEG-IFN α ²³⁶. 175 patients chronically infected with HBV and HDV were divided into four cohorts and either treated with PEG-IFN α monotherapy for 48 weeks with a post-treatment follow-up of 48 weeks, or PEG-IFN α plus 2 mg/day of Bulevirtide for 48 weeks followed by Bulevirtide 2 mg monotherapy, or PEG-IFN α plus 10mg/day of Buleviritle followed by Bulevirtide 10 mg for 48 weeks, or treated with 10 mg/day Bulevirtide monotherapy for 96 weeks. After 24 weeks, an higher HDV RNA decline was observed in 88% and 92% of patients treated with either Bulevirtide 2 mg or 10 mg plus PEG-IFN α compared to Bulevirtide monotherapy (72%). In addition, a decline of HBsAg (>1 log) was only observed in patients receiving the combination therapy

In an ongoing Phase III clinical trial (named MYR301), 150 patients chronically infected with HBV and HDV were treated either for 144 weeks with 2 or 10 mg followed by 96 weeks of off-treatment follow-up or no treated during 48 weeks and then treated with 10 mg for 96 weeks (the control group) to assess the efficacy and safety of Bulevirtide monotherapy²³⁷. 10mg of Bulevirtide did not provide an efficacy advantage over 2 mg. A decline of HDV RNA by more than 2 log at week 48 was achieved for 45% and 48% of patients treated with 2 and 10mg of Bulevirtide respectively, compared to the group control with only 2%.

Bulevirtide treatment was well tolerated without serious adverse events or treatment discontinuations. Bulevirtide was shown to interfere with the original function of the NTCP receptor, *i.e.* bile acid transport, only at a dose significantly higher than that required to inhibit HDV and HBV^{163,232}. However, interfering with the bile acid transporter NTCP, a transient increase of total bile acids was reported in all studies. The blocking of NTCP may also have metabolic effects.

On July 31, 2020, Bulevirtide was granted conditional marketing authorization for the treatment of chronic HDV infection under the trade name Hepcludex pending the results of ongoing clinical trials.. The optimal dose and duration of treatment have not yet been defined, but long-term treatment with 2 mg once daily may be considered²³⁸.

c- The international guidelines for the management of CHD and the development of new anti-HDV therapeutics

According to European Association for the Study of the Liver (EASL) clinical practice guideline on HDV, all patients with CHD should be considered for antiviral treatment on an individualized basis after careful evaluation²³⁸. Indeed, the choice of treatment is influenced by the stage of liver disease as well the phase of HBV infection, IFN α contraindication, tolerability and patient's will and compliance to treatment. Currently, the therapeutic approaches used in CHD to control the infection and disease rely on the use of PEG-IFN α and Bulevirtide in combination or monotherapy for patients with CHD and compensated liver disease (Figure 18). Currently, there are no approved treatments available for patients with decompensated cirrhosis related to CHD. They should undergo evaluation for liver transplantation. In cases where transplantation is not feasible, it is recommended to consider a best-supportive-care strategy. To prevent post-transplantation relapses, a combination therapy with a high genetic barrier NUCs and hepatitis B immunoglobulin (Ig-HBsAg) can be envisaged²³⁸.

For the assessment of clinical trials concerning new anti-HDV treatment, both the FDA and the EASL-AASLD have established primary endpoints, focusing on two major strategies: the maintenance treatment and finite treatment, aimed at controlling and curing the infection respectively. For the maintenance treatment, it is expected that the treatment will lead to a decline in HDV RNA levels by more than 2 log for EASL-AASLD, and ATL normalization for FDA. On the other hand, for finite treatment, undetectable HDV RNA levels and ATL normalization is expected for FDA, and, ideally, HBsAg loss in addition to the above endpoints for EASL-AASLD.

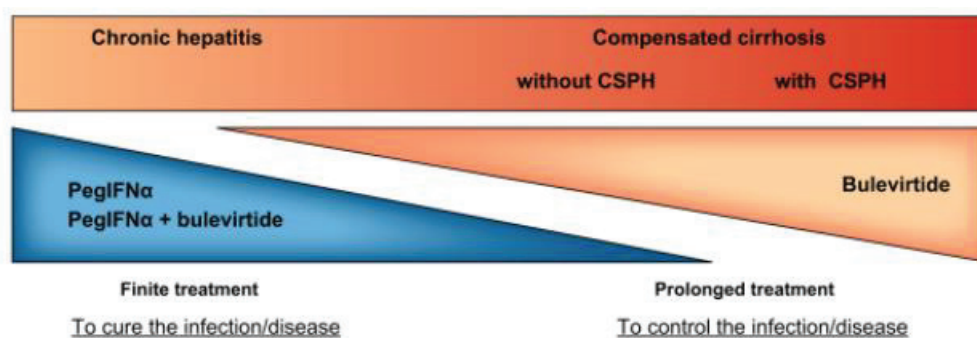


Figure 18: *The management of antiviral treatment for CHD. CSPH: clinically significant portal hypertension. From EASL clinical practice guideline on HDV, 2023.*

2- The new therapeutics in development

a- The inhibitor of farnesylation: lonafarnib

As previously described, farnesylation of L-HDAg is essential for the formation of HDV particles, enabling the recruitment of envelope proteins. In the absence of this post-translational modification, virions are not secreted. Targeting this key step in the HDV viral cycle, therefore, appears to be an interesting avenue for the development of new antiviral strategies to combat HDV. As with Bulevirtide, this therapeutic strategy is based on the progressive elimination of the virus by inhibiting its propagation to uninfected cells, combined with hepatocyte renewal and elimination of intracellular HDV RNAs by immune response. It, therefore, relies on the use of farnesylation inhibitors (FTIs). It should be noted that, unlike Bulevirtide, only HDV virus

is expected to be targeted. FTIs were initially tested in clinical phases as an antitumor treatment, but their use was abandoned for lack of efficacy^{239,240}. However, some FTIs, such as FTI-277 and FTI-2153, have shown an antiviral effect on HDV infection by inhibiting viral particle production *in vitro* and *vivo*^{241,242}. Currently, one of the FTIs, Lonafarnib, is undergoing clinical evaluation for the treatment of chronic hepatitis delta. A Phase IIa clinical trial involved 14 patients who were either treated as monotherapy with Lonafarnib 100mg or 200mg twice daily or with a placebo for 28 days²⁴³. A modest decrease in viral load of around 1 log was reported. In addition, a rebound effect was observed after treatment discontinuation. Side effects were also described. In other clinical trials, LOWR HDV-1 to -4, the efficacy and tolerability of Lonafarnib at different concentrations were studied in combination with Ritonavir and PEG-IFN α -2a^{244,245}. In triple therapy, Lonafarnib showed greater efficacy, where a synergy of the three treatments was observed. However, side effects were still observed. A Phase III clinical trial (D-LIVR) is currently underway.

b- The inhibitors of secretion : The nucleic acid polymers (NAPs)

Another therapeutic approach that could be used against HDV and HBV infection is to decrease HBsAg secretion and thus block the release of particles from the two viruses that share the same envelope. Nucleic acid polymers (NAPs) are phosphorothiolated oligonucleotides with amphipathic properties that can interact with the equally amphipathic α -helices of type 1 surface glycoproteins of a large number of infectious agents such as HIV or HCV. This interaction is not sequence-dependent, but rather length-dependent. NAPs are also known to concentrate in the liver.

Two studies showed that NAP REP-2055 exhibited antiviral activity at entry and post-entry stages *in vitro* and *in vivo* models infected with DHBV²⁴⁶⁻²⁴⁸. This inhibition of the virus did not appear to result from immunostimulation. In addition, there was a marked reduction in the amount of DHBsAg secreted into the blood of all treated ducks. However, the accumulation of

DHBsAg in the liver and the persistence of DHBV DNA in the serum suggested that REP-2055 blocked DHBsAg secretion. Preliminary open-label but non-randomized clinical trials were conducted in HBV-infected patients in Bangladesh. Administration of REP-2055 led to a 2-7 log reduction in HBsAg levels, as well as a reduction in HBV DNA levels in serum²⁴⁹. To ensure better tolerability, REP-2055 has been modified to obtain REP-2139.

Another preliminary clinical evaluation was conducted on 12 patients chronically infected with HDV²⁵⁰. After 15 weeks of treatment with REP-2139, followed by 15 weeks of dual therapy with PEG-IFN α -2a and 33 weeks of PEG-IFN α -2a monotherapy, a significant reduction in the amount of HBsAg secreted and in HDV viremia was observed.

c- The IFN lambda (IFN λ)

More broadly, the use of immunomodulators to restore the functions of innate and adaptive immunity is also being considered in the fight against HDV infection. Type III IFNs offer an interesting therapeutic alternative to IFN α , as they are generally better tolerated. It has been demonstrated that IFN λ can effectively inhibit HBV replication¹⁰⁵. IFN λ has been shown to display similar antiviral effects on HDV as IFN α both *in vitro* and *in vivo* in humanized mouse models²⁵¹. A Phase II clinical trial involving 14 patients chronically infected with HDV who were treated with either 120 μ g or 180 μ g per week by subcutaneous injection for 24 weeks²⁵². After 24 weeks of treatment, a marked reduction in HDV RNA was reported in 96% of patients, 42% of whom had an undetectable viral load. Moreover, after treatment discontinuation, a decrease of more than 2.3 log in HDV RNA was still observed²⁵³.

Further clinical trials are underway to assess the efficacy and tolerability of IFN λ monotherapy with different concentrations, as well as in combination. A phase III trial, named LIMT-2, is ongoing where patients are treated during 48 weeks with weekly 180 μ g of IFN λ ²⁵⁴.

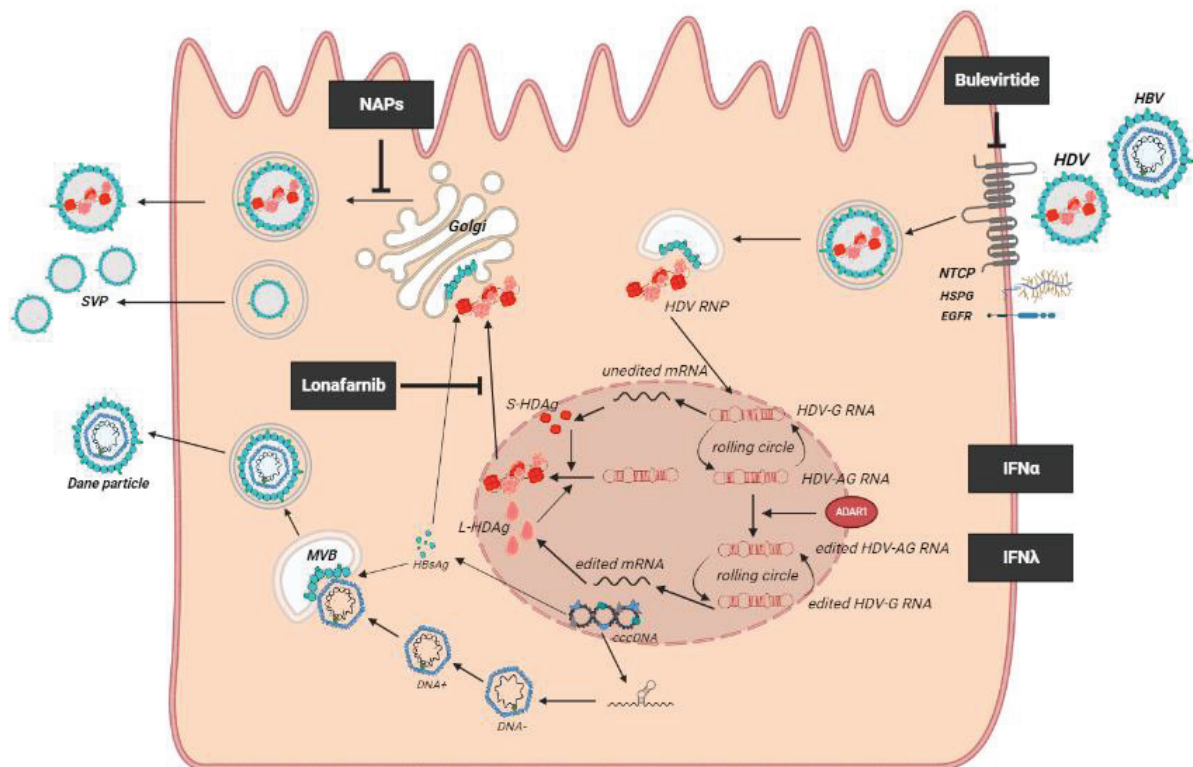


Figure 17 : *The therapeutic strategies deployed against HDV. Several therapeutic approaches have been developed to target the HDV life cycle, the entry inhibitors (Bulevirtide), the farnesylation inhibitors, the secretory inhibitors (NAPs), and the immunomodulators (IFN α and IFN λ). Adapted from²⁰.*

Part 3 : The bile acids metabolism and hepatropic viruses

I- The liver and its multiple functions

1- The liver organization

The liver, located in the upper abdomen, plays a crucial role in maintaining overall bodily functions. It consists of two main lobes, the right and left lobes, which are further divided into smaller segments. It is the most densely vascularized organ in the human body, with around 1.5 liters of blood flowing through it every minute in an adult. It receives blood from the hepatic artery and portal vein, which carry substances from the stomach, intestines, spleen, and pancreas. The liver is also crossed by a large number of bile ducts, which evacuate the bile secreted by the liver into the digestive tract.

The liver is composed of various cell types, with hepatocytes being the most abundant, accounting for 70% of the liver's cellular composition²⁵⁵. Hepatocytes play a pivotal role in numerous metabolic processes within the liver, including the detoxification of harmful substances. Alongside hepatocytes, the liver also houses other cell types, such as endothelial cells, biliary epithelial cells (cholangiocytes), Kupffer cells (liver-resident macrophages responsible for phagocytosis of foreign substances), stellate or Ito cells (involved in fat storage and fibrous tissue production in the event of inflammation), as well as dendritic cells and intrahepatic lymphocytes. This diverse cellular population collectively contributes to the liver's essential functions and overall hepatic homeostasis.

3- The liver functions

As an exocrine gland, it is responsible for the continuous production of bile, which is essential for the digestion and absorption of lipids and micronutrients. Bile is composed of bile acids, bilirubin, cholesterol and lecithin. It is stored in the gallbladder for release during fat digestion. Beyond its role in digestion, the liver is also involved in many physiological processes essential to the body.

The liver is known as a "metabolic hub", as it plays a crucial role in regulating macronutrient metabolism²⁵⁶. As far as lipids are concerned, the liver is involved in the synthesis of lipoproteins responsible for the blood transport of cholesterol, triglycerides, and phospholipids. It is also responsible for the production of cholesterol, which is essential for the synthesis of

bile acids, steroid hormones and vitamin D. The liver plays a role in carbohydrate metabolism. It is capable of producing, storing, metabolizing and releasing glucose according to the body's energy requirements. It is also essential in the metabolism of amino acids and proteins, particularly blood proteins such as albumin, globin and coagulation factors.

In addition to its metabolic functions, the liver acts as an organ of detoxification. By its organization, the liver filters blood from the gastrointestinal tract and the systemic circulation, via the portal vein and hepatic arteries respectively. It eliminates endogenous substances resulting from cellular metabolism, as well as exogenous or xenobiotic substances, such as toxins, drugs, or alcohol, which can be toxic to the body in high concentrations, and red blood cells. By draining portal blood from the digestive tract, the liver is continuously exposed to food antigens, bacterial products as well as possible pathogenic microorganisms circulating in the blood that should trigger a massive immune response²⁵⁷. However, this is not the case, as studies of allogeneic transplants in rats, mice, and pigs have shown²⁵⁸. These experiments revealed that, in some species, liver grafts were accepted and tolerated by the recipient organism, unlike other tissues from the same donor. The liver is the site of complex immune mechanisms whose purpose is to maintain a state of immune tolerance to food antigens, to avoid a general immune response to the slightest food antigen, while still being able to mount an effective response against pathogens. Despite the establishment of immunotolerance, liver transplanted human patients receive immunosuppressive treatment.

II- The hepatic metabolism and hepatotropic viruses

The liver is a target for many human pathogens, including viruses that can cause liver damage, such as cytomegalovirus (CMV), herpes simplex virus (HSV), and yellow fever virus. However, among these many viruses, the five alphabetic hepatitis viruses (A, B, C, Delta, and E) stand out for their truly almost exclusive hepatic tropism.

Due to evolutionary processes, these viruses have developed a reliance on hepatic metabolism for their replication. The hepatotropic tropism of these viruses seems to be determined more by the specific functional properties of the liver than by the expression of unique receptor molecules, with the exception of the entry receptor for HDV and HBV, which exhibits liver-restricted expression²⁵⁹. These functional properties include various physiological processes present in the liver, which provide viruses with a cellular environment conducive to replication. The liver plays a crucial role in providing a large amount of energy, as well as synthesizing the biomolecules required for viral replication.

For example, the hepatitis C virus (HCV) is closely linked to carbohydrate and lipid metabolism in liver cells as demonstrated by the occurrence of metabolic disorders associated with a chronic HCV infection. Individuals with chronic infection have an increased risk of developing insulin resistance and diabetes mellitus, with prevalence ranging from 10% to 30%. HCV infection interferes with glucose metabolism, specifically by reducing the phosphorylation of insulin receptor substrate 1 (IRS-1), a key molecule involved in insulin signaling, and by decreasing the expression of glucose transporter 2 (GLUT2)²⁶⁰. Moreover, the natural history of HCV infection is associated with dyslipidemia and the development of hepatic steatosis. Indeed, HCV interferes with the lipid metabolism and especially with the lipoproteins metabolism. It uses lipovirions, formed by viral envelope proteins E1 and E2, and nucleocapsid associated with low-density or very low-density cellular lipoproteins (LDLs or VLDLs), to circulate in the bloodstream and evade the immune system^{261,262}. HBV, in its case, has the ability to recruit transcription factors and coactivators to its genome that are commonly involved in the regulation of essential metabolic genes in the liver. Among these factors, there are liver-enriched nuclear receptors that play an active role in the regulation of hepatic genes. *In vitro* and *in vivo* studies have shown that the nuclear receptors, peroxisome proliferator-activated receptor alpha (PPAR α), hepatocyte nuclear factor 4 (HNF4 α), and farnesoid x receptor alpha (FXR α) interact with the viral genome. These nuclear receptors are above all pivotal regulators of various metabolic processes in the liver, including lipid, glucose and cholesterol metabolism. They bind to specific promoter and enhancer regions and have various effects on the expression of different viral genes. For example, the PPAR α / retinoid X receptor alpha (RXR α) complex binds to three specific sequences in the promoter regions of the Core gene, PreS1, and in the EnhI region, thereby regulating the expression of pgRNA and preC RNA. HNF4 α binds to the promoter region of the Core gene and the EnhI and EnhII regions, favoring the expression of pgRNA. Specific sequences for the FXR nuclear receptor have also been identified on the HBV viral genome. FXR binds either as a monomer or as a heterodimer with RXR α , to two sequences in the viral genome that resemble FXR receptor response elements. These elements are found in the Core gene promoter and the enhancer II region²⁶³. Several studies have demonstrated that the transcription and replication of HBV in non-hepatic cell lines such as 293T cells, can only occur upon the expression of activated PPAR4 α and HNF4 α ^{264,265}. This suggests that HBV uses these liver-specific transcription factors to hijack the host cellular machinery and ensure its own replication and gene expression. These *in vitro* observations are consistent with those made *in vivo*, where in transgenic mice, the transcription and replication of HBV are limited to certain organs such as the liver and kidneys,

likely due to the expression of certain of these transcription factors²⁶⁶. The dependence of HBV for this nuclear receptors et their expression restricted to the liver are elements that likely contribute to the limited tropism of HBV.

This unique interaction between HBV and host factors has led to HBV being referred to as a « metabolovirus »²⁶⁷. Indeed, it has been supposed that by mimicking hepatic metabolic genes, the virus could regulate its own gene expression and replication in a way that aligns with the host genes metabolism, potentially minimizing host responses and thus favoring its persistence as well as facilitating the use of host resources.

Among these metabolic pathways involved in HBV replication, the bile acid metabolism seems to play an important role, with NTCP playing a role in viral entry and FXR being involved in viral genome transcription. The subsequent sections of this manuscript will be dedicated exclusively to elucidating the intricate connection between the bile acid metabolism and hepatitis B and D viruses.

III- The bile acids metabolism and FXR

1- The discovery of FXR α and generalities

In 1995, FXR α (NR1H4) was discovered following the screening of a complementary DNA (cDNA) library from a mouse liver by two-hybrid. This study aimed to identify the interaction partners of the RXR α ²⁶⁸. This study identified two FXR α isoforms initially named RXR-Interacting Proteins 14 (RIP14) and 15 (RIP15), which were classified as orphan receptors. Expression of the two isoforms differed. RIP15 was ubiquitously expressed, while RIP14 was mainly expressed in the liver and kidney²⁶⁸. A few months after the discovery of the two isoforms in mice, a homologous sequence of RIP14 was identified in rats²⁶⁹. The same study revealed that farnesol and its metabolites act as ligands for this receptor. As a result, the receptor was renamed farnesoid X receptor alpha. Subsequent in-depth research into FXR α led to the identification of bile acids as the main endogenous ligands of FXR α ²⁷⁰⁻²⁷². This discovery has led to a better understanding of the role of FXR α in the regulation of bile acid homeostasis and has opened up new perspectives for the study of FXR α functions in other biological processes.

A second coding sequence for FXR and more specifically FXR β (NR1H5) has been identified²⁷³. However, FXR β is a pseudogene in humans and primates, *i.e.* it does not code for a functional protein, unlike in mice²⁷³. In humans, FXR α encodes four different isoforms resulting from the use of two different promoters and alternative splicing of the FXR α transcript

at exon 5: FXR α 1, 2, 3, and 4²⁷⁴. Their expression differs according to the cell or tissue type studied. In humans, FXR α 1 et FXR α 2 isoforms are preferentially expressed in the liver and adrenal glands while all isoforms are expressed in the small intestine. FXR α 3 et FXR α 4 are predominantly expressed in the colon and kidney. A recent study showed that FXR is also expressed in the brain. Although in this manuscript, the only hepatic role of FXR will be developed, it is important to note that FXR also plays significant extra-hepatic roles. This includes the regulation of adipose tissue where it promotes adipogenesis and enhances peripheral insulin sensitivity²⁷⁵. FXR is also involved in enteroprotection by preventing bacterial overgrowth in the intestine. Furthermore, it has a role in renal physiology by promoting water reabsorption and ensuring urine concentration²⁷⁶. Additionally, FXR activation has been implicated in cell survival under hyperosmotic stress. FXR has effect on the cardiovascular system, including the activation of platelets and atherosclerosis. The structure of FXR α

As a member of the nuclear receptor superfamily, FXR shares common structural features with other nuclear receptors. It comprises a central DNA-binding domain (DBD), close to the functional N-terminal ligand-independent activation domain (AF-1), and a C-terminal ligand-binding domain (LBD) encompassing the ligand-dependent activation domain (AF-2). The LBD and DBD are linked by a hinge region (*Figure 19*).

a- The domain A/B

The N-terminal A/B domain is highly variable and therefore not highly conserved between NRs, either in terms of length or sequence. It contains the AF-1 domain, which contributes to receptor activation independently of ligand binding. This AF-1 domain interacts with co-regulatory or co-repressor proteins and is subject to post-translational modifications such as phosphorylation, acetylation, and SUMOylation, which affect the receptor's transactivator potential. Multiple AF-1 domain isoforms exist for FXR via an alternative splicing process. FXR α 3 and FXR α 4 have an extended N-terminus compared with FXR α 1 and FXR α 2.

b- The domain C

The C domain, also known as the DBD, is a highly conserved 66-amino-acid region that binds the receptor to response elements in the regulatory regions of target genes. It consists of two α -helices, named H1 and H2 respectively, two adjacent zinc fingers, and a terminal -COOH motif. Each zinc finger contains four cysteine residues that string together a zinc ion for binding to DNA, and more specifically to the large groove of the DNA double helix. FXR binds to

specific DNA sequences called FXR response elements (FXREs). FXREs consist mainly of an inverted repeat (IR) of the AGGTCA consensus sequence or a related sequence separated by a base pair (IR-1). However, FXR can bind other response elements of various geometries, such as IR-0 (no nucleotides separating two consensus sequences), everted repeats (ER) separated by two or eight nucleotides (ER-2 or ER-8) as well as direct repeats (DR) separated by one, four or five nucleotides (DR-1, DR-4 or DR-5). A study using ChIP-seq technique characterized the genome-wide FXR binding in PHH identifying the most commonly sequence motifs presented in the **Figure 20**²⁷⁷. It has been demonstrated that FXR isoforms can bind different specific DNA sequences. All isoforms bind to IR-1 motif whereas only FXR α 2 and FXR α 4 bind to the ER-2 motif²⁷⁸.



Figure 20: *The motif analysis of the most commonly identified sequences motifs from the top 500 FXR binding sites in PHH using multiple EM for motif elicitation (MEME). The height of letter is directly proportional to their frequency. From ²⁷⁷.*

c- The domain D

The D domain is the hinge region providing a flexible link between the DBD and LBD domains. It also contains a NLS. FXR α 1 and FXR α 3 have a four-amino acid MYTG insert in this domain affecting their transcriptional activity on part of their target genes. The D domain is also thought to be involved in interaction with co-repressor proteins.

d- The domain E

The E domain consists of the ligand-binding domain, LBD, which is also a highly conserved region between NRs. LBD is composed of 12 α -helices (H1 to H12) organized in three parallel layers forming a helical α -sandwich encompassing a hydrophobic ligand-binding pocket (LBP). The E domain also contains the ligand-dependent AF-2. AF-2 is a region involved in the regulation of FXR transcriptional activity in response to specific ligand binding via the recruitment of co-activators or co-repressors.

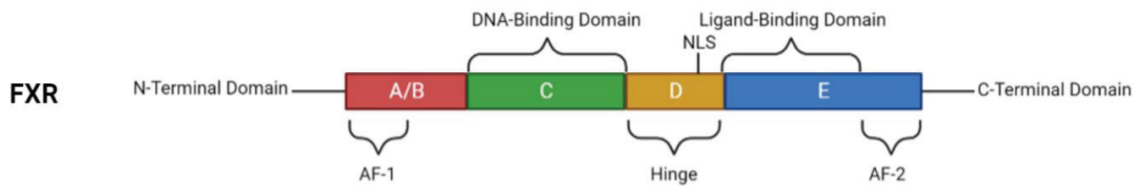


Figure 19: The schematic representation of farnesoid X receptor (FXR). A/B: domain A/B, C: domain C, D: domain D, E: domain E, *AF-1*: activation domain 1, *NLS*: nuclear localization signal, *AF-2*: activation domain 2.

2- The mode of action of FXR α

a- The genomic effects of FXR

FXR controls the expression of its target genes using both transactivation and transrepression mechanisms.

When activated, FXR can stimulate the expression of numerous target genes via a simple transactivation mechanism. Ligand binding to FXR, associated as a heterodimer with another nuclear receptor such as RXR, induces a conformational change in the entire FXR/RXR heterodimer (**Figure 21A**). On the one hand, this conformational change induces the release of the corepressor complex consisting, for example, of nuclear receptor corepressor 1 (NCOR1) or silencing mediator retinoic acid and thyroid hormone receptors (SMRT)^{279,280}. In the basal state, *i.e.* in the absence of ligand, corepressors are bound to FXR and inhibit its activity via the recruitment of histone deacetylase proteins, known as HDACs, which modify chromatin structure by condensing it, thereby inhibiting the recruitment of the transcription machinery. On the other hand, coactivators are recruited to support the release of corepressors. Numerous coactivators have been identified as interacting with FXR and playing a role in histone modification, such as the methyltransferase-active protein PRMT1 or the acetyltransferase-active proteins CBP/p300, chromatin remodeling, such as switch/sucrose non-fermentable (SWI/SNF) related matrix associated actin dependent regulator of chromatin subfamily A

member 4 (SMARCA4) of the SWI/SNF complex, and in the recruitment of other cofactors, such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 α) or steroid receptor coactivator 1 (SRC1), or in the recruitment of the transcriptional machinery²⁸¹⁻²⁸⁴. It has also been suggested that FXR regulates the expression of target genes via a monomeric transactivation mechanism where FXR binds as a monomer to DNA, as proposed for the expression of the glucose transporter type 4 (GLUT4), as well as via so-called “composite” transactivation, *i.e.* a mechanism that involves synergistic cooperation between FXR and other nuclear receptors or transcription factors such as cooperation between FXR and liver receptor homolog-1 (LRH-1) or FXR and HNF4 α ²⁸⁵⁻²⁸⁷.

FXR is not only a transcriptional activator once activated, it can also repress the expression of certain target genes. For example, FXR has been reported to repress the expression of ApoAI and apolipoprotein CIII (ApoCIII)^{288,289}. Several corepressors have been identified as being involved in FXR’s repressive activity, such as the NAD-dependent deacetylase sirtuin-1 (SIRT1), the nuclear receptor 0B1 (DAX1/NR0B1), the Ku80 and 70 proteins, the transcriptional corepressor SMRT and the nuclear receptor 1 corepressor NCOR1²⁹⁰⁻²⁹². However, the direct repression of gene expression by FXR is a rare event.

b- The non-genomic effects of FXR

The genomic effects of nuclear receptors are characterized by a complex series of events that require multiple intermediaries, resulting in a longer response time often taking several hours. On the other hand, some nuclear receptors exhibit non-genomic effects that occur rapidly, within minutes, and are not dependent on transcriptional activity or direct interactions with gene promoters. These non-genomic effects involve several mechanisms like the activation of secondary messengers and various signaling cascades. However, non-genomic effects are still less understood. An example of a non-genomic effect is the ability of FXR to enhance the phosphorylation of serine/threonine residues on protein kinase AKT²⁹³. Moreover, FXR could interact directly with proteins and regulate their activity without involving genomic transcription.

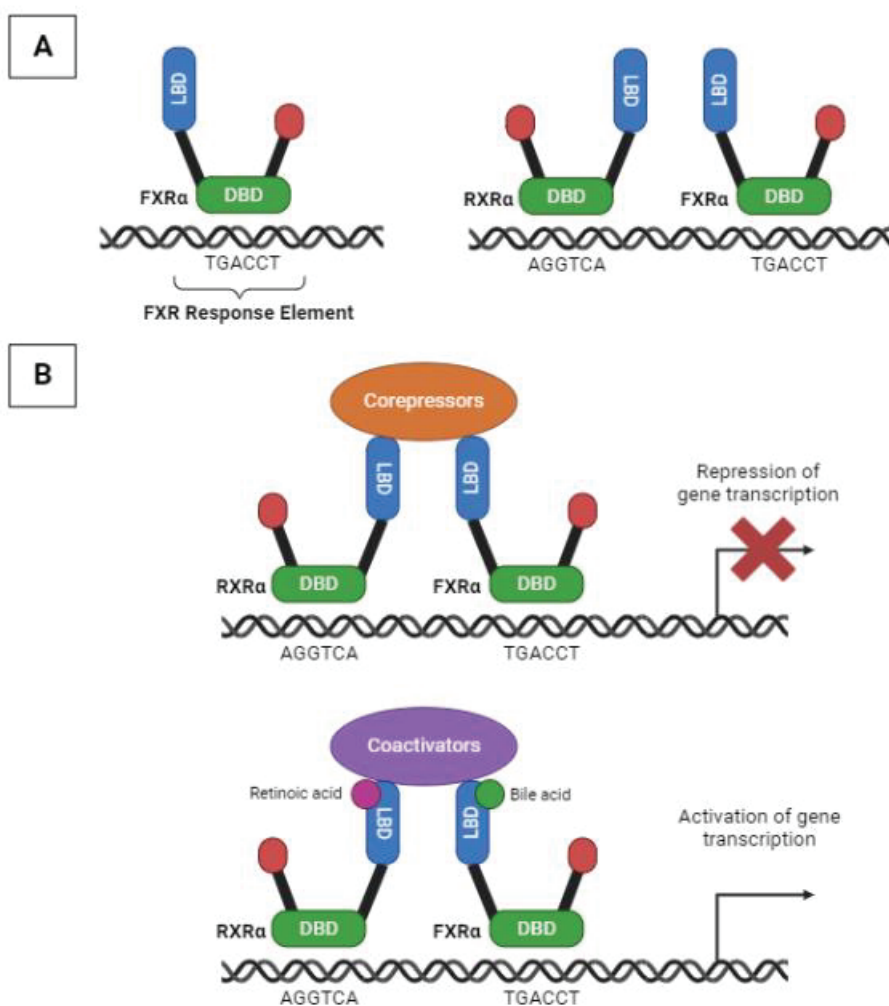


Figure 21 : The mode of actions of FXRα in the regulation of genes expression. (A) The different association of FXR on specific DNA sequences. LBD: ligand binding domain , DBD: DNA binding domain. (B) The schematic representations of genomic effects of FXR. LBD: ligand binding domain , DBD: DNA binding domain.

3- The pleiotropic functions of FXRα

a- The role of FXR in the regulation of bile acids metabolism

i. The definition and role of bile acids

Bile acids (BAs) are amphipathic molecules derived from the catabolism of cholesterol in the liver. BAs have a molecular structure composed of a steroidal core made up of 24 carbons forming 4 steroidal rings, including three 6-carbon rings (named A, B, and C respectively) and a 5-carbon ring (D), as well as a short 5-carbon side chain (**Figure 22**). This structure gives bile acids detergent properties, with a hydrophobic face and a hydrophilic phase.

BAs are the main components of bile, along with water, phospholipids, bilirubin, cholesterol, heavy metals, minerals, and vitamins. Thanks to their detergent properties, bile acids facilitate the absorption of lipids, fats, and fat-soluble vitamins (vitamins A, D, E, and K), as well as digestion.

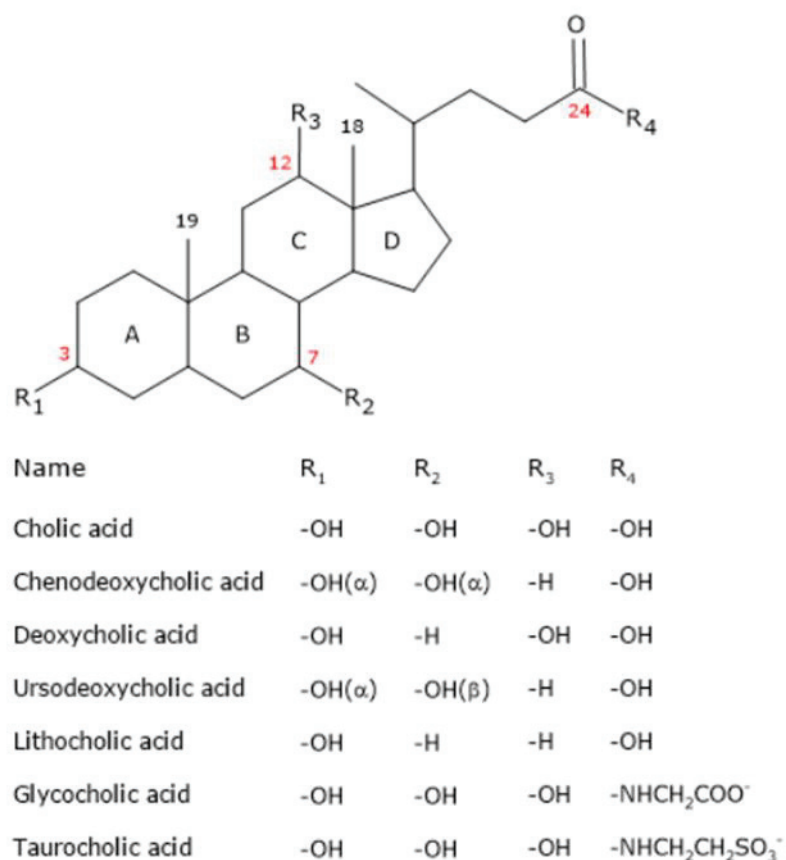


Figure 22: The schematic representation of general structure of bile acids. (From ²⁹⁸)

ii. *The synthesis of bile acids*

BAs are synthesized in hepatocytes through a series of specific enzymatic reactions involving approximately twenty enzymes distributed across different cellular compartments, such as the ER, peroxisomes, mitochondria, and cytoplasm. Two main pathways coexist in humans for bile acid synthesis: the classical (or neutral) pathway and the alternative (or acidic) pathway. In both pathways, the synthesis of bile acid involves four main steps: the initiation of synthesis, modification of the steroid core, oxidation and shortening of the side chain and

finally, conjugation with an amino acid²⁹⁴. The different steps of BAs synthesis presented in this part are summarized in *Figure 23*.

The initiation of synthesis in the classical pathway proceeds with the hydroxylation of cholesterol at the C-7 position by the enzyme cholesterol 7 α -hydroxylase (CYP7A1). In contrast, the alternative pathway starts with the hydroxylation of the cholesterol side chain at C-24, C-25 or C-27, catalyzed by specific enzymes like cholesterol 24-hydroxylase (CYP46A1), cholesterol 25-hydroxylase (CH25H), or sterol 24-hydroxylase (CYP27A1). The 7 α -hydroxycholesterol and the modified oxysterols from the alternative pathway then converge into the common pathway.

The next step involves the modification of the steroid core. The 7 α -hydroxyl derivatives of cholesterol are converted to 7 α -hydroxy-4-cholesten-3-one, known as C4, by the enzyme hydroxysteroid dehydrogenase 3B7 (HSD3B7). C4 is the precursor for both cholic acid (CA) and chenodeoxycholic acid (CDCA). At this stage, C4 can take two different routes: it can be converted to CA through 12 α -hydroxylation catalyzed by the enzyme sterol 12 α -hydroxylase (CYP8B1) or to CDCA without this modification.

Following the modification of the steroid core, the synthesis proceeds to the oxidation and shortening of the side chain. The intermediates undergo oxidation, and the last three carbons of the side chain are removed via reactions similar to the β -oxidation of fatty acids. This process is catalyzed by several enzymes, including bile acid CoA synthase (BACS). The side chain shortening leads to the formation of CA-CoA and CDCA-CoA.

The final step involved the conjugation with an amino acid, glycine, or taurine, depending on their availability. This conjugation is catalyzed by the enzyme amino acid N-acyltransferase (BAAT) and enhances the solubility of BAs, preventing their passive absorption and cleavage.

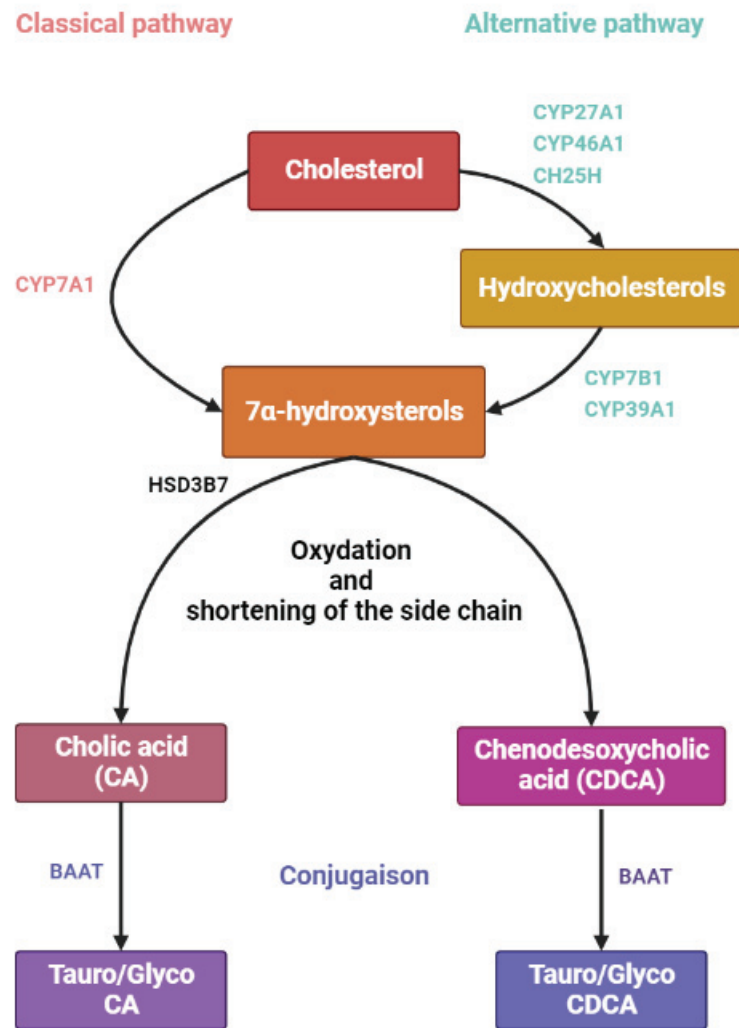


Figure 23: The schematic representation of primary bile acids synthesis in human.

- *The enterohepatic circulation of bile acids*

The enterohepatic circulation of bile acids corresponds to the cyclic circulation of BAs between the liver and the small intestine (**Figure 24**)²⁹⁴. It begins with the synthesis of bile acids in the liver from cholesterol, as described above. BAs are then secreted into the bile and stored in the gallbladder. Following a meal, some of the gallbladder's contents are discharged through the duodenal lumen into the ileum, where bile acids emulsify fats to facilitate digestion and absorption. Bile acids are then reabsorbed by the epithelial cells of the intestine, the enterocytes, and directed into the bloodstream, returning to the liver via the portal vein. BAs that escape reabsorption are converted into secondary bile acids, such as lithocholic acid (LCA) or deoxycholic acid (DCA) by the intestinal microbiota. Back in the liver, they are secreted into the bile to continue the cycle. During this cycle, around 5% of bile acids will not be reabsorbed and are excreted in the feces.

Enterohepatic circulation of conjugated bile acids requires the presence of active transporters²⁹⁴. Conjugated bile acids cannot diffuse passively, unlike unconjugated bile acids. The bile salt export pump (BSEP), expressed on the surface of hepatocytes, ensures the export of conjugated bile acids from their place of synthesis to the bile ducts leading to the gallbladder. Multidrug-resistance-associated protein 2 (MRP2) and multidrug-resistance 3 P-glycoprotein (MDR3) are also involved in the movement of bile acids to the gallbladder but also act as transporters for a wide range of substrates such as drugs, metabolites, hormones, and toxins. Bile acids are reabsorbed into enterocytes by apical sodium-dependent bile salt transporter (ASBT) and the heterodimer organic solute transporter α/β (OST α/β). The reabsorption of bile acids from the bloodstream to the liver involves the NTCP receptor, expressed on the basolateral surface of hepatocytes, as well as various members of the organic-anion-transporting polypeptide (OATP) family.

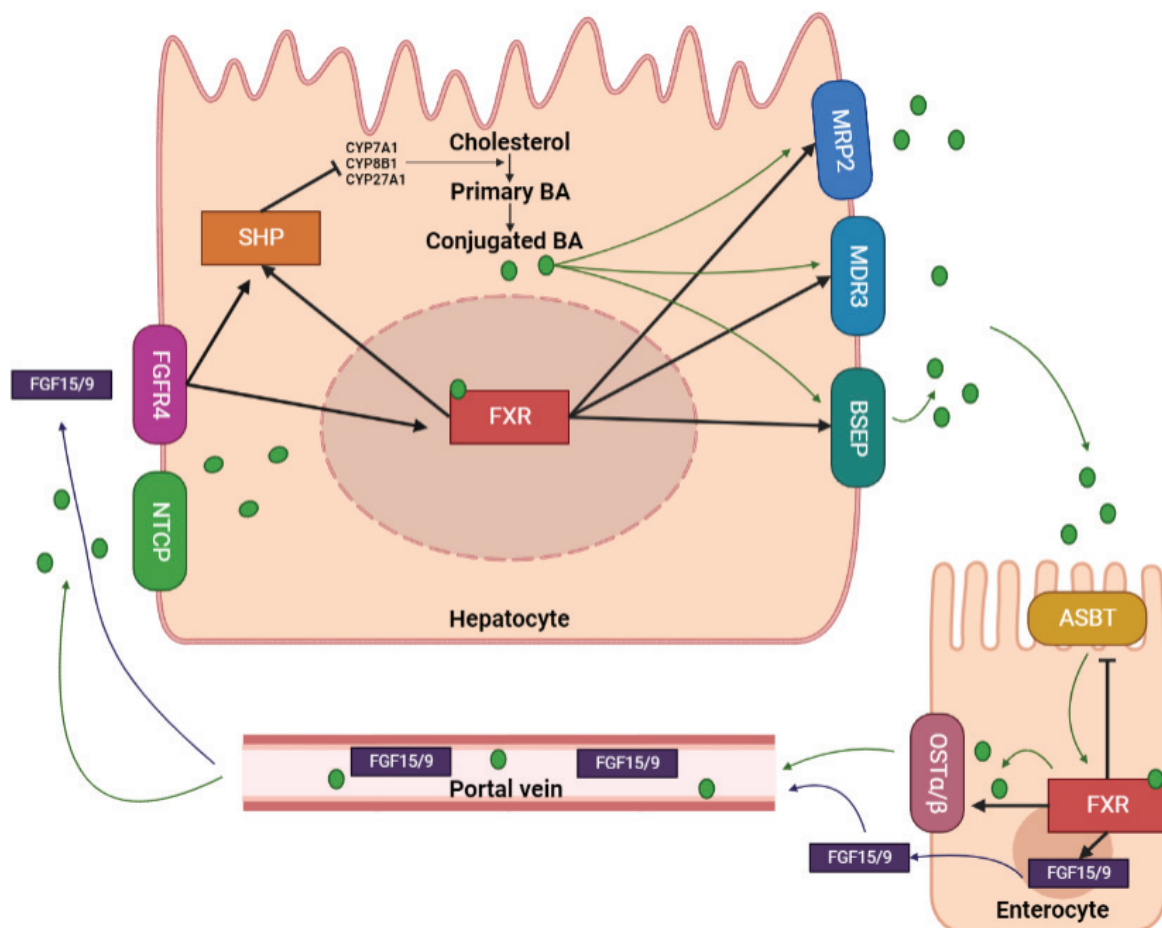


Figure 24: The enterohepatic circulation of bile acids is regulated by FXR. The green arrows represent the circulation of bile acids. The violet arrows represent the circulation of FGF15/9. BA: Bile acids.

- *The regulation of bile acids homeostasis by FXR*

FXR plays a major role in regulating the homeostasis of BAs, which in high concentrations are toxic, hence the need to maintain a constant pool of BAs. When bile acid concentrations rise in the liver and intestine, FXR is activated and regulates the expression of genes involved in bile acid synthesis and transport.

- iii. *The regulation of bile acids synthesis by FXR*

In the liver, FXR activation leads to decreased expression of the CYP7A1 and CYP8B1 enzymes involved in the synthesis of BAs from cholesterol (**Figure 24**). FXR induces expression of the orphan nuclear receptor small heterodimer partner (SHP), which acts as a transcriptional repressor, not by binding to DNA but by binding to other regulatory proteins to inhibit the expression of target genes. SHP inhibits the expression of LRH-1 and HNF4 α , two nuclear receptors, which in turn reduce the expression of CYP7A1 and CYP8B1^{295,296}. The hepatic effect of FXR in regulating bile acid synthesis in the liver is complemented by the action of FXR in the intestine. Activation of FXR in the intestine positively regulates the expression and secretion of the fibroblast growth factor 15 (FGF15) in mice and FGF19 in humans^{297,298}. Circulating from enterocytes to the liver via the portal vein, FGF15/19 acts endocrinally by binding to the membrane receptor fibroblast growth factor receptor 4 (FGFR4)²⁹⁹. Activation of FGFR4, in conjunction with β -Klotho protein, represses CYP7A1 and CYP8B1 expression via the extracellular signal-regulated kinases (ERK) and c-Jun-N-terminal kinases (c-Jun/JNK) pathways^{297,300}.

- iv. *The regulation of bile acids transport by FXR*

In the liver, FXR activation decreases bile acid import into hepatocytes by inhibiting NTCP expression via SHP-dependent mechanisms³⁰¹. SHP interferes with the transcriptional activity of RAR α /RXR α heterodimer and glucocorticoid receptor (GR) leading to the decrease of NTCP gene³⁰². Conversely, FXR activation stimulates the export of bile acids to bile ducts by directly increasing the expression of BSEP³⁰³. FXR also positively regulates MRP2 and MDR3 transcription^{304,305}. The presence of FXR helps prevent the toxic effects of bile acid accumulation in the liver.

In the intestine, the effect of FXR on ASBT expression is debated. Some studies have shown that in both humans and mice, a negative feedback control of ASBT expression was

observed after FXR activation, in contrast to rats, where no change in expression was observed^{306,307}. FXR positively regulates the expression of OST α/β to facilitate the export of bile acids to enterocytes.

b- The role of FXR in the regulation of lipid metabolism

In addition to bile acid homeostasis, FXR α is also involved in the regulation of lipid metabolism in the liver, and more specifically in the regulation of triglyceride, lipoprotein, and cholesterol metabolism (**Figure 25 and 26**). Indeed, an increase in plasma and hepatic triglyceride and cholesterol levels was observed in FXR α mice^{-/-308}.

i. The regulation of metabolism and transport of triglycerides

In the liver, triglycerides are synthesized from two main sources of fatty acids: exogenous fatty acids from other tissues and fatty acids produced by lipogenesis, the process of producing fatty acids from glucose. FXR reduces triglyceride synthesis, via a SHP-dependent mechanism, by inhibiting the expression of sterol regulatory element binding protein 1-c (SREBP-1c)³⁰⁹. SREBP-1c is a key regulator of lipogenesis enzymes, activating their expression. FXR is also involved in triglyceride transport via the regulation of lipoprotein metabolism, particularly very low-density lipoproteins (VLDLs), which transport triglycerides from the liver to peripheral tissues. Indeed, treatment of HepG2 liver cells with CDCA inhibited the expression of microsomal triglycerides transfer protein (MTP), which is involved in the assembly and secretion of VLDLs via the expression of SHP³¹⁰. FXR also reduces the expression of apolipoprotein B (ApoB), a major structural protein of lipoproteins. Triglyceride clearance is also controlled by FXR. FXR promotes the hydrolysis of triglycerides into fatty acids contained in VLDLs by inducing expression of apolipoprotein CII (ApoCII), which activates lipoprotein lipase (LPL), and inhibiting expression of ApoCIII^{289,311}. Fatty acids are released and captured by adipose tissue, depleting VLDL and becoming intermediate-density lipoproteins (IDL) and

then low-density lipoproteins (LDL)^{312,313}. In addition, FXR increases expression of the VLDL receptor (VLDLR), as well as syndecan-1, which plays a role in VLDL clearance^{314,315}.

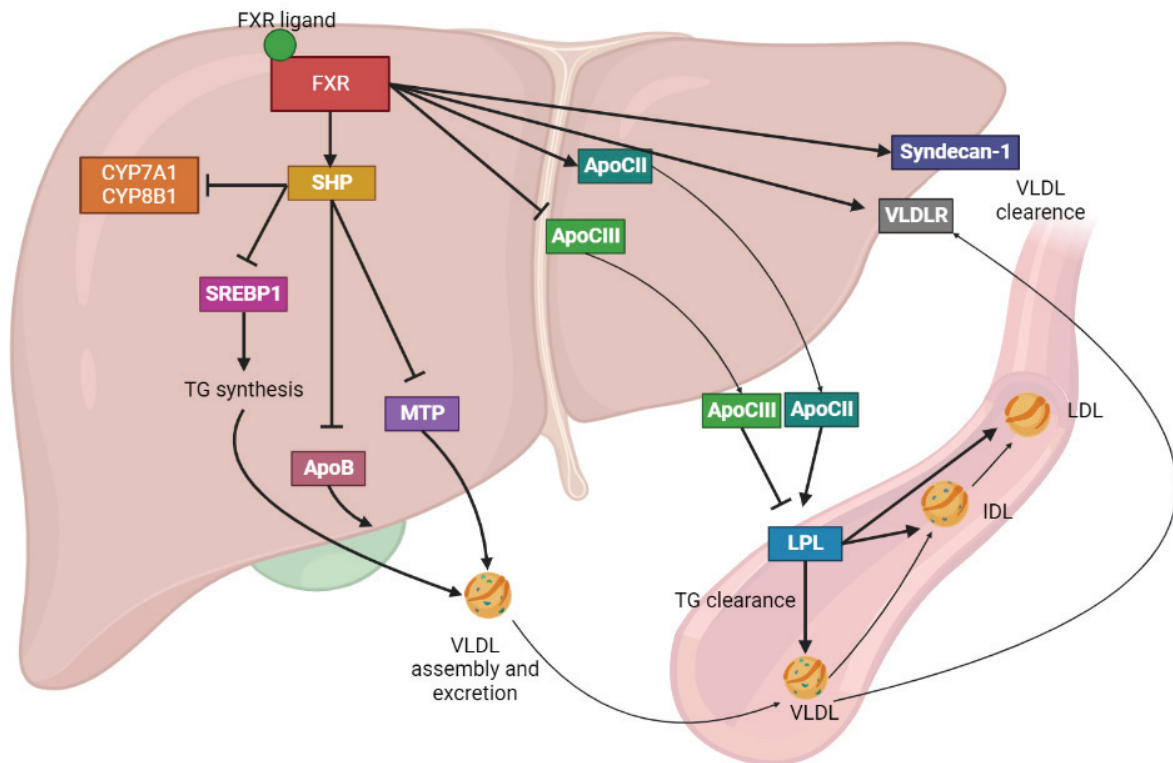


Figure 25: *The regulation of triglycerides synthesis and transport by FXR.* TG : triglycerides.

ii. *The regulation of the metabolism and transport of cholesterol*

As previously mentioned, FXR plays a major role in bile acid homeostasis, which is closely linked to that of cholesterol, since the latter is the precursor of the bile acid synthesis pathway. Cholesterol can be synthesized by the liver or derived from the food. It is transported from peripheral tissues to the liver via high-density lipoproteins (HDL), composed mainly of apolipoprotein AI (ApoI). FXR has been shown to inhibit ApoAI expression, either by binding directly to the promoter of this gene or via an SHP-dependent mechanism^{288,316}. Cholesterol is then transferred from HDLs to VLDLs, LDLs, and IDLs by the cholesterol-ester transfer protein (CETP) enzyme, and the reverse transfer is catalyzed by the phospholipid transfer protein (PLTP) enzyme. FXR has been shown to inhibit CETP expression but increase PLTP expression, reorganizing cholesterol exchanges between the different lipoproteins^{317,318}. Cholesterol-enriched LDLs bind to LDL receptors (LDLRs) expressed on the hepatocyte surface and are endocytosed, releasing cholesterol into the cell. Cholesterol accumulation induced by FXR inhibition of CYP7A1 expression leads to a decrease in LDLR expression,

thus reducing the uptake of cholesterol-rich HDL³¹⁹. Cholesterol can also be taken up directly by the liver via the scavenger receptor class B member 1 (SR-B1) receptor. The effect of FXR on SR-B1 expression is contradictory in mouse models. In one study, SR-B1 expression is stimulated after FXR activation, whereas in the second study, FXR represses SR-B1 expression^{320,321}.

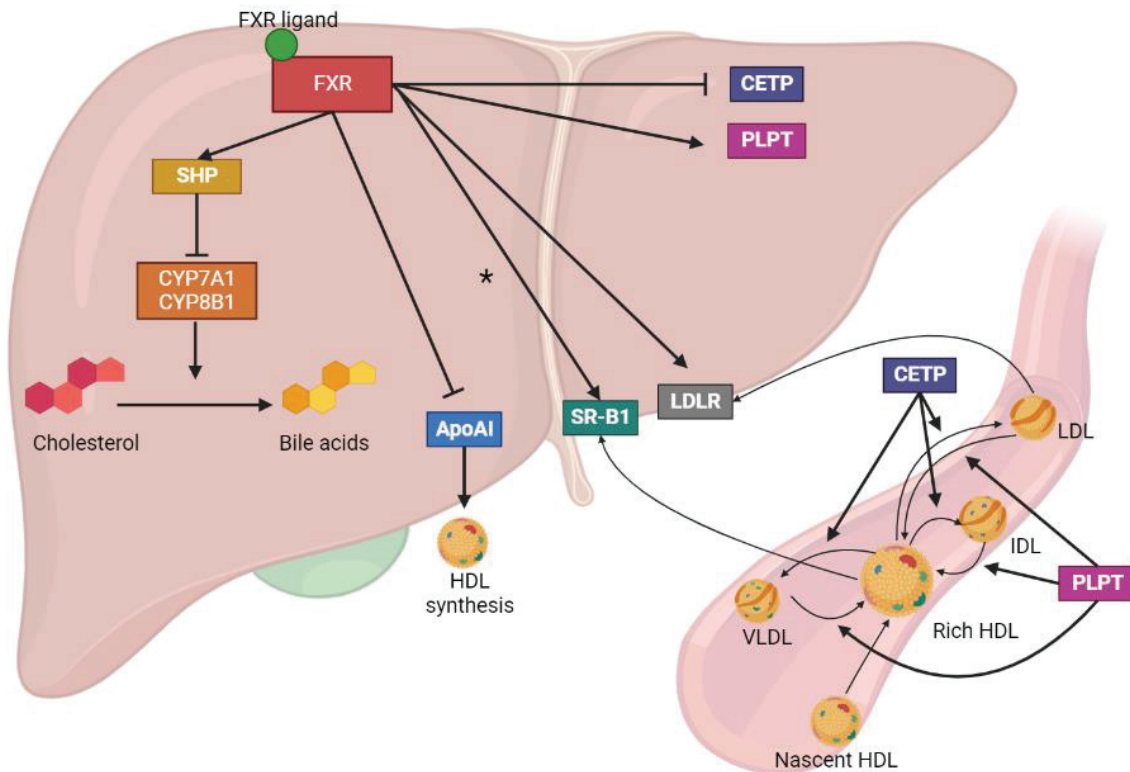


Figure 26 : *The regulation of cholesterol metabolism by FXR. *: The effect of FXR on SR-B1 is still debated.*

c- The role of FXRα in the regulation of glucose metabolism

The liver plays a major role in controlling blood glucose levels by modulating gluconeogenesis and glycogen synthesis (**Figure 27**). Several studies have established a correlation between bile acid metabolism, FXR, and glucose metabolism. In murine models of type I and type II diabetes, FXR expression was reduced but increased after treatment with insulin³²². This suggests that glucose and insulin regulate FXR expression. In addition, alterations in the bile acid profile were found in patients with type II diabetes, as well as in diabetic Wistar rats^{323,324}. In parallel, studies have shown that treatment with GW4064 or overexpression of the activated form of FXR led to a reduction in blood glucose levels in diabetic or wild-type mice, suggesting that FXR regulates blood glucose levels³²⁵. To achieve this, FXR inhibits the expression of genes involved in glycolysis, including the pyruvate kinase

L-type pyruvate kinase (LPK), by inhibiting the transcriptional activity of ChREBP and the recruitment of the SMRT corepressor, which binds to the LPK gene promoter *in vitro*^{322,326}. Some studies have also highlighted the role of FXR in the regulation of gluconeogenesis. Hepatic gluconeogenesis mobilizes several enzymes such as phosphoenolpyruvate-carboxykinase (PEPCK), fructose 1,6-bis phosphatase (FBP1), or glucose-6-phosphate (G6P). The role of FXR in the control of gluconeogenesis seems contradictory according to various studies but might be dependent on the physiological context. On the one hand, it has been shown that activation of FXR by CDCA induces a decrease in the expression of PEPCK, FBP1, and G6P, thus inhibiting gluconeogenesis in HepG2 cells or *in vivo* in mice^{327,328}. On the other hand, other studies showed that, conversely, activation of FXR by CDCA induces an increase in PEPCK expression *in vitro* in two hepatoma-derived cell lines, H-4-II-E and M1HC1, in primary rat hepatocytes and *in vivo* in mice³²⁹.

In addition, FXR^{-/-} mice have been shown to develop insulin resistance, suggesting that FXR also plays a role in regulating insulin sensitivity and activation of FXR by GW4064 in diabetic mice induced an increase in glycogen synthesis³²⁵. Indeed, FXR activation induces an increase in phosphorylated glycogen synthase kinase-3 β (GSK3 β) levels, which in turn increases glycogen synthase activity ensuring glycogen production.

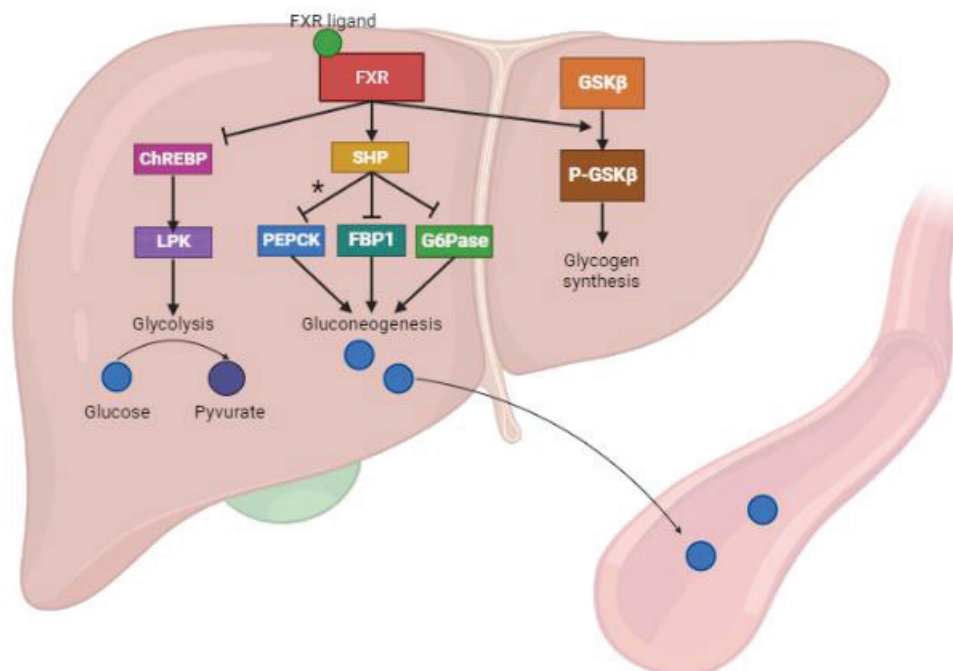


Figure 27: *The regulation of glucose metabolism by FXR. *: The effect of FXR on PEPCK depends on feeding or fasting conditions diets.*

d- The role of FXR in the cellular proliferation regulation

The FXR receptor plays a crucial role in liver protection and regeneration. A study carried out on FXR-deficient mice showed a reduced capacity for liver regeneration after partial liver removal (70% of the liver was removed), resulting in increased mortality in these animals³³⁰. In addition, FXR expression was observed to increase following the induction of liver damage, reinforcing the idea that FXR is involved in the liver regeneration process³³¹. Several studies have demonstrated that FXR stimulates liver cell proliferation. One of the mechanisms by which FXR exerts its effect is the induction of expression of the transcription factor forkhead box m1b (FOXM1b), a key regulator of the cell cycle³³².

e- The role of FXR in inflammation and immunity

Studies on FXR-deficient mice have shown that they develop liver damage, inflammation, and spontaneous tumors³³³. In addition, these mice showed elevated expression of genes associated with inflammation in the liver³³⁴. These observations suggest that FXR may have a direct role in modulating hepatic inflammation.

The NF- κ B signaling pathway plays an essential role in regulating the expression of genes involved in immune regulation and inflammation. The NF- κ B proteins, p50 (formed from the precursor p105), p52 (formed from the precursor p100), c-rel, p65 (RelA), and RelB, are transcriptional activators that act as dimers. The most common transactivator complex is the p50/p65 heterodimer. Studies have shown negative cross-talk between FXR and the NF- κ B signaling pathway. After treatment with FXR agonists, a decrease in the expression of genes under the control of the NF- κ B pathway, such as TNF α , cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS), was observed in HepG2 cells. FXR inhibits NF- κ B signaling, thereby reducing NF- κ B transcriptional activity by decreasing its DNA binding, as demonstrated by electrophoretic mobility shift assay (EMSA)³³⁵. Conversely, overexpression of p65 has been shown to inhibit GW4064-induced FXR activation³³⁵.

4- The ligands of FXR α

a- The natural ligands of FXR α

Bile acids are the major ligands of FXR α . They agonize FXR α with more or less variable efficacy in the following order: CDCA > DCA > LCA > CA²⁷⁰. Bile acids can function as signaling molecules to regulate their synthesis and affect various biological processes. However, bile acids are not exclusively FXR ligands. Some can also activate other nuclear

receptors such as pregnane X receptor (PXR), vitamin D receptor (VDR), as well as the G protein-coupled membrane receptor TGR5^{336,337}. Other molecules can also activate FXR α , such as androsterone, ethiocholanolone, and polyunsaturated fatty acids (EC₅₀ ranging from 0.9 to 4.7 μ M according to the polyunsaturated fatty acid considered)^{338,339}.

Some of these natural ligands antagonize the transcriptional activity of FXR by either destabilizing the receptor/coactivator complex or stabilizing the receptor/coreceptor complex, thereby inhibiting gene expression. Among them, some bile acids seems to have antagonist properties such as ursodeoxycholic acid (UDCA) (IC₅₀ >50 μ M) in humans as well as tauro- α MCA and tauro- β MCA (IC₅₀ 40 μ M) in rodents only which induce the decrease of FXR target genes expression such as SHP, GFG15/19, IBABP and OST β ³⁴⁰⁻³⁴².

b- The synthetic ligands of FXR α

The discovery of FXR as a key regulator in the control of metabolism makes it a therapeutic target of choice in some metabolic diseases. Because of the low selectivity and affinity of FXR α 's natural ligands for the latter, new molecules with greater affinity and specificity have been developed. GW4064 or 3-(2,6-dichlorophenyl)-4-(3'-carboxy-2-chlorostilben-4-yl)oxymethyl-5-isopropylisoxazole was the first synthetic agonist to be developed³⁴³. GW4064 has an EC₅₀ of 65nM. It is used as a reference molecule in the study of FXR α functions both *in vitro* and sometimes *in vivo* (**Figure 28**). Because of its relatively limited bioavailability, its use in clinical trials is limited. One study showed that GW4064 could also activate the estrogen-related receptor alpha (ERR α)³⁴⁴. Other ligands were therefore subsequently developed, two of which, 6-ECDCA and Tropicifexor, were used during this thesis project in addition to GW4064 (**Figure 28**). 6-ECDCA or 6 α -ethyl-chenodeoxycholic acid or obeticholic acid is a semi-synthetic FXR agonist developed in 2002³⁴⁵. Its structure is derived from that of primary bile acids and it has an EC₅₀ of 99nM. Tropicifexor, formerly known as LJN452, was developed by Novartis.³⁴⁶ It is a highly selective and potent agonist with an EC₅₀ of 0.2nM. Tropicifexor has been evaluated for its efficacy in patients with nonalcoholic steatohepatitis (NASH)³⁴⁶.

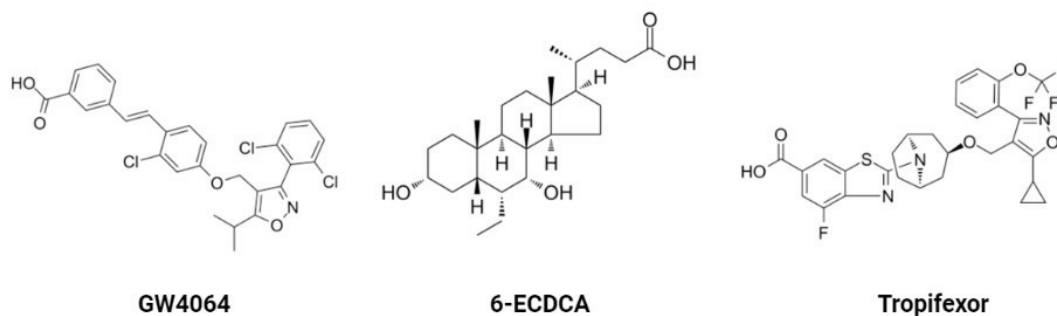


Figure28: The molecular structure of FXR agonists GW4064, 6-ECDCA and Tropifexor.

In contrast to the aforementioned FXR agonists, only few selective FXR antagonists have been characterized, despite the potential therapeutic of FXR as a target for metabolic disorders. Indeed, FXR antagonists could be used as therapeutic agent for hypercholesterolemia by inducing the expression of CYP7A1 reducing levels of serum cholesterol for example. Guggulsterone, a nonselective FXR ligand, is the first known antagonist of FXR (IC_{50} $4.1\mu M$)^{347,348}. It is derived from the gum resin of the *Commiphora mukul* tree and seems to act by reducing the activation of FXR through displacement of coactivator complexes. However, guggulsterone has been reported to exhibit both antagonist and agonist effects depending on the specific target gene³⁴⁹. Different selective antagonist have been developed as DY268 (IC_{50} $7.5nM$) and NDB^{350,351}. NDB has been shown to act as a specific antagonist of FXR by effectively inhibiting the interaction between FXR and RXR. This inhibition results in the downregulation of FXR target genes including SHP and BSEP³⁵⁰. Further research is needed to fully characterize and explore the potential therapeutic applications of FXR antagonists.

c- The current and potential clinical applications of FXRa ligands

FXR, discovered as a key regulator of hepatic metabolism, has emerged as a therapeutic target for various metabolic disorders. The development of bile acids and derivatives as potential drugs holds promise for the treatment of cholestatic liver diseases, gallstone disease, type 2 diabetes, obesity, and nonalcoholic fatty liver disease (NAFLD).

Cholestatic liver diseases are characterized by dysfunction in the normal formation and flow of bile. Primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are the most common chronic cholestatic liver diseases. The restoration of proper bile formation and/or enhancement of adaptative mechanisms to manage the overload of cholestatic bile acids are key therapeutic strategies. FXR agonists have emerged as promising anti-cholestatic agents.

The use of obeticholic acid (OCA) for the treatment of PBC was approved in 2016³⁵². It has anti-cholestatic, anti-inflammatory and anti-fibrotic effects.

NAFLD is the most prevalent chronic liver disease worldwide, characterized by the accumulation of lipids in hepatocytes, known as hepatic steatosis. NAFLD can be further classified into two subtypes, nonalcoholic fatty liver (NAFL) and NASH. NASH is distinguished by the presence of steatosis, lobular inflammation and hepatocyte ballooning, with or without hepatic fibrosis. NASH poses a significant risk for disease progression, leading to fibrosis, cirrhosis, and an increased likelihood of developing hepatocellular carcinoma. The complex pathophysiology of NASH makes its treatment challenging, with no approved drugs specifically targeting the disease. However, in recent years, FXR agonists have emerged as promising therapeutic agents for NASH. Obeticholic acid (OCA), an FXR agonist, has shown efficacy in improving NASH scores and reducing fibrosis in clinical trials, particularly in patients with PBC³⁵³. However, OCA is associated with side effects such as pruritus and dyslipidemia^{354,355}. Other FXR agonists, such as cilofexor, EDP-305, MET409, and tropifexor, are currently being evaluated in clinical studies, demonstrating potential for reducing hepatic steatosis^{356–358}.

Several potential therapeutic targets have been identified for various liver-related conditions, including, portal hypertension, alcoholic steatohepatitis, hepatic encephalopathy, diarrhea caused by primary bile acid malabsorption, kidney injury, and primary liver cancer.

IV- The bile acids metabolism and HBV infection

The discovery of NTCP as an entry receptor for HBV and HDV has emphasized analyzing the involvement of bile acid metabolism in HBV infection.

1- The effect of HBV infection on bile acids metabolism

HBV infection has been shown to alter bile acid and cholesterol metabolism^{359,360}. This is reflected in increased serum and hepatic bile acid levels. A study carried out on humanized mice showed an intrahepatic increase in CYP7A1 expression (by 12-fold induction), NTCP (by 1.4-fold), and a decrease in SHP expression (by 0.6-fold)³⁶¹. Similar observations have been reported in chronically HBV-infected patients. An increase in FXR expression (by 1.8 log) was also reported in humanized mice and liver biopsies from HBV-infected patients³⁶¹. During HBV infection, FXR activity appears to be maintained in a state of low activity, allowing the

expression of genes that are normally repressed. This repression of FXR activity, caused by an intracellular decrease in its natural ligands, may be explained by the existence of competition between HBV and bile acids for NTCP^{362,363}.

2- The FXR ligands exhibit antiviral effects on HBV

FXR α has been shown to bind, as a monomer or heterodimer with RXR α , to two FXREs present on the HBV viral genome²⁶³. These FXREs are located in the Core promoter and the other in the enhancer II region. Furthermore, experiments have demonstrated that ectopic expression of the FXR α /RXR α heterodimer, in non-hepatic cell lines, is capable of inducing HBV replication, suggesting that FXR α has a proviral role in the context of HBV^{263,364}. These observations are reinforced by results showing that silencing FXR expression leads to a reduction in pool size of cccDNA and secretion of HBsAg and HBeAg^{365,366}.

This proviral effect of FXR α on HBV is abolished in the presence of FXR ligands³⁶⁵. Indeed, FXR α agonists GW4064 and 6-ECDC are potent inhibitors of HBV replication *in vitro*, in dHepaRG and PHHs. This results in reduced transcription of all viral RNAs, specifically pgRNA and preC RNA by around 88%, protein translation, and secretion of HBV particles by around 90% in HepaRG cells. A decrease in cccDNA was observed only after early treatment, suggesting that FXR α may play a role in cccDNA establishment. The mechanisms underlying the antiviral effect of FXR ligands on HBV have not yet been identified but may be explained in part by the retrocontrol of FXR α expression by its ligands. This inhibition is also observed *in vivo* in C3H/HeN adult mice infected by a recombinant AAV2/8-HBV virus³⁶⁵. No effect of FXR antagonists on HBV has been reported³⁶⁶.

3- The FXR α ligands as potential therapeutic strategies against HBV infection

Because of the *in vitro* and *in vivo* results, targeting FXR α appeared to be a therapeutic target of choice. A highly specific FXR agonist, Vofanexor (EYP001), has been developed and is currently in Phase II clinical development for the treatment of chronic hepatitis B. Preliminary results from phase Ib clinical trial have demonstrated that Vofanexor, when administered as monotherapy or in combination with PEG-IFN α , is safe and exhibits potential antiviral effects. Indeed, after 4 weeks of Vofanexor monotherapy of 400 mg once daily, a modest decrease in HBsAg levels has been observed (0.10 log IU/mL), whereas no change was seen with entecavir and 100 mg and 200 mg of Vofanexor³⁶⁷. In combination with PEG-IFN α , Vofanexor induced a decrease of HBsAg by 0.17 log IU/mL and 0.12 log IU/mL with 150 mg and 300 mg of Vofanexor respectively. Besides, a decrease of HBV DNA by around 1.80 log

IU/mL associated with a decline of pgRNA and HBcAg levels were observed. Vonafexor need to be further studied in combination with PEG-IFN α over an extended duration of time.

Part 5 : Research project

I- Hypothesis and aims

As HBV, HDV tropism is limited to the liver, a key organ regulating important metabolic pathways. Bearing the same envelope, HDV and HBV share also the same entry receptor, which is NTCP. NTCP is a bile acid transporter expressed at the surface of hepatocytes involved in the enterohepatic circulation of bile acids as it ensures the uptake of bile acids into hepatocytes. NTCP is the first link between HBV and HDV with the bile acids metabolism. Therefore, it was investigated if other(s) link(s) existed, with, for example, the important regulator of bile acid metabolism, FXR.

In a previous study, the team investigated the involvement of FXR in HBV infection. It was discovered that FXR interacts with the HBV genome and acts as a proviral factor for HBV, promoting HBV replication. Additionally, it was found that FXR ligands exhibit strong inhibitory effects on HBV. However, apart from the role of NTCP in HDV entry, the potential impact of bile acids metabolism on HDV infection has not been yet explored.

The objectives of this doctoral project are to further **investigate the effects of FXR ligands inhibitors on HDV infection and the underlying mechanisms.**

II- *In vitro* models used in this study

1- *Primary human hepatocytes (PHH)*

Primary human hepatocytes (PHH) are considered as the gold standard model for physiologically relevant *in vitro* liver studies due to their ability to main *in vivo*-like functions and morphology (**Figure 29**). They provide a suitable platform for investigating hepatic metabolism, drug toxicity, and xenobiotic effects *in vitro*³⁶⁸. PHH are an ideal model for studying HBV and HDV as they can be naturally infected by both viruses, allowing the establishing of an infection system³⁶⁹. These cells are obtained from liver resections of patients

undergoing surgery to remove liver tumors mostly. However, there is a significant variability in the susceptibility to viral infection among different donors due to genetic diversity and different metabolic backgrounds³⁷⁰. Additionally, the isolation procedures and technical limitations associated with PHH culture present challenges, as the cells require specific growth conditions and have limited life duration, making their isolation and long-term culture a complex task. Due to aforementioned limitations and the low availability of PHH, alternative *in vitro* models have been sought.

2- Differentiated HepaRG cells (dHepaRG)

Hepatocyte cell lines commonly used, derived from tumor cells or obtained through oncogenic transformation, lack many liver-specific functions and are therefore not suitable for accurately mimicking *in vivo* liver cells in contrast with HepaRG cells. These cells, first discovered in 2002, are derived from liver tumor tissues of a female with HCV infection and secondary liver cancer (but do not express HCV). HepaRG cells exhibit bipotent characteristics, capable of differentiating into mature hepatocyte-like cells and cholangiocytes(**Figure 29**)³⁷¹. To do this, a differentiation protocol involving a 15-day proliferation phase following by a 15-day differentiation phase in the presence of DMSO has been established³⁷². The hepatocyte-like cells constitute approximately 50-55% of total cell population. Differentiated HepaRG (dHepaRG) cells express key enzymes involved in hepatic metabolism as well as nuclear receptors (FXR, PPAR α , HNF4 α , PXR and CAR). Moreover, dHepaRG cells display immunocompetence by expressing innate immune receptors and are fully functional for the IFN and NK- κ B pathways³⁷³. The differentiation of HepaRG cells into hepatocyte-like cells makes them susceptible to infection by providing access to the basolateral HBV and HDV-specific receptor, NTCP³⁷⁴. These cells are capable of supporting the complete life cycle of HBV (including the formation of cccDNA) and HDV¹⁴⁵. However, despite attempts to optimize the

culture conditions, the efficiency of virus infection in dHepaRG cells remains relatively low, at approximately 10-15%.

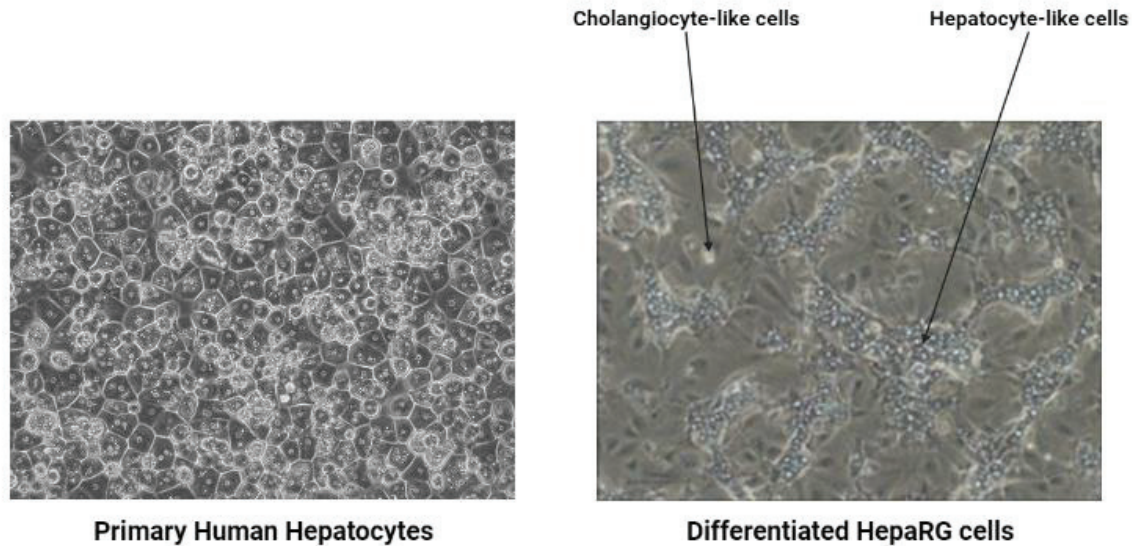


Figure 29: The representative images of the two in vitro models employed in this research study are presented above.

3- HuH7.5-NTCP

HuH7.5-NTCP cell line is derived from HuH7 cells line, a is hepatocyte-derived carcinoma cell line originally isolated from a Japanese male in 1982. Compared to HuH7 cells, HuH7.5 cells carry a missense mutation in the *RIG-I* gene (also known *DDX58* gene), making them highly permissive to HCV infection. However, HuH7.5 cells are not naturally susceptible to HBV and HDV infection. To enable HBV and HDV infection, these cells were stably transfected with NTCP (called HuH7.5-NTCP cell line), which allows them to support infection by HBV and HDV. In this research project, HuH7.5-NTCP cells were exclusively used for infectivity experiments due to differences in the regulation of FXR expression by its ligands compared to dHepaRG cells and PHH.

III- Results

1- The research article

ORIGINAL ARTICLE

OPEN

Farnesoid X receptor alpha ligands inhibit HDV *in vitro* replication and virion infectivity

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Abstract

Background and Aims: HDV, a satellite of HBV, is responsible for the most severe form of human viral hepatitis, for which curative therapy is still awaited. Both HBV and HDV use the hepatic transporter of bile acids (ie, Na⁺-taurocholate cotransporting polypeptide) to enter hepatocytes. We have previously shown that ligands of the farnesoid-X-receptor alpha (FXR), a master regulator of bile acids metabolism, inhibit HBV replication. Here we asked whether FXR ligands can also control HDV infection.

Approach and Results: *In vitro* HDV mono-infections or HDV/HBV coinfections and superinfections were performed in differentiated HepaRG cells (dHepaRG) and primary human hepatocytes. Following treatment with FXR ligands, HDV RNAs and antigens were analyzed by RT-qPCR, northern blot, immunofluorescence, and western blot. Virus secretion was studied by RNA quantification in supernatants, and the infectivity of secreted HDV particles was measured by reinfection of naive HuH7.5-Na⁺-taurocholate cotransporting polypeptide cells. In HDV/HBV superinfection models, a 10-day treatment with FXR ligand GW4064 decreased intracellular HDV RNAs by 60% and 40% in dHepaRG cells and primary human hepatocytes, respectively. Both HDV genomic and antigenomic RNAs were affected by treatment, which also reduced the amount of intracellular delta antigen. This antiviral effect was also observed in HDV mono-infected dHepaRG cells, abolished by FXR loss of function, and reproduced with other FXR ligands. In

Abbreviations: BA, bile acids; BSEP, bile salt export pump; CYP7A1, cytochrome P450 family 7 subfamily A member 1; dHepaRG, differentiated HepaRG cells; FXR, farnesoid X receptor alpha; HDAg-L, large hepatitis delta antigen; HDAg-S, small hepatitis delta antigen; NR, nuclear receptor; NTCP, Na⁺-taurocholate cotransporting polypeptide; Peg-IFN- α , pegylated interferon alpha; PHH, primary human hepatocyte; SoC, standard of care; SVR, sustained virologic response; TR, tetracycline repressor; vge, viral genome equivalent; 6-ECDCA, 6 α -ethylchenodeoxycholic acid.

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David Durantel and Patrice André contributed equally to this work.

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HBV/HDV coinfecting dHepaRG cells, HDV secretion was decreased by 60% and virion-specific infectivity by > 95%.

Conclusions: FXR ligands both inhibit directly (ie, independently of anti-HBV activity) and indirectly (ie, dependently of anti-HBV activity) the replication, secretion, and infectivity of HDV. The overall anti-HDV activity was superior to that obtained with interferon- α , highlighting the therapeutic potential of FXR ligands in HDV-infected patients.

INTRODUCTION

HDV is a defective and satellite virus of HBV, requiring HBsAg for its propagation. HDV coinfection or superinfection in HBV carriers is characterized by a more rapid progression to liver fibrosis and an increased risk of end-stage and lethal liver diseases.^[1,2] The exact prevalence of HDV infection is unknown. According to recent meta-analyses, it is estimated that HDV infects between 12 and 60 million people worldwide, which corresponds to 4.5%–13% of HBV-infected patients, with significant geographic variations of prevalence.^[1,3,4]

Current therapies relying mainly on pegylated interferon-alpha (Peg-IFN- α) are unsatisfactory, as a sustained virologic response (SVR) following cessation of treatment is only obtained in a very limited number of patients.^[5,6] Recently, the HDV entry inhibitor Buleviride (also widely known as Myrcludex) was approved in the European Union for the treatment of HDV patients with compensated liver disease, but optimal treatment duration and SVR remain to be determined.^[7] A few investigational antiviral approaches are currently being tested, but it is widely admitted that novel specific antiviral strategies will be necessary to foster an HDV cure.

Replication of the HDV genome, a 1.7 kb single-stranded negative-sense circular RNA, takes place in the nucleus of infected hepatocytes. Antigenomic RNAs are synthesized by a rolling circle mechanism and serve as templates for the synthesis of new genomic RNAs. Viral mRNAs are also synthesized and encode the small and large delta antigens (HDAg-S and HDAg-L) from a unique open reading frame.^[8] Compared with HDAg-S (195 amino-acid long), HDAg-L contains an additional domain of 19–20 AA at its C-terminus, which results from an adenosine deaminase RNA-specific 1-mediated RNA editing of the antigenomic HDV RNA at a location corresponding to the stop codon of the HDAg-S gene.^[9] This additional domain contains a CXXX-box motif, allowing the addition of a farnesyl group to the cysteine by cellular farnesyltransferase activity. While HDAg-S is essential to HDV replication, farnesylated HDAg-L inhibits the replication step but favors virus egress.^[10,11] HDV assembly is initiated by the interaction between HDAg-L and newly synthesized

genomic RNAs. These neo-formed ribonucleoproteins, which also contain HDAg-S moieties,^[12] are then exported from the nucleus to bud into HBsAg empty subviral particles (ie, devoid of HBV nucleocapsids), supposedly at the endoplasmic reticulum, to generate HDV infectious particles.^[13] Aside from the crucial role of HBV envelope proteins in the production of infectious HDV particles, the other steps of the HDV life cycle are not dependent on HBV.

Bearing HBV envelope proteins at their surface, HDV infectious virions use the same entry receptor as HBV to enter hepatocytes, that is the sodium taurocholate cotransporting polypeptide (NTCP).^[14] NTCP is the main transporter of bile acids (BA) at the basolateral membrane of hepatocytes and several studies have suggested that HBsAg-containing particles and BAs compete for binding to NTCP. Indeed, it has been shown that high concentrations of taurocholate inhibit both HBV and HDV entry into differentiated HepaRG cells (dHepaRG). Inversely, binding of the HBsAg preS1 domain to NTCP blocks NTCP-mediated BA uptake in HepG2-NTCP cells.^[15,16] Even though HDV uses NTCP to enter hepatocytes, the link between BA metabolism and HDV life cycle remains to be explored. BA metabolism homeostasis is maintained by a complex interplay between the liver and the gut, in which the farnesoid-X-receptor alpha (FXR), the liver-enriched nuclear receptor (NR) of BA, plays a key role by directly or indirectly regulating the transcription of numerous genes. FXR activation in response to increased intracellular BA concentrations leads to its downregulation (through a negative feedback loop), a decreased BA entry into enterocytes and hepatocytes, as well as an increased BA excretion into bile ducts through the regulation of expression of several BA transporters (eg, the bile salt export pump, BSEP). In addition, synthesis of primary BA from cholesterol is inhibited in the liver, in particular following repression of cytochrome P450 Family 7 Subfamily A Member 1 (CYP7A1) enzyme expression.^[17]

In our previous work, we have shown that FXR can bind to 2 FXR response elements on the HBV genome.^[18] Other studies have shown that HBV infection modified the expression of FXR and its target genes in the humanized mouse model and liver

biopsies from chronically infected patients.^[19] Importantly, we and others have shown that some FXR agonists inhibit HBV replication *in vitro*.^[20,21] This led to the clinical evaluation, in monotherapy or combination with standards of care (SoC), of the FXR agonist Vonafexor, as a potential anti-HBV asset.^[22]

Here, we address the question of whether FXR and BA metabolism could also play a role in the HDV life cycle, and whether this could be of therapeutic interest. Using relevant cell culture models [primary human hepatocytes (PHH) and dHepaRG cells], we showed that treatment with FXR ligands leads to a moderate inhibition of HDV intracellular replication; irrespective of the HBV presence, there is a strong decrease of HDV virion secretion, and a very potent reduction of their specific infectivity. These data open perspectives for the treatment of hepatitis delta with FXR ligands.

METHODS

Plasmids and viruses

HDV-1 replication-competent plasmid (pSVLD3) and HBV envelope protein (genotype D) encoding the pT7HB2.7 plasmid were kind gifts from Camille Sureau (INTS). HDV viral stocks were obtained by transfection of HuH7 cells with plasmids pSVLD3 and pT7HB2.7, as previously described.^[23] HBV viral stocks were produced from HepAD38 cells as described before.^[24]

Chemicals

FXR agonist GW4064 was purchased from Sigma-Aldrich, 6 α -ethylchenodeoxycholic acid (6-ECDCA) from MedChemExpress, and tropifexor from ProbeChem.

Interferon-alpha (IFN- α)-2a was purchased from PBL Assay Science and lamivudine from Selleckchem.

Cell culture and infections

HepaRG cells were cultured, differentiated, and infected by HBV and HDV as described.^[25,26]

PHH were freshly prepared from a human liver resection obtained from the Centre Léon Bérard (Lyon) with French ministerial authorizations (AC 2013-1871, DC 2013-1870, AFNOR NF 96 900 September 2011) as described.^[27]

A polyclonal HepaRG-TR-Cas9 cell line was generated by dual lentiviral transduction (T-Rex system from Invitrogen/Thermo Fischer) leading to the stable chromosomal integration of 2 transgenic expression cassettes, one constitutively coding the tetracycline repressor protein (TR) and another coding upon tetracycline induction (2 binding sequences for TR in

the promoter/+1 transcription region) the *Streptococcus pyogenes* Cas9 protein.

HuH7.5 cells were kindly provided by C.M. Rice (Rockefeller University). The derived HuH7.5-NTCP cells were generated by lentiviral transduction.^[28]

Analysis of specific infectivity and density of secreted HDV particles

Supernatants from dHepaRG infected with both HBV and HDV were concentrated using 8% PEG 8000. HDV RNA was quantified by RT-qPCR in concentrates and HuH7.5-NTCP cells were infected using the same viral genome equivalents for each condition of treatment. At indicated times, total cellular RNA was extracted, and HDV RNA was quantified by RT-qPCR.

In parallel, concentrated viruses were characterized by analysis of fractions from 20% to 44% iodixanol gradients. Twelve fractions of 1 mL were collected and used for the quantification of HDV RNA and HBV DNA by qPCR, HBsAg dosage by ELISA, and HDAg and HBsAg detection by western blot.

Methods for nucleic acid quantification, small interfering RNA transfection, HBs and HBe quantification by ELISA, and western blot and immunofluorescence analyses are described in the Supplemental Methods section (<http://links.lww.com/HC9/A175>). Antibodies used for western blot and immunofluorescence experiments are listed in supplementary table 1.

RESULTS

FXR ligand GW4064 decreases intracellular levels of HDV RNAs and proteins in both HBV-infected dHepaRG cells and PHH superinfected with HDV

The anti-HDV activity of the synthetic FXR ligand GW4064 was first evaluated in dHepaRG cells infected with HBV and superinfected with HDV; such a protocol was initially used as it best mimics *in vivo* infections. IFN- α , which can be considered as the current SoC for HDV in its pegylated form, was used as a control. In superinfected dHepaRG cells, a 10-day treatment with GW4064 decreased the amount of total intracellular HDV RNAs in a dose-dependent manner, reaching 66% of inhibition at 10 μ M of GW4064 (Figure 1A). Of note, the same levels of inhibition were obtained with a supraphysiological dose of IFN- α (1000 IU/mL), thus suggesting a superiority of the FXR agonization over type-I IFN receptor engagement.

Then, we analyzed intracellular HDV antigens, that is HDAg-S and HDAg-L by western blot and immunofluorescent staining. Treatment with GW4064 decreased intracellular amounts of both HDAg-S and HDAg-L in the

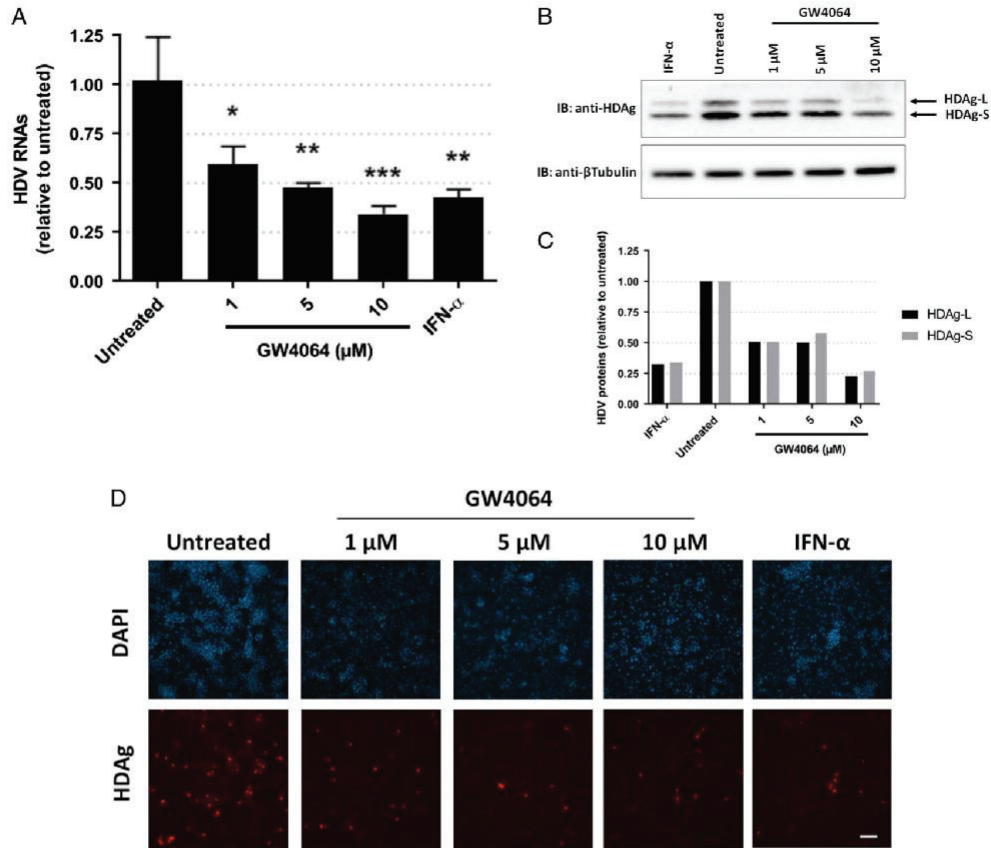


FIGURE 1 Farnesoid X receptor alpha ligand GW4064 decreases the levels of intracellular HDV RNAs and proteins in HBV-infected differentiated HepaRG superinfected with HDV. Differentiated HepaRG cells were infected with HBV at an MOI of 100 viral genome equivalent (vge) per cell and 7 days later with HDV at an MOI of 10 vge per cell. Three days after HDV infection, cells were treated with 1, 5, or 10 μ M of GW4064, IFN- α (1000 IU/mL), or not. Cells were harvested 10 days after treatment for cellular RNA and protein extraction or fixed with formaldehyde for immunofluorescence analyses. Intracellular HDV RNAs were quantified (A). Results are the mean \pm SD of 3 experiments each performed with 3 biological replicates. Student *t* test **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Analysis of the levels of HDAg by western blot analyses was performed using anti-HDAg antibodies and anti-B-tubulin antibodies as a loading control (B). Densitometry analyses are presented as ratios of HDAGs normalized to the levels of B-tubulin (C). Immunofluorescence analyses were performed using anti-HDAg antibodies and nuclei DAPI staining (D). Scale bar: 200 μ m.

same proportions, by 50% at 1 and 5 μ M of GW4064 and by 75% for 10 μ M (Figure 1B, C). Once again, 10 μ M of GW4064 led to the same inhibition as with IFN- α . Moreover, we observed less HDAG-positive cells following treatments (Figure 1D).

The effect of GW4064 on HDV was also analyzed in PHH infected with HBV and superinfected with HDV. Following treatment with FXR ligand GW4064, a 44% reduction of intracellular HDV RNA levels was observed at 10 μ M (Figure S1A, <http://links.lww.com/HCG9/A176>). Western blot and IF analyses also showed a decrease of HDV antigens in infected PHH (Figure S1B–D, <http://links.lww.com/HCG9/A176>). The anti-HDV activity of GW4064 was slightly lower in PHH than in dHepaRG cells, most likely reflecting a much higher replication of HDV in PHH.^[28]

HBV infection was also monitored by measuring total intracellular HBV RNAs and HBeAg levels secreted in

cell culture supernatants. As expected based on previous studies,^[20] GW4064 strongly inhibited both markers of HBV replication in either superinfected dHepaRG cells or PHH (Figure S2, <http://links.lww.com/HCG9/A176>).

Overall, these results showed an anti-HDV activity of the FXR ligand GW4064 both at the RNA and protein level in two relevant *in vitro* models of HBV/HDV superinfection.

FXR ligands inhibit early phases of HDV infection in dHepaRG cells and PHH monoinfected with HDV

To further describe the inhibitory effect of FXR ligands on HDV infection, we analyzed the impact of FXR ligands whether treatment was initiated at early times

postinfection or after the peak of HDV replication, to determine whether FXR modulation affected infection establishment or later phases of the HDV life cycle. To this end, HDV mono-infection experiments were performed in dHepaRG cells, thus freeing a potential effect of FXR ligands on HBV infection. Following infections, cells were treated at day 1 postinfection (early treatment) or at day 5 postinfection (late treatment). Moreover, to rule out putative off-target effects of GW4064, the cells were treated for 10 days with 3 structurally different FXR ligands, a BA derivate (6-ECDCA) and 2 nonsteroidal synthetic ligands (GW4064 and tropifexor). Quantification by RT-qPCR at the end of treatment showed that GW4064, 6-ECDCA, and tropifexor reduced the amount of total intracellular HDV RNAs by around 50% following early treatment (Figure 2A). A decrease in HDV RNAs was also observed after late treatment. However, this decrease was overall less pronounced and not significant with GW4064. For both early and late treatments, FXR ligands significantly decreased the level of FXR mRNA and strongly increased the level of BSEP mRNA as expected^[29] (Figure 2B, C). As the different forms of viral RNAs produced in HDV-infected cells could not be discriminated by our RT-qPCR, we verified by northern blot analysis the effect of FXR ligands on genomic and antigenomic RNAs. Results showed that the levels of both genomic and antigenomic RNAs were decreased following treatment with all 3 FXR ligands (Figure S3, <http://links.lww.com/HC9/A176>). Similar experiments of early and late treatments were performed in PHH. Contrary to dHepaRG cells, an antiviral effect of FXR ligands was only observed when treatment was initiated at early phases of HDV infection. The maximal inhibition was obtained with tropifexor with a 60% decrease of HDV RNAs compared with a 30% decrease with GW4064. When infection was established, FXR ligands were not efficient to impede HDV replication (Figure S4, <http://links.lww.com/HC9/A176>). As PHH maintain detoxification capacities, these differences may result from different kinetics of FXR ligands catabolism in these cells. Importantly, treatment did not significantly modify hepatocyte nuclear factor-4 mRNA expression compared with untreated cells, suggesting that the evolution of the differentiation state of PHH was not strongly affected by FXR ligands (Figure S5, <http://links.lww.com/HC9/A176>). Together, these results showed that 3 FXR agonists displayed antiviral activity against HDV, especially on early HDV infection phases in 2 relevant *in vitro* models. An antiviral effect of a late treatment was only observed in dHepaRG cells. The use of 3 structurally different FXR ligands showed that the anti-HDV activity of GW4064 was not the result of an off-target effect. Moreover, the inhibitory effect of FXR ligands did not require the presence of HBV.

Antiviral activity of FXR ligands is specific and relies on the presence of FXR

FXR ligands used in these experiments, in particular GW4064 and tropifexor, are considered highly specific for FXR. However, to confirm that the antiviral activity of these molecules was indeed FXR-specific, we silenced FXR in HDV-mono-infected cells. We took advantage of an HepaRG cell line expressing an inducible Cas9 (described in Figure S6, <http://links.lww.com/HC9/A176>) to use the CRISPR-Cas9 technology to stably repress FXR expression. Two synthetic crRNA targeting distinct exons of the FXR gene were used. Transfection of either of the 2 guide RNAs led to a strong decrease in FXR protein level, indicating that both guides were efficient to block FXR protein synthesis (Figure 3A). In line with our previous results, a 6-day treatment with GW4064 moderately, but significantly, reduced the amount of total intracellular HDV RNAs in nontransfected cells and in cells transfected with a control crRNA (Figure 3B). In FXR-silenced cells, the inhibitory effect of GW4064 was either completely (case of guide #1) or greatly (case of guide #2) reverted, confirming that its anti-HDV activity was indeed dependent on FXR expression. To confirm these results, we also silenced FXR in dHepaRG cells coinfecting with HBV and HDV by using small interfering RNA technology. The antiviral effect of 3 FXR ligands was reverted in the presence of small interfering RNA targeting FXR, whose expression was decreased by more than 80% (Figure S7A, B, <http://links.lww.com/HC9/A176>). The induction of BSEP expression was also reduced in the presence of siFXR after FXR ligand treatment compared with the siCTRL untreated condition (Figure S7C, <http://links.lww.com/HC9/A176>). Partial or complete reversion of the antiviral impact of FXR ligands on HBV was also observed following FXR silencing (Figure S7D, <http://links.lww.com/HC9/A176>). Overall, these results demonstrated that the inhibition of HDV markers induced by FXR ligands was dependent on the presence of FXR, its specific target.

FXR ligands strongly decrease the secretion of HDV particles in dHepaRG cells and PHH coinfecting with HBV and HDV

Next, we wanted to evaluate the effect of FXR ligands on HDV particle secretion corresponding to the late step of the HDV life cycle. To this end, HBV/HDV coinfections were performed in dHepaRG cells. Following infection, cells were treated at day 1 (early treatment) or at day 5 postinfection (late treatment). Following a 10-day treatment, cells and supernatants were collected for further analyses. As previously observed in HDV-mono-infected cells, all 3 FXR ligands decreased by around 50% the amount of intracellular HDV RNAs

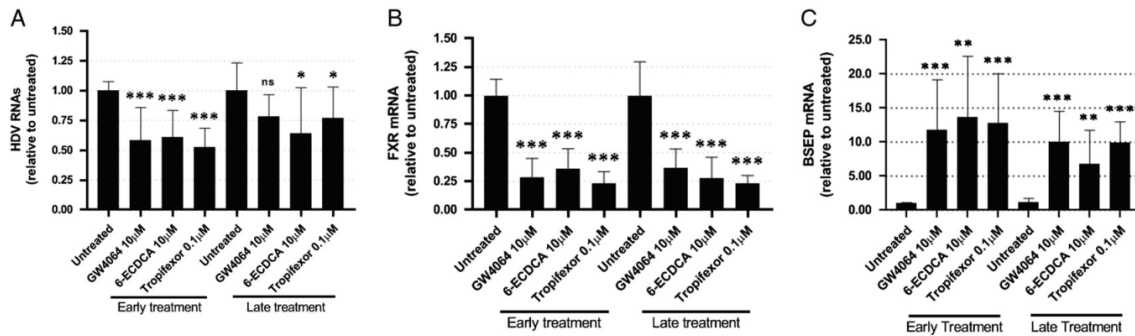


FIGURE 2 Farnesoid X receptor alpha (FXR) ligands inhibit early and late phases of HDV infection in HDV-monoinfected dHepaRG cells. Differentiated HepaRG cells were infected with HDV at an MOI of 10 viral genome equivalent per cell. Cells were treated with 10 µM of GW4064 or 6-ECDCA or 0.1 µM of tropifexor, 1 day postinfection for early treatment, and 5 days postinfection for late treatments. Cells were harvested 10 days after treatment. Total cellular RNAs were extracted and intracellular HDV RNAs (A), FXR mRNA (B), and BSEP mRNA (C) were quantified by RT-qPCR. Results are the mean ± SD of 3 experiments each performed with 3 biological replicates. Data are normalized to untreated conditions for early and late treatments. Student *t* test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Abbreviations: BSEP, bile salt export pump; ns, not significant.

following early treatment, whereas after late treatment, this decrease was lower or not significant (Figure 4A).

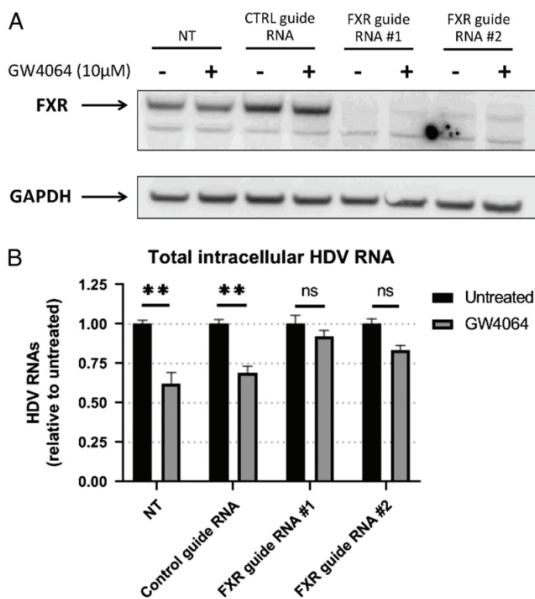


FIGURE 3 FXR ligands decrease the levels of intracellular HDV RNAs in an FXR-dependent manner. Differentiated HepaRG-TR-Cas9 cells were transfected twice with indicated RNA guides 4 days and 1 day before infection with HDV at an MOI of 25 viral genome equivalent per cell. From day 3 to day 9 postinfection, cells were treated with 10 µM of GW4064 or vehicle. Cells were harvested at day 9 postinfection. (A) Levels of FXR proteins were analyzed by the western blot. GAPDH detection was used as a loading control. (B) Total cellular RNAs were extracted and the levels of total intracellular HDV RNAs were quantified by RT-qPCR. Results are the mean ± SD of 3 experiments each performed with 3 biological replicates. Data are normalized to the untreated cells for each condition of transfection. Two-way ANOVA, ***p* < 0.01. Abbreviations: FXR, Farnesoid X receptor alpha; ns, not significant; NT, nontransfected.

Analysis of secreted viral parameters showed that a decrease of secreted HDV RNAs in supernatants was observed following early treatment with FXR ligands by around 60%. In contrast to intracellular HDV RNAs, secreted HDV RNAs were reduced following late treatment in the same proportions as early treatment (Figure 4B). A decrease in total HBV RNAs and secreted HBV DNAs was reported by more than 50% after early and late treatments with FXR agonists (Figure 4C, D). As expected, a decrease of FXR mRNA and an induction of BSEP mRNA were observed following treatment with FXR agonists (Figure S8, <http://links.lww.com/HC9/A176>). Overall, these results showed that treatment with FXR ligands affected the secretion step of the HDV life cycle, even when treatment was performed late at the peak of HDV replication.

FXR ligand GW4064 decreases specific infectivity of HDV particles

Next, we wanted to determine the impact of FXR ligands on specific infectivity of secreted HDV particles. This was particularly important to investigate as the continuous infection of naive or already infected hepatocytes by HBs-bearing infectious HDV virions is thought to play a major role in HDV persistence in patients, as opposed to HBV for which persistence is a less dynamic process associated with long-lived cccDNA.^[30] To this end, HBV/HDV coinfection experiments were performed in dHepaRG cells according to the protocol depicted in (Figure 5A). Cells were treated for 10 days with either GW4064, IFN-α, or a specific HBV-polymerase inhibitor (lamivudine). At the end of treatment, cells and supernatants of dHepaRG cells were collected for further analysis. First, western blot

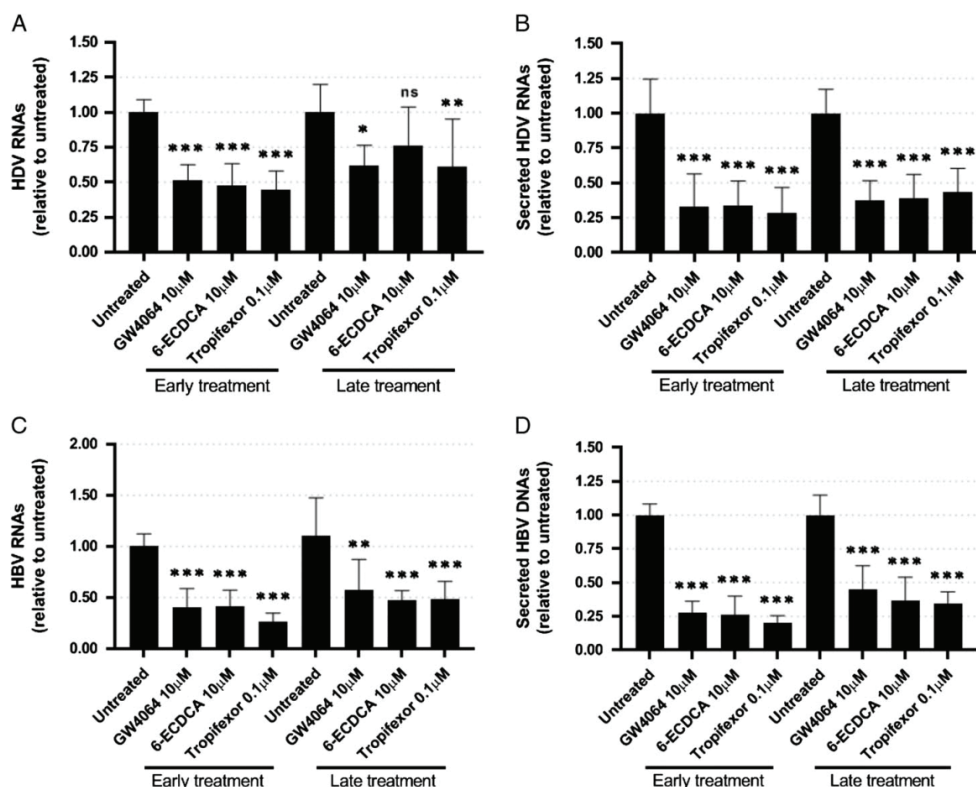


FIGURE 4 Farnesoid X receptor alpha ligands inhibit secretion of HDV RNA in differentiated HepaRG cells coinfecting with HBV and HDV. dHepaRG cells were coinfecting with HBV and HDV using 100 and 10 viral genome equivalent per cell, respectively. Cells were treated with 10 μ M of GW4064 or 6-ECDC or 0.1 μ M of tropifexor, 1 day postinfection for early treatment and 5 days postinfection for late treatment. Cells and supernatants were harvested 10 days after treatment. Total cellular RNAs and viral nucleic acids in supernatants were extracted. Intracellular (A) and secreted (B) HDV RNAs were quantified by RT-qPCR. Intracellular HBV RNAs (C) and secreted HBV DNA (D) were also quantified by RT-qPCR and qPCR, respectively. Results are the mean \pm SD of 3 experiments each performed with 3 biological replicates. Data are normalized to the untreated conditions for early and late treatments. Student *t* test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Abbreviation: ns, not significant.

analysis of the levels of intracellular HDV antigens was performed to confirm the antiviral effect of FXR ligands. As previously observed, GW4064 and IFN- α treatment strongly decreased the amount of intracellular HDV Ag-S and HDV Ag-L, while lamivudine had no effect, as expected (Figure 5B). Quantification of HDV RNA in supernatants by RT-qPCR revealed that the secretion of HDV genomes decreased by 65% following GW4064 treatment, and 52% with IFN- α treatment (Figure 5C). Of note, lamivudine slightly and unexpectedly stimulated HDV viral secretion through a mechanism that still needs to be clarified.

To determine the specific infectivity of secreted HDV, viral particles in supernatants were concentrated using PEG precipitation, and naive HuH7.5-NTCP cells were infected with the same viral genome equivalents for each condition. Six days postinfection, intracellular HDV RNA was quantified by RT-qPCR, and HDV antigen production was evaluated by IF staining. Total intracellular HDV RNAs in HuH7.5-NTCP cells decreased by

98% and 71% when the cells were infected with supernatants from infected dHepaRG cells treated with GW4064 and IFN- α , respectively (Figure 5D). By contrast, treatment with lamivudine did not affect specific infectivity of HDV particles. Interestingly, when infecting naive HuH7.5-NTCP cells with a different inoculum (ie, 100 and 500 viral genome equivalent per cell), a dose response was observed with supernatants from dHepaRG cells treated with either IFN- α , lamivudine, or vehicle, whereas this was not the case for GW4064 (Figure 5E). Finally, IF staining confirmed the RT-qPCR data as HDV Ag-positive cells could hardly be detected among HuH7.5-NTCP cells infected with supernatants from dHepaRG cells treated with GW4064 (Figure 5F).

Supernatants were also subjected to isopycnic centrifugation on iodixanol density gradients. Fractions collected were tested for density, HDV RNA, and HBV DNA by qPCR, HBsAg by ELISA, and HDV Ag and HBsAg by western blot. No modification in the

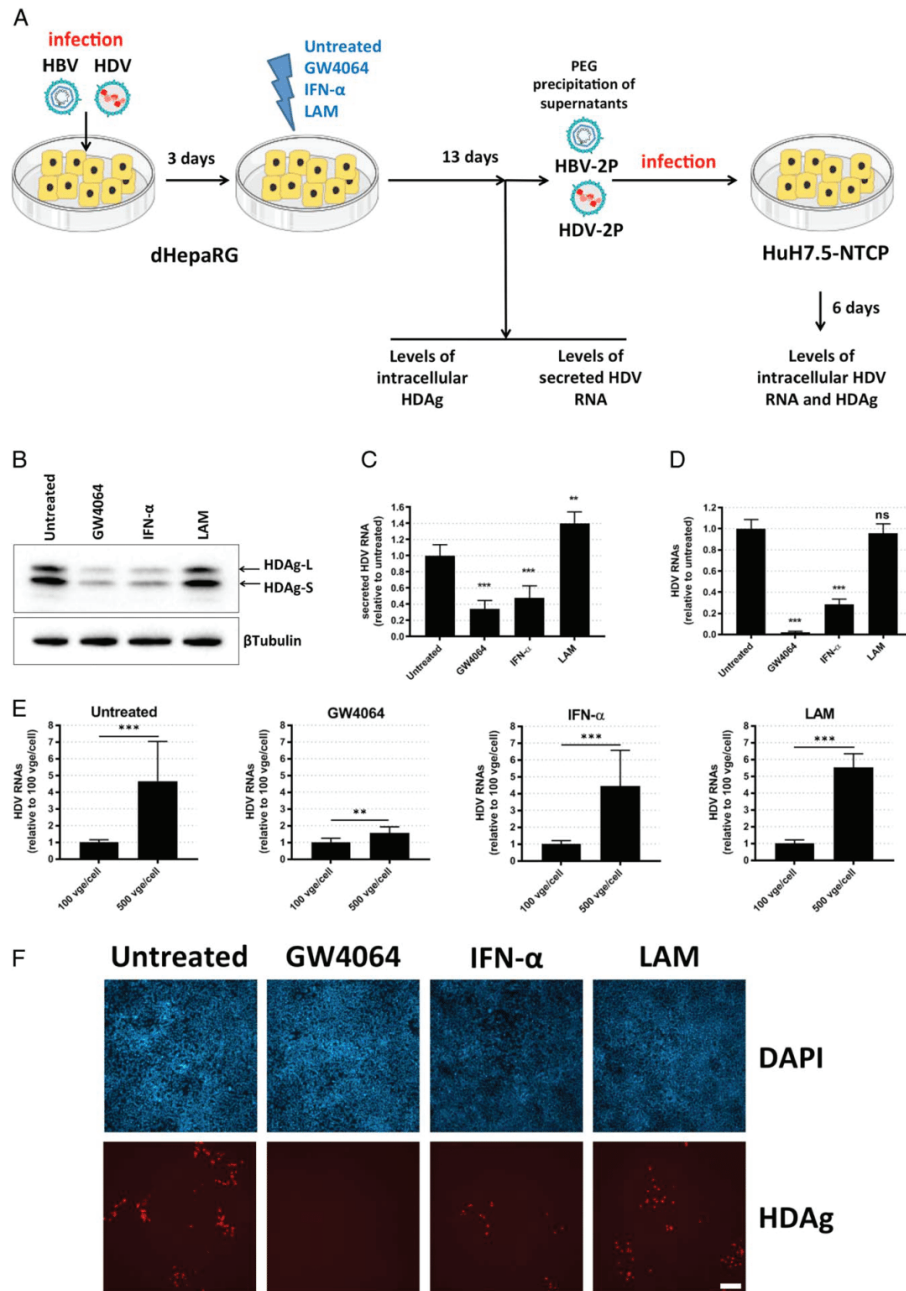


FIGURE 5 GW4064 reduces the infectivity of HDV particles. Differentiated HepaRG cells were coinfecting with HBV and HDV with 500 viral genome equivalent (vge) per cell for HBV and 50 vge per cell for HDV. Cells were treated or not 3 days later with GW4064 (10 μ M), IFN- α (500 IU/mL), or lamivudine (LAM, 10 μ M) for 10 days. (A) Schematic representation of the experimental procedure. (B) Differentiated HepaRG (dHepaRG) cells were collected and the level of intracellular HDAg was analyzed by the western blot. (C) Supernatants of infected dHepaRG cells were collected, concentrated by PEG precipitation, and the levels of extracellular HDV RNAs (called HDV-2P for second passage) were assessed by qRT-PCR analyses. (D–F) Naive HuH7.5-NTCP cells were infected with the different concentrated supernatants (HDV-2P) with (D) 500 vge cell or (E) the indicated vge per cell. Six days later, (D, E) levels of intracellular HDV RNAs were assessed by RT-qPCR analyses; (F) cells were stained with DAPI and anti-HDAg antibodies. Scale bar: 200 μ m. Results of RT-qPCR are the mean \pm SD of 3 independent experiments each performed with 3 biological replicates. Student *t* test, ***p* < 0.01, ****p* < 0.001. Abbreviations: IFN, interferon; ns, not significant; NTCP, Na⁺-taurocholate cotransporting polypeptide.

distribution and densities of fractions containing HDV RNA, HBV DNA, HDAg, and HBsAg was observed following treatment with GW4064, IFN- α , and lamivudine compared with untreated cells (Figure S9, <http://links.lww.com/HC9/A176>). Similar experiments were performed with FXR ligands 6-ECDCA and tropifexor to confirm the results obtained with GW4064. Following infection of naïve HuH7.5-NTCP cells with viruses produced in dHepaRG cells treated with 6-ECDCA and tropifexor, a decrease of intracellular HDV RNAs by more than 95% was also observed, indicating that the specific infectivity of secreted HDV particles was affected by all 3 FXR ligands used in this study (Figure S10A, B, <http://links.lww.com/HC9/A176>). A comparable decrease of infectivity was observed when naïve dHepaRG cells were infected with HDV viruses produced in the same conditions of treatment (Figure S10C, <http://links.lww.com/HC9/A176>). Altogether, these results showed that FXR ligands not only decreased secretion of HDV particles in infected dHepaRG cells but also impaired their specific infectivity.

DISCUSSION

This study shows that in the most relevant *in vitro* models of HDV infection, treatment with FXR ligands moderately reduces early steps of HDV intracellular replication, more efficiently inhibits secretion of HDV virions, and drastically inhibits the specific infectivity of secreted viral particles. Target engagement was confirmed by the use of several ligands with different chemical structures and FXR loss of function experiments.

In HDV monoinfected dHepaRG cells, the amount of genomic and antigenomic HDV RNA, which are key markers of HDV replication, was reduced by the treatment with FXR ligands. This demonstrates that, at least for the HDV genome replication steps, the inhibitory effect of FXR ligands is unrelated to their anti-HBV activity, which has been previously described.^[20,21] In HBV/HDV coinfection models, it is shown that FXR ligands induced a decrease in HDV RNA secretion. This impaired secretion of hepatitis delta virions may result from the decrease of all markers of HDV replication (HDV RNAs and proteins), combined with the inhibition of HBs synthesis, not excluding other mechanisms that remain to be identified.

One major impact of FXR activation is the pronounced decrease in specific infectivity (by more than 95%) of virions produced by coinfecting dHepaRG cells. HDV RNA-containing particles were indeed poorly efficient to infect and/or initiate viral replication in naïve HuH7.5-NTCP cells, suggesting key modifications of virion composition following treatment. No shift in the density of RNA-, HDAg-, or HBs-containing fractions

secreted upon FXR engagement could be observed, but a detailed analysis of virion composition and structure is warranted (ie, proteomic, lipidomic, atomic-force microscopy). Importantly, this reduction of specific infectivity obtained with GW4064, 6-ECDCA, and tropifexor was superior to that obtained with the SoC IFN- α .

One limitation of the study is that all experiments were performed using a unique HDV strain of genotype 1 with a particular history.^[31] This strain is commonly used in HDV research; however, it would be of great interest to study the antiviral effect of FXR ligands on other HDV genotypes.

FXR belongs to the NR superfamily, a group of transcription factors sharing a common modular structure that includes a DNA-binding domain and a ligand-binding domain. Binding of ligands on FXR may result in FXR activation and modulation of FXR target gene expression, in modifications of FXR conformation leading to either promotion or destabilization of interactions with nucleic acids or protein partners and finally in repression of FXR expression through a negative feedback loop. HDV RNAs, HDAg-S, and host components associate to form HDV viral replication/transcription complexes. In particular, it was shown that host DNA-dependent RNA polymerase II could be recruited onto HDV RNA genome and drive transcription, thus indicating that HDV can hijack host factors known to interact with dsDNA and not dsRNA.^[32] To our knowledge, FXR activation does not directly modulate the expression of DNA-dependent RNA polymerase II, but it is well established that ligand binding induces a conformational change of NRs, leading to the recruitment of various coactivators, some of them with acetylase and methylase activities. Histone acetylation and methylation status is then modified by these coactivators with consequences on the transcription of target genes. Whether this could apply to the regulation of HDV transcription remains to be explored, as the regulation of RNA polymerase II on the HDV genome is far from being fully understood. However, one can speculate that FXR may interact or interfere with viral or cellular components within HDV replication complexes, and that this interaction may be modified by FXR ligands.

Alternatively, FXR activation by ligands may specifically modulate the expression of genes involved in pathways playing a key role in the HDV life cycle. Such modulations may lead to altered replication and transcription of the HDV genome and/or modifications of the structure and composition of secreted particles during the virion assembly. FXR is an essential regulator of several liver metabolic pathways, its role in regulating lipid metabolism (in particular BA and lipoprotein metabolism) and glucose metabolism being extensively studied. However, its role is not limited to these pathways, as FXR has also been linked to the regulation

of liver regeneration and innate immunity.^[17,33] Cellular pathways essential for the HDV life cycle are still poorly known to date. Recently, a screening based on RNA interference identified several cellular pathways involved in HDV replication such as HIF-1 signaling pathway, pyrimidine biosynthesis, insulin resistance, or glycosaminoglycan biosynthesis.^[34] Protein prenylation pathway is also known to play a key role during HDV virion assembly as farnesylation of HDAg-L by cellular farnesyl-transferases favors HDAg-L localization to the ER and association with HBsAg. Interestingly, some links exist between prenylation and BA pathways. Besides geranylgeranyl, farnesyl can also be transformed into squalene, an essential biochemical precursor for steroids (including cholesterol and BA) or farnesol. Farnesol was the first ligand identified for FXR^[35] but, following the identification of BA as new FXR ligands, studies on relationships between FXR and prenylation pathway were given less priority. Given the major impact of FXR ligands on HDV virion infectivity, studying the impact of FXR activation on HDAg-L may be of great interest. Moreover, overexpression of HDAg-L has also been shown to inhibit HDV viral replication,^[11] and FXR-induced putative modulations of HDAg-L activity might also interfere with the replication step of the HDV cycle.

In conclusion, our results revealed that FXR agonists inhibit early steps of intracellular HDV genome replication in *in vitro* infected dHepaRG and PHH, with an antiviral activity comparable to that of IFN- α . In HBV/HDV coinfecting cells, FXR ligation decreases HDV secretion and even more significantly reduces the specific infectivity of secreted HDV virions, leading altogether to an antiviral phenotype far superior to that obtained with IFN- α , the current SoC in HDV patients. After HBV, HDV is the second hepatotropic virus that seems to be very sensitive to therapeutic FXR agonization, which suggests that this NR may be broadly involved in host antiviral responses. The majority of studies focus on FXR metabolic functions, and a more detailed analysis of mechanisms underlying FXR antiviral activity has to be pursued.

To date, the therapeutic options for patients infected with HDV are limited to Peg-IFN- α and bulevirtide, an entry inhibitor. Other molecules are currently under evaluation: Peg-IFN- λ 1, lonafarnib, a prenylation inhibitor that impairs HDV secretion, and REP 2139, a nucleic acid polymer that inhibits HBs, HBV, and HDV secretion. Combined therapies will likely be necessary to achieve an SVR in the majority of patients. As constant reinfection of hepatocytes is thought to play a major role in HDV persistence, the strong inhibition of virion production and spreading following treatment with FXR ligands opens promising therapeutic perspectives for these molecules in the treatment of hepatitis delta. FXR is an attractive target

for the development of antiviral molecules as some FXR ligands have already been approved for patients with primary biliary cholangitis or are currently in clinical evaluation in patients with NASH.^[36] Moreover, one FXR ligand is currently in a phase II clinical trial in patients with chronic HBV infection (Clinical Trial identifiers NCT04365933 and NCT04465916). In this context, the clinical evaluation of FXR modulators in HBV/HDV coinfecting patients, alone or in combination, could be a successful strategy.

AUTHOR CONTRIBUTIONS

Christophe Ramière, Julie Lucifora, Patrice André, and David Durantel: study concept and design. Anne-Flore Legrand, Julie Lucifora, Benoît Lacombe, Maud Michelet, Anna Salvetti, Camille Ménard, Adrien Foca, Pauline Abrial, and Christophe Ramière: acquisition and analyses of data. Christophe Ramière, Anne-Flore Legrand, Julie Lucifora, Benoît Lacombe, David Durantel, Patrice André, and Vincent Lotteau: interpretation of data. Christophe Ramière, Anne-Flore Legrand, Julie Lucifora, David Durantel, Benoît Lacombe, and Vincent Lotteau: drafting of the manuscript: CR, AFL, JL, DD, BL, VL. Christophe Ramière, Julie Lucifora, David Durantel, Vincent Lotteau, and Patrice André: funding acquisition. Michel Rivoire: material support.

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CONFLICTS OF INTEREST

The authors have no conflicts to report.

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2- The supplementary data of the research article

a- Supplementary methods

i- Nucleic acid quantification

Total intracellular RNAs were extracted using the NucleoSpin RNA II kit according to the manufacturer's instructions (Macherey-Nagel). DNAs and RNAs from HBV or HDV particles were isolated from cell supernatants using the NucleoSpin RNA Virus kit (Macherey-Nagel) according to the manufacturer's instructions. RNA was reverse-transcribed using either High-Capacity RNA-to-cDNA kit (ThermoFisher Scientific) or Maxima RT (Life Technologies). Quantitative PCR for HDV and HBV were performed as previously described^{375,376}. FXR and BSEP mRNAs were quantified by qPCR as previously described³⁷⁷. Statistical analyses were performed using Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not significant.

Northern blot for detection of HDV genomic and antigenomic RNAs was essentially performed as previously described³⁷⁶. Briefly, purified RNA was denatured at 50°C for one hour with glyoxal (Life Technologies), subjected to electrophoresis through a phosphate 1.2% agarose gel and transferred to a nylon membrane (Amersham Np, GE). Membrane-bound RNA was hybridized to digoxigenin(DIG)-labeled HDV-specific probes. Quantitative analysis of HDV RNA was achieved by phosphorimager scanning (Typhoon Fla 9500, GE); 18S and 28S rRNA detection was used as loading control.

ii- ELISA

Commercial immunoassay kits (Autobio Diagnostics Co., China) were used for HBsAg and HBeAg quantification in cell culture supernatants. Results are presented as a ratio to a control sample, described for each experiment. Cut-offs for these ELISA were 0.05 IU/mL for HBsAg and 0.1 PEIU/mL for HBeAg.

iii- Western blotting

For the detection of FXR protein by western blot, cells were lysed using buffer containing 10 mM HEPES, 10mM NaOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 0.3% NP40, protease inhibitor mixture cocktail (P8340-Merck) and Pierce™ universal nuclease for cell lysis (#88701, Thermo Fisher Scientific). Samples were then diluted with 4X Laemmli sample buffer (Biorad, #1610747) and supplemented with 1mM dithiothreitol. Mouse anti-FXR (clone A9033A, ThermoFisher Scientific) and anti-GAPDH (clone 1E6D9, Proteintech) antibodies were used, followed by horseradish peroxidase (HRP) detection with SuperSignal™ West Pico or Femto chemiluminescent substrate (Thermo Fisher Scientific) according to manufacturer's instructions. HRP signal detection was determined electronically using the Syngene PXi Image system (Ozyme).

For the detection of HDV antigens, cells were washed with Phosphate buffered saline (PBS) and harvested in RIPA lysis buffer (Tris-HCl pH 7,5 10mM, NaCl 140mM, EDTA 1mM, EGTA 0,5mM, 1% Triton X100, 0,1% SDS, 0,1% Na-Deoxycholate) containing protease inhibitors (Protein Cocktail Inhibitors from Sigma-Aldrich). Clarified lysates were subjected to SDS-PAGE and Western Blot transfer onto nitrocellulose membranes using the iBlot2 apparatus according to the manufacturer (Thermofisher Scientific). The polyclonal anti-HDAg serum, obtained by rabbit immunization (with a proprietary strategy), was a kind gift from Janssen. Anti-human β Tubulin was purchased from Abcam and used as a loading control. Detection was performed with Gel Doc XR β System (BioRad) and images were analyzed with ImageJ software.

Anti-HBsAg (H166), used for HBs detection in gradient fractions, has been previously described³⁷⁸.

The references of antibodies used in this study are provided in Supplementary Table 1.

Immunofluorescence (IF) experiments were performed as previously described³⁷⁶. Cells were fixated with formaldehyde 4% and permeabilized by Triton 0.1%. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The anti-HDAg antibody used was the same as for western blots. Secondary antibody used was goat anti-human Alexa Fluor 555 (Invitrogen). Images were obtained by epifluorescence microscopy (Nikon eclipse TE2000-E; Nikon).

b- *Supplementary references*

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c- Supplementary Table 1

Antibodies	Application	Description	Source	Catalog/Clone number
FXR	WB	Mouse monoclonal	Thermo Scientific	Fisher clone A9033A
GAPDH	WB	Mouse monoclonal	Proteintech	clone 1E6D9
HDAg	WB, IF	Rabbit polyclonal	Janssen	proprietary strategy
β Tubulin	WB	Rabbit polyclonal	Abcam	ab15568
HBsAg	WB	Mouse monoclonal	Abbott Laboratories	H166

d- Supplementary figures

Figure S1

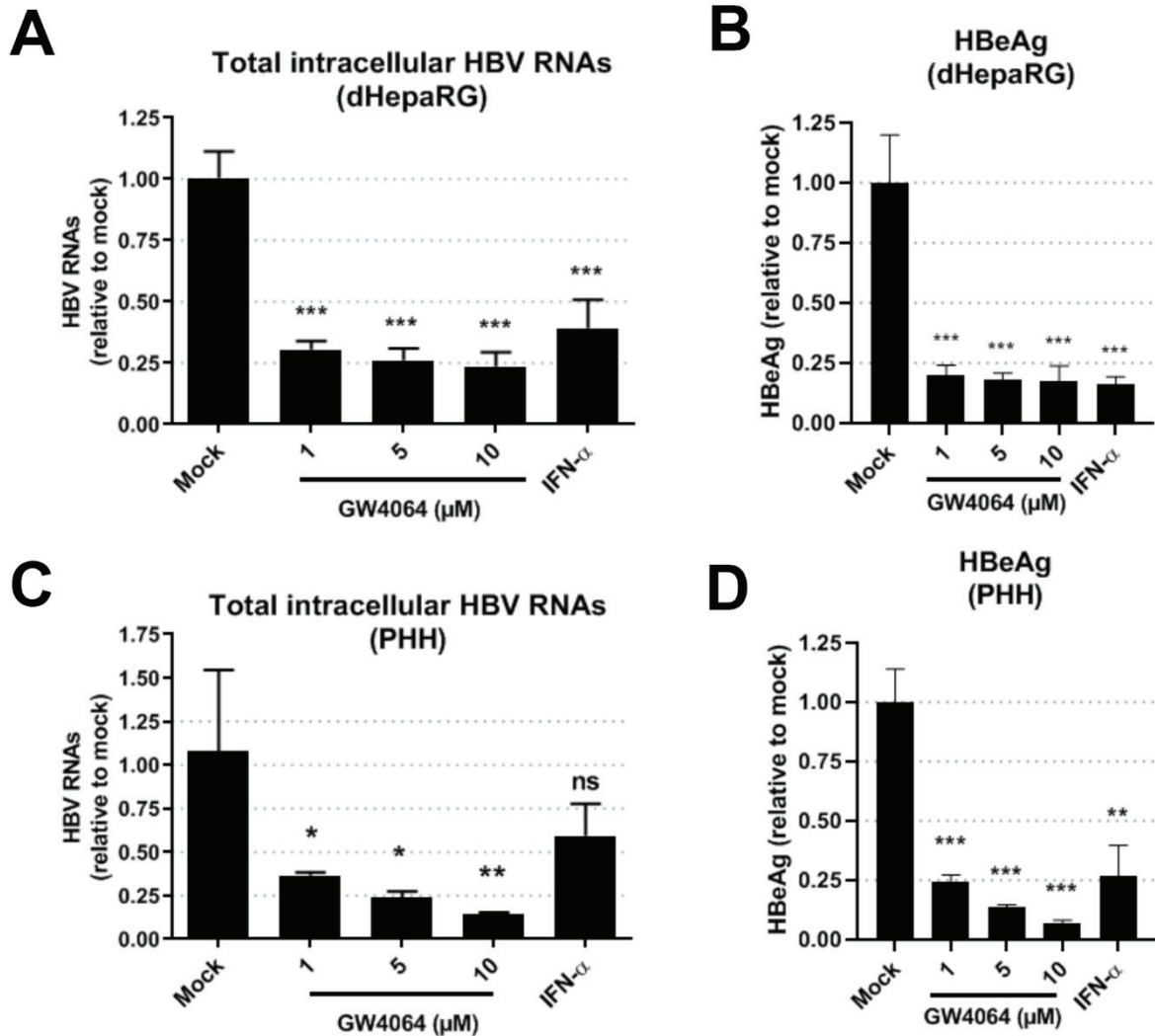


Figure S1. FXR ligand decreases the levels of HBV replication markers. dHepaRG cells or PHH were infected and treated as described for Fig. 1. Cells and supernatants were harvested 10 days post-treatment for quantification of intracellular HBV RNA (A: dHepaRG cells ; C: PHH) and secreted HBe antigens (B: dHepaRG cells ; D: PHH). Results are the mean +/- SD of three experiments (dHepaRG) and one experiment (PHH) each performed with three biological replicates. Student's t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not significant.

Figure S2

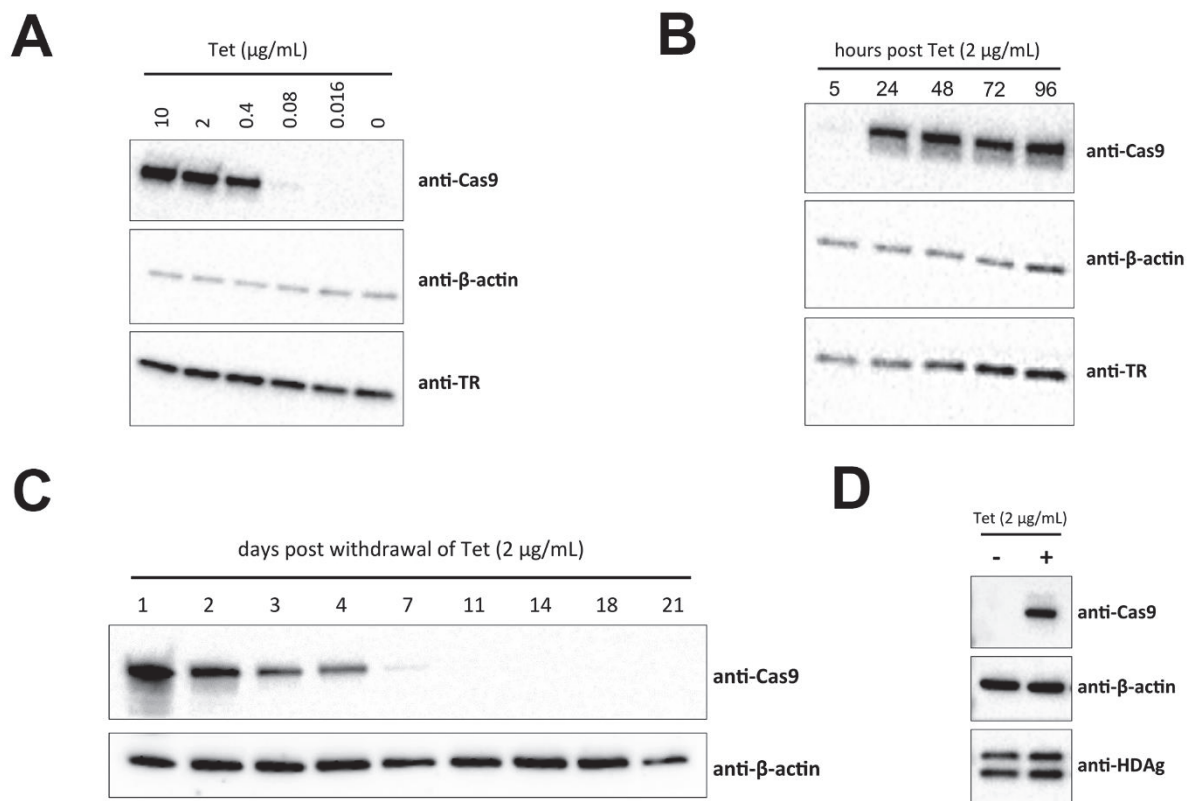


Figure S2. Characterization of HepaRG-TR-Cas9 cells. (A) dHepaRG-TR-Cas9 cells were treated with the indicated concentrations of Tetracyclin (Tet) for 3 days. (B) dHepaRG-TR-Cas9 cells were treated with Tet and collected at the indicated time. (C) dHepaRG-TR-Cas9 cells were treated with Tet for 24h and cells were collected at the indicated time after withdrawal of Tet. (D) dHepaRG-TR-Cas9 cells were treated or not with Tet for 1 day before infection with HDV at 100 vge/cell. Cells were collected at day 6 post-infection. (A, B, C, D) Western blot analyses were performed using the indicated antibodies.

Figure S3

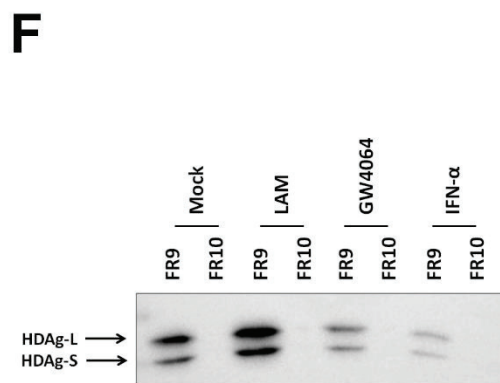
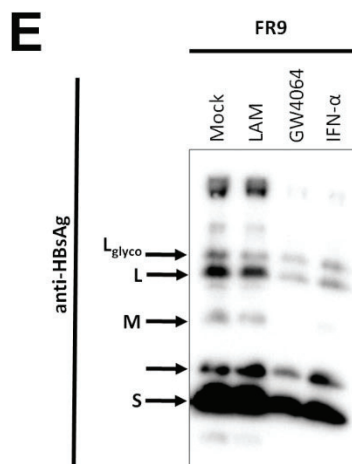
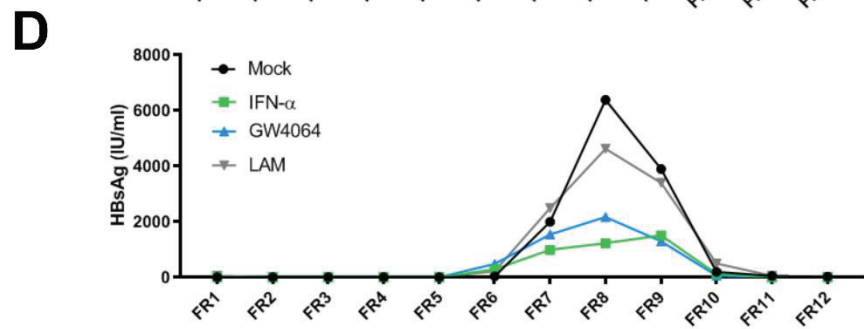
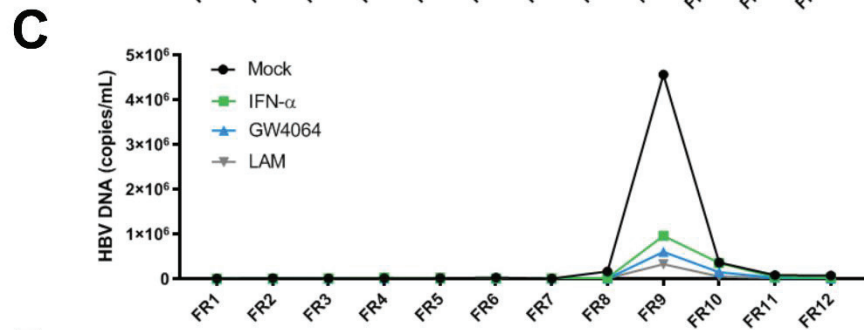
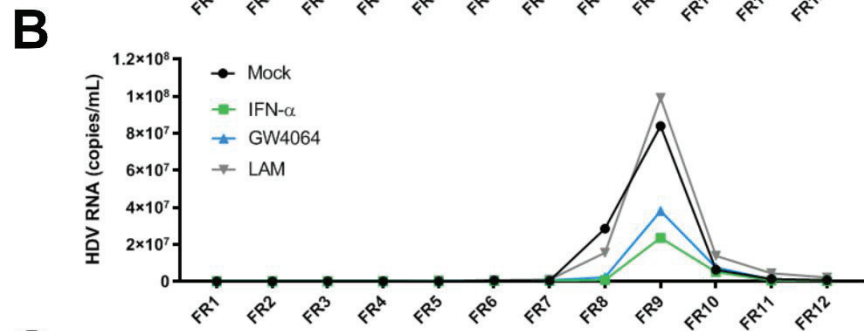
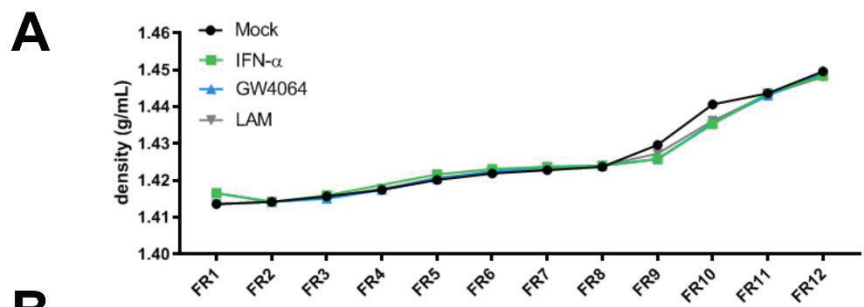


Figure S3. FXR ligand GW4064 does not modify buoyant density of secreted HDV particles. dHepaRG cells were coinfecting with HBV and HDV with 500 vge/cell for HBV and 50 vge/cell for HDV. Cells were treated or not 3 days later with GW4064 (10 μ M), IFN- α (500 IU/mL) or LAM (10 μ M) for 10 days. Supernatants were collected at day 13 post infection. Supernatant were collected, concentrated by PEG precipitation and submitted to iodixanol gradients overnight. Fractions (FR) were collected and (A) the density, the levels of (B) HDV RNAs, (C) HBV DNA and (D) HBsAg were analysed by RT-qPCR, qPCR and ELISA. (E, F) WB analyses were performed in selected fractions using anti-HBsAg (E) and anti-HDAg (F) antibodies . LAM: lamivudine.

Part 6 : Discussion

In this study, FXR ligands were shown to inhibit HDV replication *in vitro* in two relevant cell models, HepaRG cells and PHHs. Firstly, FXR ligands induced a modest reduction in intracellular HDV replication in the early phases of infection, resulting in a decrease in all intracellular viral markers (HDV RNAs and proteins). FXR ligands also induced a strong reduction in the secretion of HDV particles. Nevertheless, the most dramatic effect of FXR ligands observed on the infectivity of these secreted particles. The effect of FXR ligands on HDV infection is specific and relies on FXR as demonstrated by the use of three structurally different FXR ligands and by silencing experiments of FXR expression.

The antiviral effect of FXR ligands on intracellular HDV replication may be attributed to their impact on viral transcription and/or replication complexes consisting of HDV RNAs, S-HDAg and associated host components. We suggested that FXR could interact with these viral complexes and that the binding of FXR ligands to their receptor could destabilize them. One possibility is a direct interaction between FXR and HDV RNAs. However, there is currently no evidence in the literature demonstrating that nuclear receptors can bind to RNA molecules. While a list of FXRE sequences has been compiled for DNA, it is challenging to extrapolate this to an RNA sequence. Nevertheless, HDV RNA is known to hijack host factors that typically interact with DNA rather than RNA. It has been shown that host DNA-dependent RNA Polymerase II can be recruited to the HDV RNA genome and drive transcription due to the unique characteristics of the HDV genome structure rather a specific sequence. Therefore, potential interaction should be considered and explored through RNA immunoprecipitation (RIP).

A second possibility is a direct interaction between FXR and HDAg, particularly with S-HDAg, destabilizing its binding to HDV RNAs. S-HDAg plays a crucial role in viral

transcription and replication by facilitating the recruitment of cellular host factors to establish transcription and replication complexes. Similar to the previously described mechanism, the binding of FXR ligands to their receptor could potentially disrupt the formation of these transcription/replication complexes, thereby inhibiting intracellular replication of HDV. On the contrary, a direct interaction between FXR and L-HDAg, which is known to inhibit HDV replication, could interfere with replication step of HDV cycle. The possibility of a direct interaction between FXR and HDAg could be investigated using standard co-immunoprecipitation techniques, whereas a reduction in HDAg binding to HDV RNAs could be investigated using RIP techniques.

A third possibility to explain the reduction in HDV replication is that FXR activation may result in a decrease in the recruitment and subsequent association of cellular factors involved in HDV transcription and/or replication complexes with HDV RNAs. Indeed, the capability of FXR as a nuclear receptor to facilitate the recruitment of transcription and/or replication machinery, transcription coactivators, and histone modifying enzymes suggests its potential involvement in HDV transcription and replication. It is noteworthy that some of the host factors recruited by FXR are also known to participate in HDV transcription and replication processes, like DNA-dependent RNA Pol II, p300, and histone H1.4. Our current knowledge suggests that FXR does not directly modulate the expression of DNA-dependent RNA Pol II, but it may affect its association with HDV RNAs. Preliminary data obtained through RNA immunoprecipitation have shown a decrease in the levels of DNA-dependent RNA polymerase II associated to HDV RNAs following FXR ligands treatments (*Annex I*). This observation may provide an explanation for the observed decrease in intracellular HDV replication. RNA immunoprecipitation could be extended to others host factors.

FXR activation by its ligands may interfere with host factor and/or modify cellular pathways associated with HDV infection, preventing the HDV replication and secretion. However, our

understanding of the specific cellular pathways essential for the HDV life cycle remains limited. A recent study utilized RNA interference screening to identify several cellular pathways and host factors involved in HDV replication such as HIF-1 signaling, pyrimidine biosynthesis, insulin resistance or glycosaminoglycan biosynthesis³⁷⁹. To investigate the potential impact of FXR activation on these pathways, three datasets obtained from RNA sequencing studies after treatment with FXR for 24 or 48 hours, two from the literature and a third which has not been published, were cross-referenced^{277,380}. 191 genes of interest were selected according to several criteria. Genes had to meet the criteria of being present in at least two datasets and exhibiting a fold change greater than 2. We conducted a loss-of-function screening using siRNA on 25 genes of those selected. Unfortunately, no conclusive results were obtained from this screening but this study could be extended to other target genes.

We observed that the effects of FXR ligands on intracellular HDV replication vary depending on whether the treatment was initiated early or late during the peak of HDV replication. When the infection was already established, FXR ligands are not efficient in inhibiting HDV replication. The antiviral effect of FXR ligands was specifically effective during the critical early stages of infection, targeting probably processes allowing the establishment of the viral population. However, when the treatment was initiated at a later stage, after the virus had already established a significant viral population, it could evade the antiviral effects induced by FXR ligands. We propose two hypotheses to explain this phenomenon. First, if the inhibition of viral replication is due to a direct interaction between FXR and a component of the replication and/or transcription complexes, the abundance of these complexes may exceed the available pool of endogenous FXR protein once the peak of viral replication is reached. A second explanation could be that FXR ligands modulate a cellular pathway, such as innate immunity pathway, and at the peak of replication, the virus may have developed effective mechanisms to inhibit this pathway.

In addition to their observed antiviral effect on intracellular HDV replication, FXR ligands also induced a decrease in the secretion of HDV viral particles. The decrease of HDV particles secretion is closely linked to the reduction of intracellular HDV replication. However, the mechanisms underlying the effects on viral secretion and replication appear to be distinct at least in part. Interestingly, a significant reduction in viral secretion was observed regardless of whether the treatment was initiated early or late, while a pronounced reduction in intracellular replication was only observed with early treatment. The decrease in HDV particles secretion can be partly explained by a decrease in the expression of HBV envelope proteins induced by FXR ligands, which are essential for HDV virion assembly. Among other putative mechanisms underlying the inhibition of secretion, a direct interaction between FXR and L-HDAg could be explored, or post-translational modifications of L-HDAg, secondary to treatment by FXR ligands. This interaction could potentially modify the subcellular localization and activity of L-HDAg, leading to impairment of HDV ribonucleoprotein assembly, transport, and/or HDV virion assembly.

FXR, as a major regulator of various metabolic pathways in the liver, has the potential to modulate cellular pathways that are essential for HDV virion assembly. However, the specific cellular pathways involved in the HDV life cycle are still not well understood. One important pathway known to play a crucial role in HDV virion assembly is the protein prenylation pathway, particularly through the farnesylation of L-HDAg. This farnesylation process enables the localization of L-HDAg to the endoplasmic reticulum and its association with HBsAg. Farnesylation of L-HDAg is catalyzed by the enzyme farnesyl transferase which uses farnesyl pyrophosphate as a substrate. There are connections between bile acids metabolism and protein prenylation. Indeed, farnesyl pyrophosphate, an essential molecule in the protein prenylation pathway, is an intermediate metabolite in the synthesis pathway of cholesterol, the precursor of bile acids. Interestingly, farnesol was the first identified FXR

ligand. However, with the subsequent discovery of bile acids as new FXR ligands, studies on the relationship between FXR and the prenylation pathway received less attention or were even halted. Some our data have indicated that treatment with Lonafarnib (**Annex 2**), a farnesyl transferase inhibitor, led to a decrease in FXR expression. This suggests a potential link between FXR activation and L-HDAg prenylation. Investigating the potential impact of FXR activation on L-HDAg prenylation could be of great interest. To explore that, chemical modulators of prenylation pathway could be used.

The predominant antiviral effect of FXR ligands was primarily observed on the specific infectivity of HDV particles secreted by dHepaRG cells upon treatment. Several hypotheses can be proposed to elucidate the mechanisms underlying the reduction of infectivity of HDV particles. One hypothesis suggests that the altered composition and properties of secreted virions, resulting from abnormal particle morphogenesis, contribute to the loss of specific infectivity, thereby linking the antiviral impact on particles secretion and infectivity. Density gradient experiments were conducted, revealing no discernible changes in the density of HDV-containing particles following treatment with FXR ligands. This suggests that the loss of infectivity is not attributable to abnormal structure of secreted particles. Furthermore, no significant modification in the L-HDAg/S-HDAg ratio were observed, indicating that an excessive abundance of the L form relative to the S form of HDAg does not account for the inhibition of replication. Notably, we demonstrated that FXR ligands also induce a decrease in the infectivity of HBV particles, suggesting that similar antiviral mechanisms induced by FXR may affect both secreted virions (**Annex 3**).

The decrease in infectivity of both HDV and HBV particles may be first attributed to the effects of FXR ligands on the synthesis of various forms of HBV surface antigens, particularly by altering the ratio of different HBsAg forms (S/M/L) as well as their post-translational glycosylation, specially their N-glycosylation status which are known to modulate

the specific infectivity of these various³⁸¹. A recent study has reported that the hepatic FXR-SHP axis appears to regulate the N-glycome in the liver³⁸². It was observed that mice lacking FXR (FXR^{-/-}) exhibited alterations in the N-glycosylation pathway, characterized by an upregulation of glycosyltransferases expression. However, our preliminary data do not appear to support this hypothesis at present (*Annex 4*).

Furthermore, it is possible that FXR, being involved in lipid metabolism and specifically lipogenesis, could induce changes in the lipid composition of secreted viral particles. However, there is currently no available information regarding the connection between FXR and membrane lipid composition. Therefore, further investigations, such as lipidomic analyses, will be conducted to explore this possibility.

Secondly, the reduction in specific infectivity of HDV particles could be attributed to the encapsidation of replication-defective viral genomes, which might result from mutations or abnormalities in the viral genome, leading to impaired infectivity. However, FXR is not generally considered to be directly involved in DNA repair mechanisms or genomic stability. However, one study has been provided evidence of an upregulation of specific deaminases family, such as members of apolipoprotein B mRNA editing catalytic polypeptide-like (APOBEC) family following FXR ligands treatment, notably APOBEC3B et APOBEC3F²⁷⁷. These deaminases are cellular enzymes that possess cytidine deaminase activity and can act on both RNA and single stranded DNA substrates³⁸³. APOBEC3 proteins are recognized for their antiviral activity, which involves inducing multiple mutations in the genome viruses such as HIV, HBV and papillomavirus but their potential to modify RNA viruses is still exploratory. To explore this hypothesis, an analysis of secreted particles was conducted by cloning individual genomes and sequencing them under different conditions (approximately 20 genomes were analyzed for each condition). An increase in the overall mutation rate was observed following GW4064 treatment (*Annex 5*). There were no evidence for APOBEC3-

induced mutations but it was noticed that FXR ligands appeared to increase A>G and T> C substitutions, which may correspond to ADAR-type mutations. However, there are no available data regarding the induction of ADAR1 by FXR in the literature and these finding must be confirmed using less-biased sequencing methods. Therefore, protocol optimization is needed, and for this reason, a deep RNA sequencing is envisaged. We have adapted and developed a protocol to amplify the complete HDV genome for sequencing, which can also be used to analyze potential alterations in the ratio between unedited and edited genomes (adapted from ³⁸⁴). An increase in the levels of secreted HDV genomes edited at the amber termination codon could result in the premature production of L-HDAg, leading to the inhibition of HDV replication. The results obtained will be further validated using droplet digital PCR (ddPCR). Additionally, investigations of potential modifications in the expression and/or activity of ADAR1 isoforms, such as p110 and p150, have been performed. Preliminary data did not indicate modifications in the expression and activity of ADAR1 following FXR ligands treatment (*Annex 6*).

The third hypothesis proposes that the loss of infectivity could be attributed to the deletion of a proviral factor or the addition of a restriction factor within the viral particles. During the assembly process, virus hijack numerous cellular factors to facilitate the establishment of new infections. The composition of several viruses, such as Influenza A virus (IAV) and HIV, has been elucidated by proteomics³⁸⁵. Virion-incorporated cellular proteins play a crucial role in wide variety of processes, including transport, exocytosis, response to stress, immune system pathways, cytoskeleton organization, translation, RNA processing, RNA stability or protein folding. These proteins facilitate the initial steps of infection by enhancing viral entry, translation initiation and viral RNA synthesis. The role of ApoE in HBV infection, particularly, in the secretion and entry of HBV, has been recently established. It has been demonstrated that ApoE-specific monoclonal antibodies can effectively capture HBV particles,

similar to anti-HBsAg antibodies, thereby blocking HBV infection³⁸⁶. Additionally, using CRISPR/Cas9 technology to silence ApoE expression resulted in a significant decrease of more than 80% in HBV particles secretion³⁸⁶. Indeed, intracellular interactions between ApoE and HBsAg have also been observed. There is evidence suggesting that FXR may regulate the hepatic expression of ApoE. Specifically, the FXR agonist GW4064 has been shown to increase the expression of ApoE intracellularly in Huh7 and HepG2 cell models³⁸⁷. Moreover, FXR activation is associated with a decrease in lipoproteins secretion. Thus, a dysregulation of ApoE expression induced by FXR activation could impact HBV infection. The effects of FXR ligands on ApoE expression and secretion require further analyses in appropriate *in vitro* models, such as dHepaRG cells and PHH. ApoE may play a similar role in the entry and secretion of HDV, but further investigations are needed to confirm this. Antiviral factors can be also incorporated into viral particles, where they interfere with the early stages of infection. To investigate this hypothesis, a proteomic analysis of the virions will be performed. However, obtaining sufficient material for the analysis is challenging in dHepaRG cells unless large quantities are produced, which is hardly feasible. Therefore, a cell line called Huh7-2C8D, which stably expresses HBsAg and HDV and produces virions in large quantities, will be utilized³⁸⁸. It was tested whether the observed antiviral effect on specific infectivity in dHepaRG cells could be also observed in Huh7-2C8D. Treatment with GW4064 also resulted in a decrease in both the secretion and infectivity of HDV particles (*data not shown*). Then, the focus shifted to optimizing the conditions for the production of virions with optimal quantity and quality for proteomic analysis (*Annex 7*). One approach considered to improve the quality was the production of infectious HDV particles without serum to avoid albumin. It was demonstrated that the production of HDV particles without serum is feasible (*Annex 7*). Efforts are currently underway to develop a purification method for the viral particles (*Annex 7*).

The majority of experiments conducted in this study used dHepaRG or PHH as *in vitro* models, which are considered to be the most relevant for studying HDV infection. However, the antiviral effects of FXR ligands have not been evaluated *in vivo*. It would be valuable to perform *in vivo* studies to confirm the results obtained *in vitro*. The use of chimpanzees has provided valuable insights into the study of HDV in the presence of HBV infection and a complete immune system. However, ethical concerns limit their use as a research model. Alternative models are needed to study the full life of both viruses. One challenge is the difference in the NTCP protein sequence between and other mammals, such as mice, which cannot be naturally infected by HDV and HBV³⁸⁹. To overcome this, immunocompetent adeno-associated vectors (AAV)-based mouse models have been used to deliver HDV and HBV replication-competent genomes³⁹⁰. While these models can mimic acute severe liver damage, HDV persistence is limited and the investigation of HDV entry is challenging. An interesting approach involves the use of human liver chimeric mouse models (uPA/SCID/beige mice), where permissive human cells for the viruses are transplanted into the mouse liver³⁹⁰. Although these models lack adaptive immune response and can be costly, they support the full life cycle of HBV and HDV, including efficient HDV spreading and long-term persistence. The identification of NTCP as the common entry receptor of HBV and HDV has opened up new possibilities for the development of immunocompetent mouse models to study HDV infection as transgenic mice exogenously expressing human NTCP. The generation of these models is relatively straightforward and is not affected by donor-to-donor variability. However, there are major limitations, including the lack of HBV infection and short duration of HDV infection³⁹¹.

Another limitation of this study is that only the predominant HDV genotype, genotype 1, is tested. It would be beneficial to conduct the same experiments with other genotypes to determine whether the antiviral effect of FXR ligands is consistent across different genotypes.

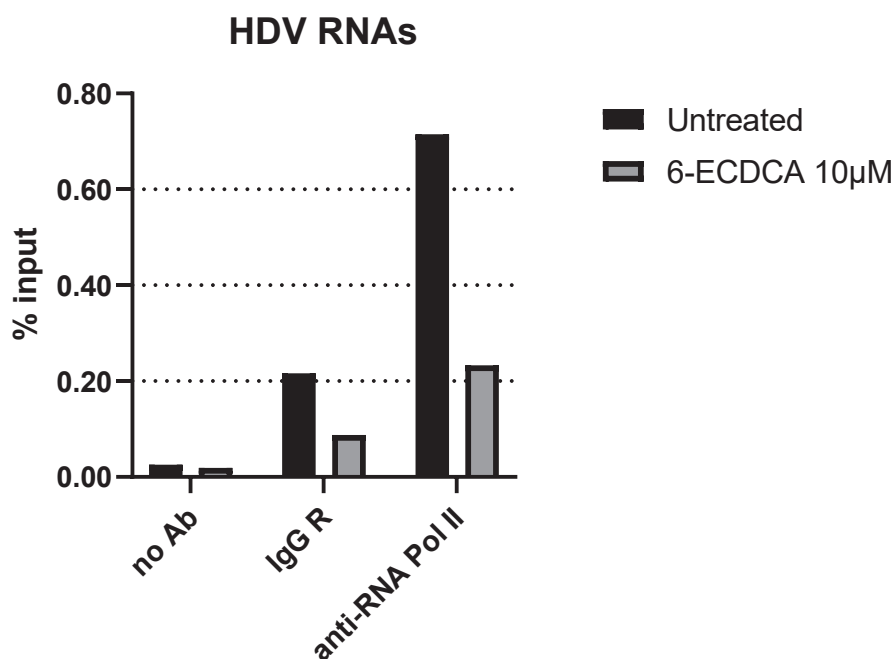
For example, it has been observed that genotype 5 shows a better treatment response to PEG-IFN-2a compared to genotype 1³⁹².

In conclusion, we demonstrated that FXR represents a promising therapeutic target against HDV infection. FXR ligands have shown inhibitory effect on HDV replication, primarily affecting the specific infectivity of HDV particles. Furthermore, FXR ligands target various steps in the HDV life cycle. Ongoing investigations aim to uncover the underlying mechanisms responsible for the antiviral effects of FXR ligands. Additionally, FXR ligands exhibit antiviral activity against HBV as well. Some FXR ligands have already received approval for the treatment of PBC and NASH, and a phase II clinical trial is currently underway to assess the potential of FXR ligands in patients with chronic HBV infection. Therefore, FXR ligands could be rapidly evaluated in patients coinfecting with HDV and HBV. The current treatments (IFN α and Bulevirtide) or those under investigation appear to be ineffective or have limited efficacy as monotherapy. Achieving HDV eradication may require a combination of molecules with additive or synergistic effects. Combining IFN therapy with FXR ligands could be a potential approach for treating patients with HDV infection and may result in complementary or synergic effects, enhancing overall antiviral efficacy. This type of combination strategies holds promise as a therapeutic approach for patients infected with HDV and HBV.

Part 7: Annex

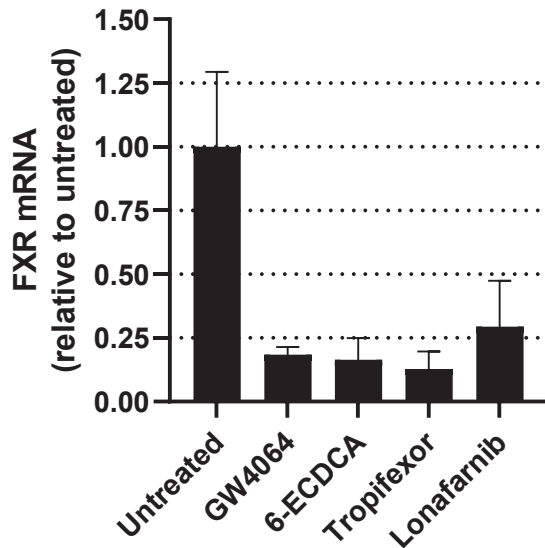
Annex 1: FXR ligands reduce the association between HDV RNAs and RNA Pol II

To investigate the impact of FXR ligands on intracellular HDV RNAs replication, we performed RNA immunoprecipitation (RIP) analysis to assess the association of RNA Pol II with HDV RNAs in the presence of FXR ligands. For this purpose, dHepaRG cells were monoinfected with HDV. After one day of infection, the cells were treated or not with 6-ECDC A for 10 days. Following the treatments, cells were lysed and subject to immunoprecipitation using either IgG rabbit, or RNA Pol II antibodies. Subsequent RNAs extractions and RT-qPCR were performed to detect HDV RNAs after immunoprecipitation. Interestingly, we observed a significant decrease of more than 50% in the levels of RNA Pol II associated with HDV RNAs upon 6-ECDC A treatment compared to the untreated condition. A reduction in the quantity of RNA Pol II associated with HDV RNAs may explain at least in part the decrease in intracellular virus replication. This observation should be confirmed by repeating the experiment several times and with other FXR ligands.



Legend: *6-ECDC*A reduces the levels of RNA Pol II associated to HDV RNAs. dHepaRG cells were coinfecteD with HDV at multiplicity of infection of 50 vge per cell. 1 day post-infection, cells were treated or not with 6-ECDC A (10 μ M) for 10 days. 11 days post-infection, cells were lysed before immunoprecipitation with the indicated antibodies (no Ab: no antibody, IgG R: IgG rabbit, anti-RNA Pol II). RNAs were extracted from the immunoprecipitated materials and the levels of HDV RNAs associated to the indicated proteins were analyzed by RT-qPCR. Results were obtained from a single experiment.

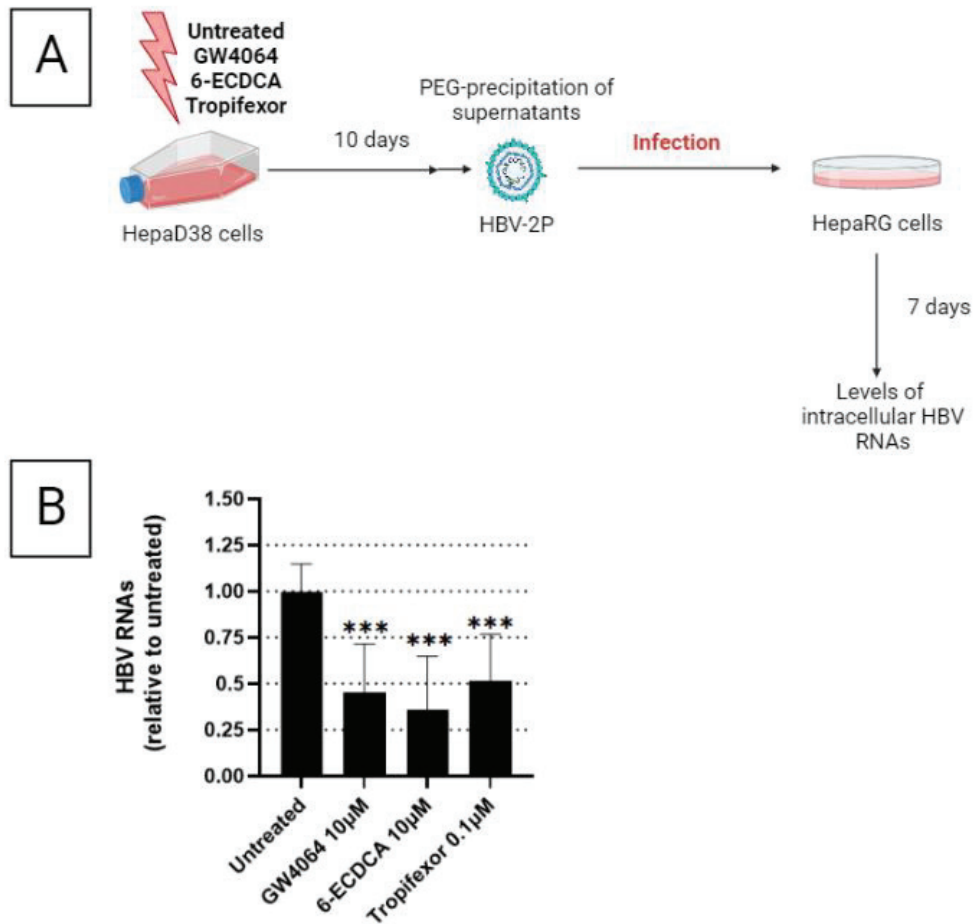
Annex 2: Lonafarnib decreases the expression of FXR



Legend : *Lonafarnib decreases the expression of FXR. dHepaRG were coinfecting with 500 or 50 vge per cell with HBV and HDV respectively. 1 day post-infection, cells were treated or not either with GW4064 (10 μ M), 6-ECDC (10 μ M), tropifexor (0.1 μ M) or lonafarnib (5 μ M). 10 days later, levels of FXR mRNA were assessed by RT-qPCR. Results of RT-qPCR are the mean of \pm SD of a single experiment performed with 3 biological replicates.*

Annex 3: FXR ligands decrease specific infectivity of HBV particles

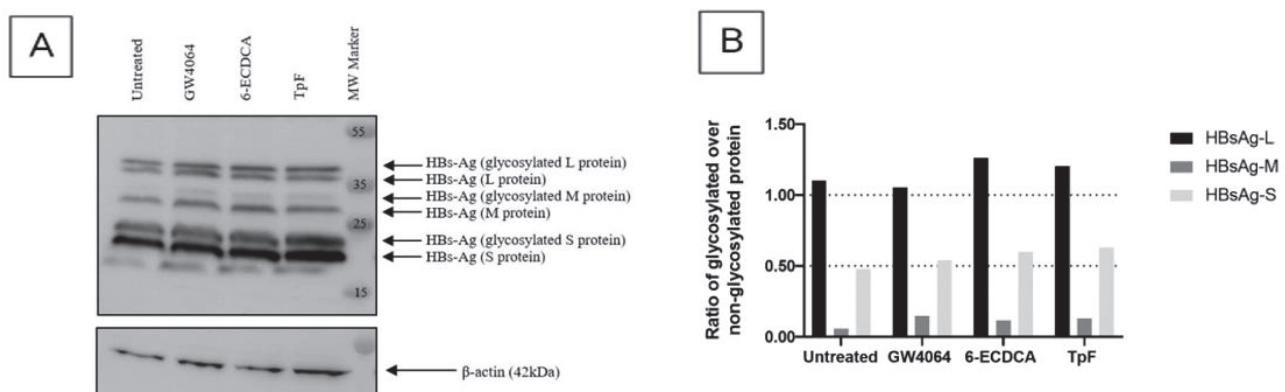
We also aimed to assess the impact of FXR ligands on specific infectivity of secreted HBV particles. To this purpose, HepaD38 cells, which constitutively express HBV and secrete infectious particles, were treated with either GW4064, 6-ECDCA or tropifexor for a duration of 10 days, following the protocol outlined in (A). At the end of treatments, the supernatants of HepaD38 cells were collected. To determine the specific infectivity of secreted HBV particles, viral particles in the supernatants were concentrated using PEG precipitation, and dHepaRG cells were infected with the same vge for each condition, *i.e.* 500 vge per cell. Subsequently, 7 days postinfection, the total intracellular HBV RNAs were quantified by RT-qPCR. Total intracellular HBV RNAs in dHepaRG cells decreased by more than 50% when the cells were infected with virus produced by HepaD38 cells treated with FXR ligands compared to untreated condition (B). Overall, these results showed that FXR ligands significantly reduced HBV virions specific infectivity. The reduction in infectivity of HBV particles after FXR ligands appears to be less pronounced than that observed for HDV particles. However, it should be noted that these differences could be attributed to the distinct cellular models used for each virus.



Legend: *Impact of FXR ligands on HBV specific infectivity.* HepaD38 cells were treated or not with GW4064 (10µM), 6-ECDCA (10µM) or tropifexor (0.1µM) for 10 days. (A) Schematic representation of the experimental procedure. (B) dHepaRG cells were infected with the supernatants (HBV-2P) with 500 vge per cell. 7 days later, levels of intracellular HBV RNAs were assessed by RT-qPCR analyses. Results of RT-qPCR are the mean of \pm SD of 2 independent experiments each performed with 3 biological replicates. Student *t* test, *** $p < 0.001$.

Annex 4: Impact of FXR ligands on intracellular HBs glycoproteins

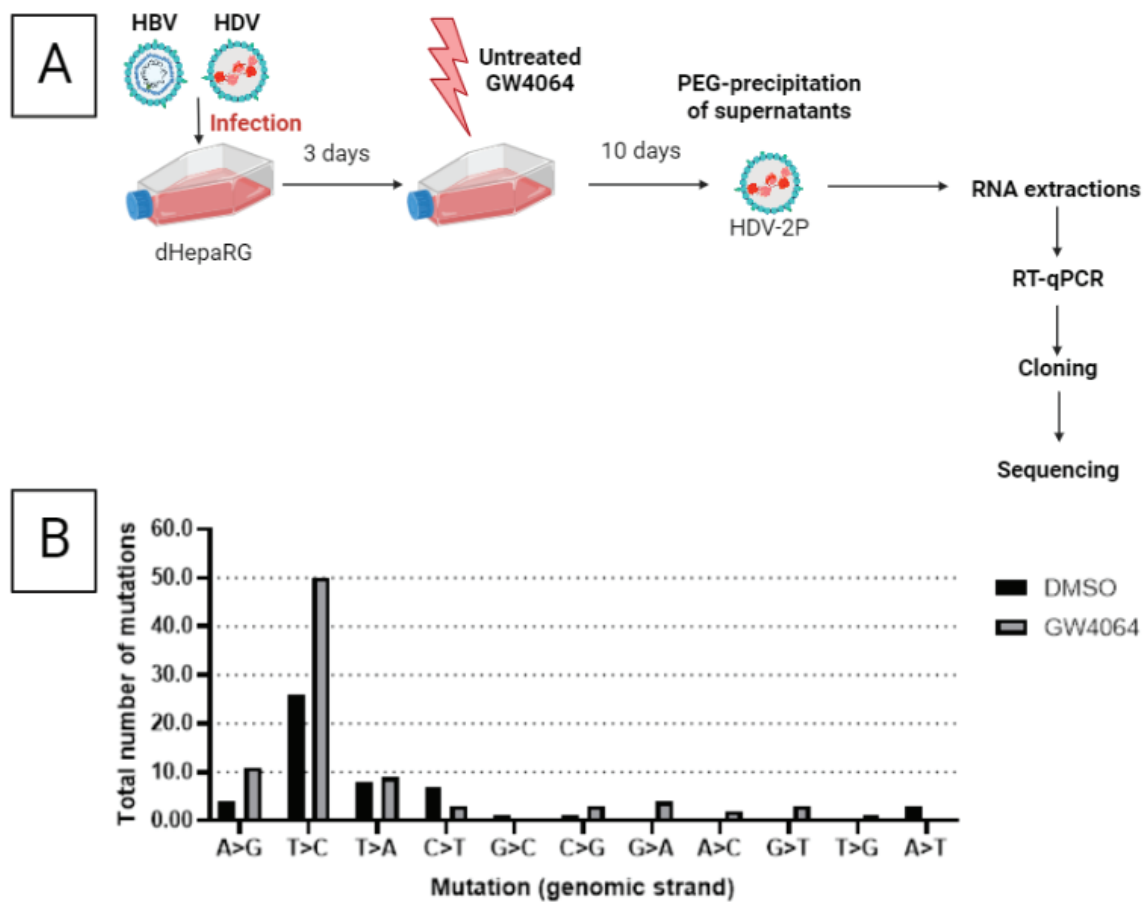
To investigate the potential impact of FXR ligands on the glycosylation status of HBsAg, HepaD38 cells were treated or not with FXR ligands for 10 days. Through Western blot analysis, we examined the intracellular levels of HBsAg expression as well their glycosylation status. (*A and B*). The ratio of glycosylated to non-glycosylated forms of each HBsAg showed no significant modifications following FXR ligands treatment.



Legend: *Impact of FXR ligands on intracellular HBsAg.* HepaD38 cells were treated with GW4064 (10 μ M), 6-ECDCA (10 μ M), or tropifexor (0.1 μ M) for 10 days. Cells were harvested for protein extraction. (A) Levels of HBsAg protein were analyzed by Western blot, using β -actin as a loading control. (B) The ratio of glycosylated to non-glycosylated forms was calculated for S-, M- and L-HBsAg by densitometry analysis using ImageQuand TL software.

Annex 5: GW4064 seems to induce mutations in HDV genome

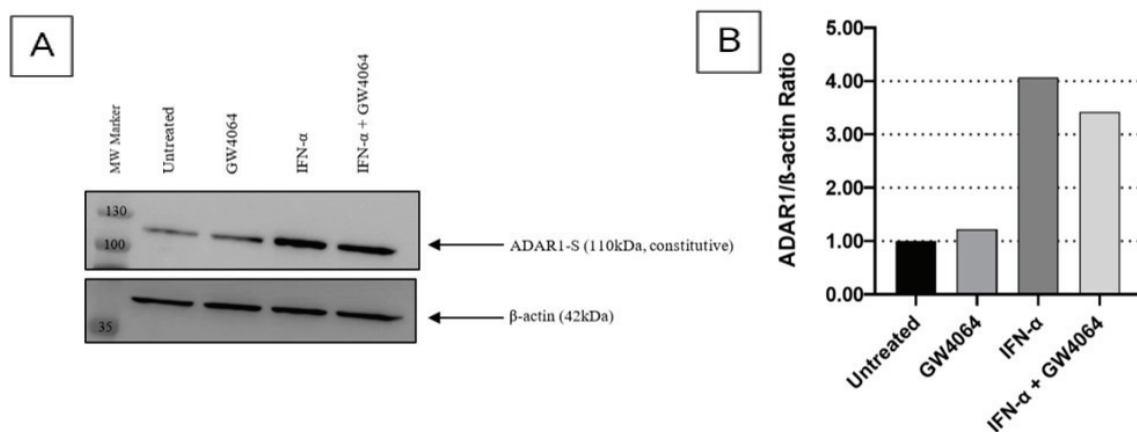
To investigate the hypothesis attributing the loss of HDV infectivity to the encapsidation of replication-defective viral genomes, supernatants from coinfecting HepaRG cells treated or not with GW4064 were collected. Secreted HDV RNAs were extracted, reverse transcribed, and amplified into three fragments covering the whole genome, which were then cloned and subjected to Sanger sequencing, as described in (A). An overall increase in the mutation rate was observed following GW4064 treatment compared to control. Two predominant types of mutations were observed, resulting in an increased number of A to G and T to C substitutions. These mutations may be related to ADAR-type mutations.



Legend: *HDV whole genome sequencing following treatment with FXR ligand GW4064. (A) dHepaRG cells were coinfecting with 500 and 50 vge per cell with HBV and HDV respectively. Cells were treated or not 1 day later with GW4064 (10 μ M) for 10 days. Supernatants were collected, secreted HDV RNAs were extracted, reverse-transcribed and amplified using a set of primers surrounding the HDAg ORF and the ribozyme region. Amplified sequences were cloned, sequenced and aligned to the HDV genotype 1 sequence (20 clones per condition). (B) Quantification of each type of mutations found on HDV genome following treatment or not with GW4064.*

Annex 6: FXR ligand GW4064 does not modify the expression of ADAR1

Our preliminary data indicated a rise in putative ADAR-type mutations in the HDV genome following treatment with GW4064. Thus, we assessed the impact of FXR ligands on ADAR1 protein expression. Non-infected dHepaRG were treated for 10 days with either GW4064, IFN α or a combination of both. Treatment with IFN α resulted in a four-fold increase in intracellular ADAR1 p110 protein levels, while treatment with GW4064 did not appear to significantly modify ADAR1 levels (*A and B*). Surprisingly, no induction of interferon-inducible p150 ADAR1 isoform was observed.

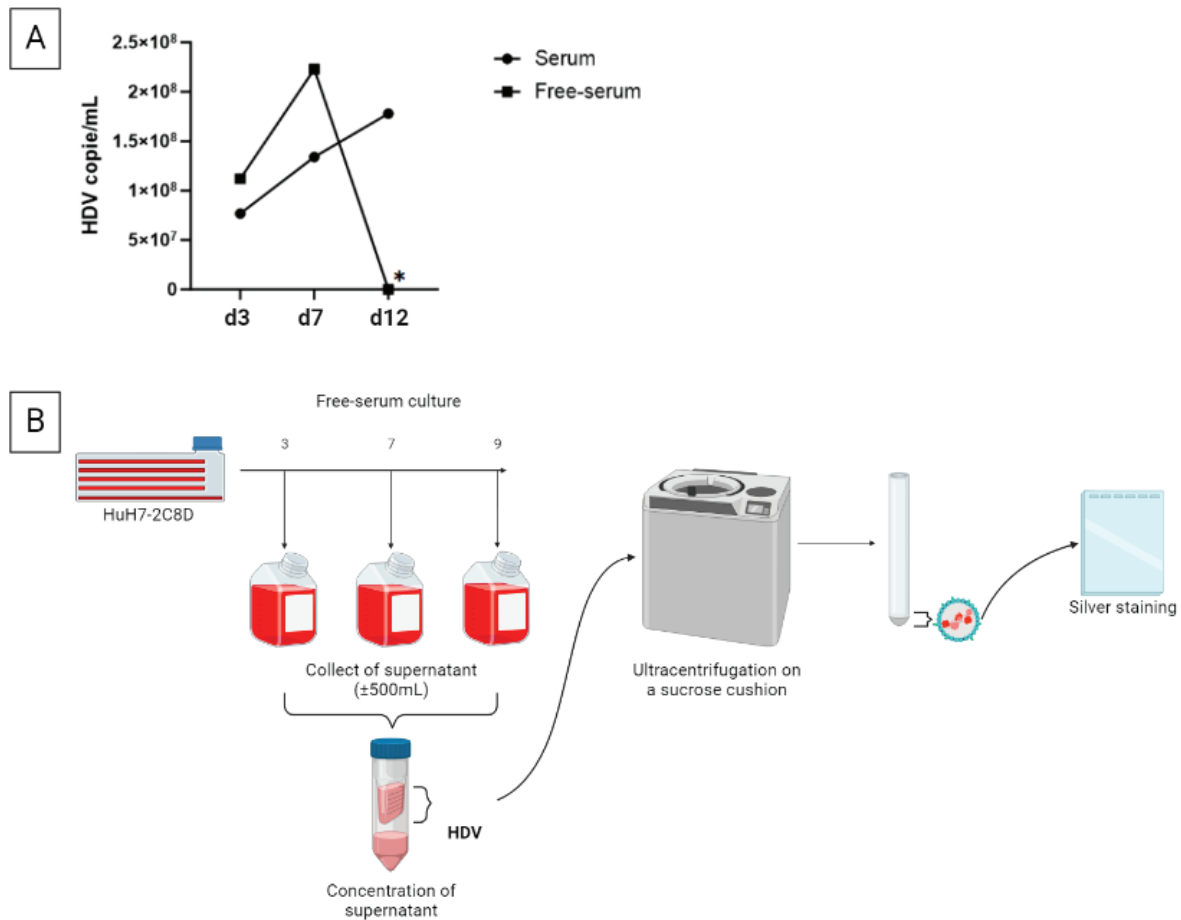


Legend: *Effect of FXR and interferon treatments on ADAR1 levels. dHepaRG cells were treated with GW4064 (10 μ M) and IFN α (100 UI/mL) alone or in combination. Cells were harvested 10 days post-treatment for protein extraction. (A) Levels of ADAR1 proteins were analyzed by Western blot using the β -actin as a loading control. (B) Densitometry analysis was performed using the ImageQuand TL software. Results from one experiment are presented as ratios of ADAR1 to β -actin intensity levels, normalized to levels in untreated cells. MW marker: Molecular weight marker.*

Annex 7: Optimizing HDV viral production for virion proteomic analysis

The primary objective for proteomic analysis is to generate a substantial quantity of virions under optimal conditions. This entails considering both the quantity and quality of the produced virions. For quality assessment, the presence of serum components such as albumin needs to be considered. Thus, the ability of HuH7-2C8D to produce viral particles without serum was investigated. Cells were cultured both with and without serum, and the supernatant from each condition was collected at different time point (day 3, 7 and 12). Secreted HDV RNAs were extracted and qRT-PCR were performed. Even in the absence of serum, the cells produced virions in equal or greater quantities compared to cells cultured in serum at day 3 and 7 (**A**) However, it is important to note that serum-free cell culture should not exceed 12 days, as prolonged culture without serum can lead to cell death.

To achieve sufficient and quality viral materials, the detailed procedures are illustrated in (**B**). It has been estimated that approximately 10^{11} viral particles are needed for proteomic analysis per condition. Optimization of the concentration and purification steps is currently underway.



Legend: Development phase for proteomic analysis. (A) Huh7-2C8 cells were cultivated in the presence or absence of serum for 12 days. The supernatants were collected at day 3, 7 and 12, and the levels of extracellular HDV RNAs were quantified by qRT-PCR analyses. * At day 12, massive cell death was observed in cells cultivated in serum-free medium. Results of qRT-PCR are derived from a single experiment with one biological replicate. (B) Schematic representation of the protocol used to produce HDV particles for proteomic analyses. Huh7-2C8D are culture in serum-free hyperflasks for a duration of 9 days. After harvesting, the supernatants undergo filtration and concentration using centrifugal filters columns. Subsequently, the concentrated HDV particles are subjected to sucrose cushion ultracentrifugation overnight to achieve purification. The purified materials are then collected and subjected to silver protein staining to evaluate both the quality and quantity of samples.

Part 8: Appendices

I- Oral presentation

- Annual meeting of the ANRS AC42 – May 2021
- Virology Specialty Day – June 2023, Lyon, France

II- Poster presentation

- International HBV Meeting - September 2022, Paris, France

III- Publications

- *Inducers of the NF- κ B pathways impair hepatitis delta virus replication and strongly decrease progeny infectivity in vitro*, 2022, *JHEP Report*

Maud Michelet^{1 †}, Dulce Alfaiate^{2 † #}, Brioux Chardès^{1 †}, Caroline Pons^{3 #}, Suzanne Faure-Dupuy⁴, Thomas Engleitner⁵, Rayan Farhat¹, Tobias Riedl⁴, **Anne-Flore Legrand**³, Roland Rad⁵, Michel Rivoire⁶, Fabien Zoulim^{1 7}, Mathias Heikenwälder⁴, Anna Salvetti^{3 #}, David Durantel^{3 ‡ #}, Julie Lucifora^{3 ‡ #}

- *Hepatitis D virus interferes with hepatitis B virus RNA production via interferon-dependent and independent mechanisms*, 2023, *Journal of Hepatology*

Julie Lucifora^{1,2#}, Dulce Alfaiate², Caroline Pons^{1,2#}, Maud Michelet², Ricardo Ramirez³, Floriane Fusil¹, Fouzia Amirache¹, Axel Rossi⁴, **Anne-Flore Legrand**¹, MéliSSanne De Wispelaere², Serena Vegna², Rayan Fahrath², Michel Rivoire⁵, Barbara Testoni², Charlotte Bach⁶, Thomas F. Baumert^{6,7}, Eloi R. Verrier⁶, Anastasia Hyrina³, Rudolf K. Beran³, Fabien Zoulim^{2,8}, Andre Boonstra⁹, Hildegard Büning⁴, François-Loïc Cosset¹, Simon P. Fletcher³, Anna Salvetti^{1,2#,*} and David Durantel^{1,2#,*}

- *Rôle du métabolisme cellulaire dans le contrôle des hépatites virales chroniques*, 2023, *Médecine / Sciences*

Diaz Olivier¹, **Legrand Anne-Flore**^{1,*,}, El-Orch Walid^{1,*,}, Jacolin Florentine^{1,*,}, Vincent Lotteau¹, Ramière Christophe^{1,2}, Vidalain Pierre-Olivier¹ et Laure Perrin-Cocon^{1,#}.

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