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Novel entry pathways for Hepatitis B virus

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Authorship Declaration

I, Devan Parmar, declare that this thesis represents
my work

Where information has been derived from other
sources, I confirm that this has been indicated in the
thesis.

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Abstract

Hepatitis B virus (HBV) is a global health problem. Patients with chronic hepatitis B (CHB) infection can develop progressive liver disease, including cirrhosis and hepatocellular carcinoma (HCC). There is still much to understand about the molecular biology of HBV, particularly viral entry. The main entry receptor of HBV is sodium taurocholate co-transporting polypeptide (NTCP); however, a variant of HBV, BT10D4 has recently been discovered, that infects cells independent of NTCP expression. This study aimed to demonstrate NTCP-independent infection by BT10D4 and to elucidate the alternative receptor involved in entry of this variant. The methodology used to investigate the entry receptor is known as pseudotyping, a technique utilised to produce 'customized' chimeric viruses that possess envelope glycoproteins that are not encoded by the viral genome. In this case, the pseudotype has an HBV glycoprotein and a HIV-1 capsid containing a luciferase reporter gene, allowing quantification of infection into susceptible cells. Pseudotypes were used to infect the hepatoma cell line Huh7, and Huh7 cells induced to express NTCP. The pseudotype assays clearly demonstrated that BT10D4 facilitated NTCP-independent infection. Furthermore, the receptor annexin A2 was identified as a candidate for BT10D4 entry. Neutralisation and binding assays using anti-annexin A2 and soluble annexin A2 were performed. Whilst statistical analyses showed no significant change in infection, Huh7.NTCP cells pre-treated with anti-annexin A2 and infected with BT10D4 showed a reduction in infectivity, as the concentration of anti-annexin A2 increased. The inhibition assay was repeated using the soluble form of annexin A2 but showed no resolvable inhibition or trend. However, it is important to note that the conformation of the soluble form of annexin A2 could be different to its conformation on the cell surface. Annexin A2 doesn't not appear to be an entry receptor for HBV and further studies should be performed to identify alternative candidates, which would aid in the development of novel therapeutic strategies to combat HBV entry that is NTCP-independent.

1 Introduction

1.1 Global Burden of disease

Infection with Hepatitis B virus (HBV) is a global threat to human health (Asrani et al., 2019). The latest statistics from the World Health Organisation estimate that 2 billion individuals have been infected with HBV (WHO, 2021), including 400 million chronic carriers, most of whom live in developing countries (Gish & Cooper, 2011 Ginzberg et al., 2018). The development of CHB infection is associated with risk of progressive liver disease, including cirrhosis and HCC. Of note is that more than 200,000 CHB patients die annually from cirrhosis and greater than 300,000 from HCC worldwide (Perz et al., 2006), making HBV the second commonest carcinogen after tobacco.

1.2 Discovery and History.

Hepatitis B surface antigen (HBsAg), the HBV envelope protein was discovered by Baruch Blumberg in Philadelphia in 1967 for which he received the Nobel Prize in 1977. Further studies by David Dane led to the discovery of the presence of intact hepatitis B virus called Dane particles in the blood of hepatitis patients (Dane et al., 1970). The Dane particle is the infectious intact virion responsible for HBV infection (Figure 1).

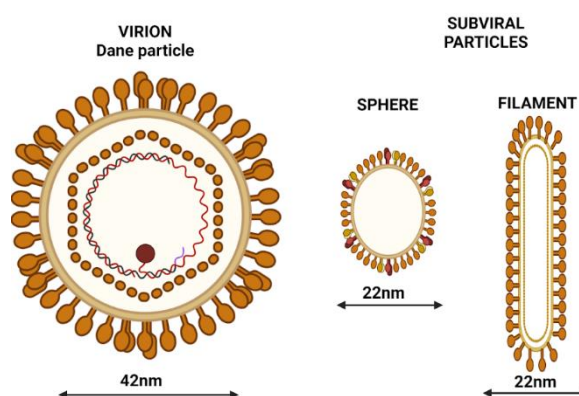


Figure 1: The Hepatitis B virion and subviral particles. HBV exists in two forms: (1) The infectious virion, the Dane particle, which is approximately 42 nm in size and contains both capsid and genome; and (2) The subviral particles, which are approximately 22 nm in size and lack capsid and genome. This illustration was created in <http://biorender.com/>.

1.3 Epidemiology, transmission, and prevention

CHB infection is thought to affect 0.3% of the UK population amounting to approximately 180,000 people (GOV.UK, 2021). The bulk of newly diagnosed cases (> 95%) arise from the immigration of people from high-prevalence areas (Martin et al., 2019). Because acute HBV infection is typically accompanied by a lack of particular symptoms, there is concern that an increasing number of people with chronic HBV infection are going undiagnosed.

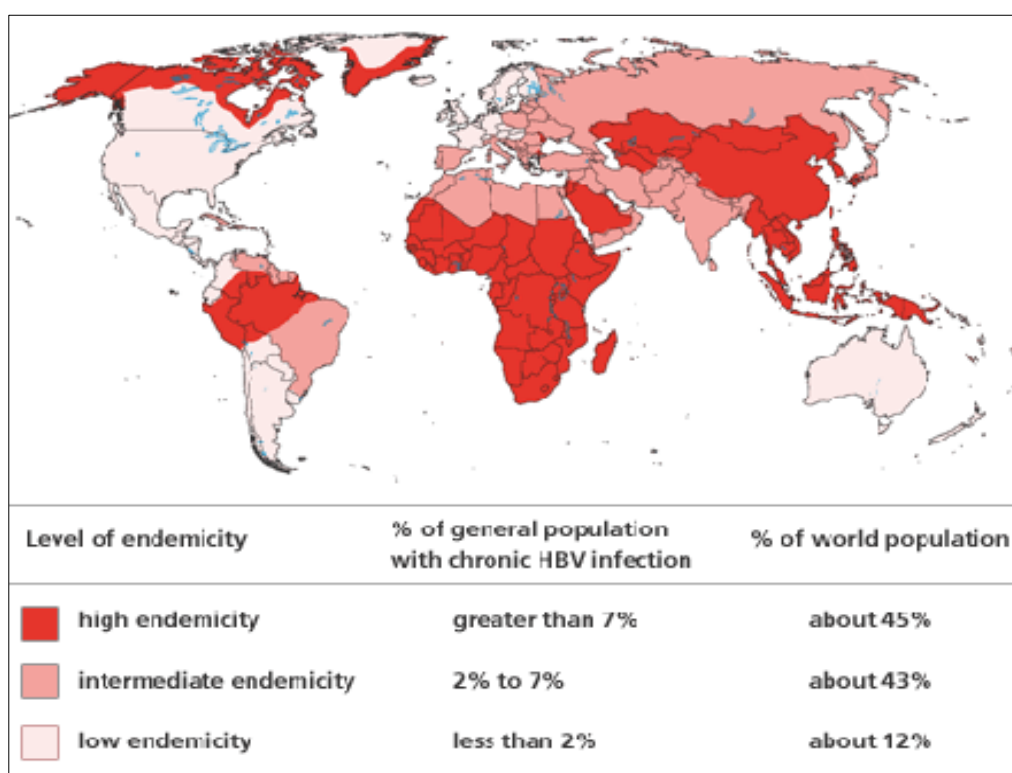


Figure 2: Global prevalence chart for chronic Hepatitis B Infection. The shades of red indicate the level of endemicity. Adapted from: (Gish and Cooper., 2011).

HBV is a highly communicable virus and is 50–100 times more infectious than HIV by (WHO, 2021), and can survive outside the body for more than 7 days. HBV is principally transmitted through contaminated blood or body fluids (Kwon & Lee, 2011). However, HBV has also been detected in semen, vaginal secretions, sweat, saliva, tears, urine, stools and breast milk (Kidd-Ljunggren et al., 2006). In endemic areas of the world, such as China & sub-Saharan Africa, HBV is mainly acquired vertically (Umar et al., 2013). In areas of low prevalence, horizontal

transmission is the most common mode of infection spread (Inoue & Tanaka, 2016). Other significant modes of transmission include occupational infection through exposure to contaminated body fluids and sharing of drug paraphernalia amongst intravenous drug users (Seal, 2000). Occasional outbreaks have also been reported as a consequence of unsterilized equipment during tattooing, body piercing and acupuncture.

The HBV vaccine currently used in the UK contains the hepatitis B surface antigen (HBsAg), a viral envelope protein that induces a protective immune response in approximately 85-90% of vaccinated individuals. Initially, it was believed that the vaccine only provided effective protection for 5-7 years, and booster doses were recommended to maintain long-term immunity (Petersen et al., 2004). However, it was later recognised that long-term immunity is maintained through immune memory that persists even after antibody levels have declined, and that booster doses are not necessary for immunocompetent individuals (Pol, 2013). Therefore, the hepatitis B vaccine is now considered to provide indefinite protection.

Presently, more than 197 countries worldwide include the hepatitis B vaccine in their national immunisation programmes. The UK adopted HBV vaccination for all new-born babies on the 1st of August 2017 and in conjunction there is a policy of pre-exposure immunisation in high-risk groups due to lifestyle, occupation (e.g., healthcare workers) or other risk factors such as travel to areas of high endemicity. Vaccination post-exposure is also recommended particularly in infants born to infected mothers or after needle stick injuries.

1.4 HBV Structure

The infectious virion is spherical with a diameter of 42nm and includes an open circular DNA genome consisting of a full-length 3.2kb minus-strand with an incomplete positive-strand whose circularity is maintained by 5' cohesive ends and a complete negative strand. The virus encodes

its own polymerase, which has reverse transcriptase/DNA polymerase and RNaseH activities. The viral genome is enclosed in an icosahedral nucleocapsid and encodes four open-reading frames (ORF): the core ORF; polymerase ORF; small, middle, and large surface ORF, and the X ORF (Scaglioni et al., 1996). The structure and organisation of the HBV genome are illustrated in Figure 3.

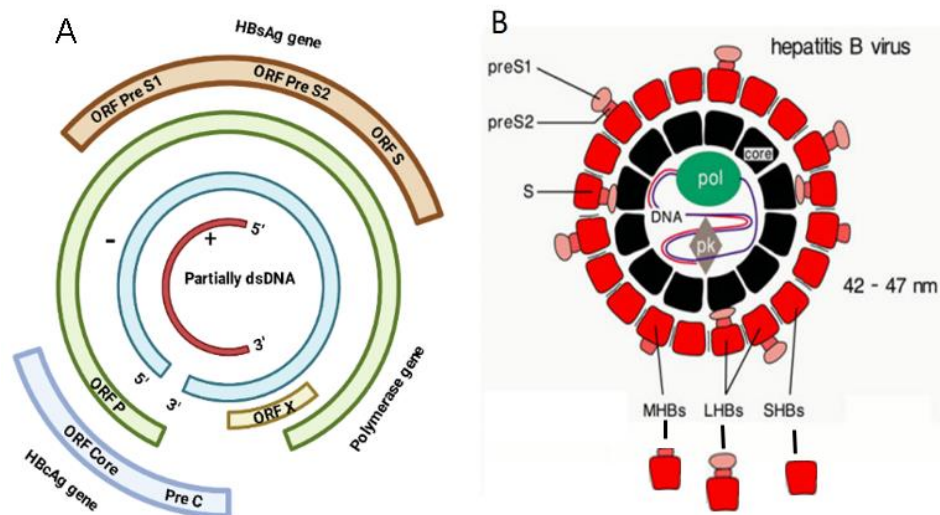


Figure 3: Structure and organisation of the HBV virion and genome. **Panel A** shows the different protein-coding genomic regions, which are represented by the semi-circular shapes. These regions are the ORF (Open Reading Frame) for pre-Core (pre-C); ORF for Core gene (C gene); the ORF for X gene and the Polymerase gene (P-gene). The other regions include the ORF pre-S1, ORF pre-S2 and the ORF S-gene. **Panel B** illustrates the structure of the HBV virion, which include the three viral envelope proteins: S (small protein), pre-S1 (L or Large protein) and pre-S2 (M or Medium). Adapted from: (Gerlich et al., 2010).

The preS-S ORF encodes three viral envelope proteins: small (SHBs), medium (MHBs) and large proteins (LHBs). The SHBs are contained within the carboxyterminal domain, is encoded in all three surface proteins. Only in LHBs are there preS1 N-terminal extensions. PreS2 is present in LHBs and MHBs (Figure 2B) (Churin et al., 2015). Of the HBV surface antigens, the S protein is one of the most abundant and is used to diagnose HBV infection. The presence of this HBsAg for >6 months in the serum of patients defines the presence of chronic infection and denotes active HBV replication (McQuillan et al., 1999) (See Section 1.9

Diagnosis). In addition to the phospholipid membrane, the HBsAg makes up the outer spherical envelope of the virion. However, HBsAg subviral particles are also released into the circulation outnumbering infectious virions by >10000 fold. (Ganem & Prince, 2004). The preC-C ORF encodes both hepatitis B early antigen (HBeAg) and hepatitis B core antigen (HBcAg). Although HBcAg forms the inner core protein shell referred to as the 'core particle' or capsid (Figure 3B.), the HBeAg does not have an essential role for replication or infection (D. Milich, 2003). It is produced during HBV replication in infected hepatocytes and secreted into the circulation. Its precise function is still unclear, but it is thought to act as a tolerogen and seems important for the persistence of HBV infection by shifting the immune response to an immunotolerant Th2 profile (R. Milich et al., 1990). The presence or absence of HBeAg and antibodies to this protein (anti-HBe) defines the stage of CHB infection (see Section 1.6 Natural History of hepatitis B infection) and is important in the optimum management of these cases (see treatment Section 1.8. Treatment) (Valsamakis, 2007). The P-ORF encodes the polymerase which is involved in viral replication. During the viral life cycle, HBV DNA enters into a pre-genomic RNA phase, which requires reverse transcriptase-polymerase produced by the P gene to reverse-transcribe the RNA back to negative-strand DNA as part of the replication process. This process is therapeutically targetable (see section on nucleos(t)ide analogues). The X ORF encodes the hepatitis B X antigen (HBxAg) and is conserved amongst all major HBV genotypes. It is believed to have multi-functional roles at different stages of HBV infection and with host processes including transactivation via DNA-binding and signalling pathways involved in the cell cycle (Hernández et al., 2012). HBx is also thought to play an important role in carcinogenesis (Lupberger, 2007).

1.5 Hepatitis B virus subtypes and genotypes

The surface antigen of HBV includes an immunodominant and immunoprotective area known as the 'a' determinant where most epitopes involved in the binding of neutralising antibodies are localised. This 'a' determinant is common to all subtypes, but additional

determinants have also been reported which include d, y, w and r (Golsaz-Shirazi et al., 2016). The difference between these determinants resides in the change of one amino acid at position 122 for d and y and position 160 for w and r. A total of 9 serotypes were described: ayw1 to ayw4, ayr, adw2, adw4, adrq- and adrq+ and have been found to be geographically distributed (Golsaz-Shirazi et al., 2016). Additionally, to the subtypes, HBV is classified into genetic subgroups known as genotypes and the correspondence between subtypes and genotypes is shown in Table 1 (Kidd-Ljunggren et al., 2002).

Table 1: Correspondence between HBV subtypes and genotypes. Serotypes in brackets are the least common. Subtype correspondence with Genotype J is unknown but closely related to Genotype C. Adapted from: (Kidd-Ljunggren et al., 2002)

Genotype	A	B	C	D	E	F	G	H	I	J
Subtypes	adw2	adw2	adr	ayw2	ayw4	agw4g-	adw	adw	adw	
	(ayw1)	ayw1	adrq-	ayw3	(adw2)					
			ayr	ayw4						

Ten different HBV genotypes (A-J) have been identified, which are different by greater than 8% at the nucleotide level (Khan, 2020). The genotypes have different geographical distribution; Genotype A predominantly in USA, Genotype B & C mainly in Asia and the Far East, D in Europe and Russia, E dominates in Central Africa, F mainly in South America, G mainly in the Mexico and France, and H is the major genotype in Central and South America (Enomoto et al., 2006). Genotype I has recently been identified in Vietnam and Laos (Figure 4) and genotype J has been discovered in Japan. (Sunbul, 2014).

The link between the geographic distribution and the viral genotypes is mainly due to the route of transmission. For instance, genotypes B and C are more prevalent in Asia, where mother-to-child transmission is the most frequent method of transmission, whereas genotypes A and D are more prevalent in Europe and North America, where sexual contact or drug injection are more prevalent (Tong & Revill, 2016).

Viral genotype is associated with the replicative 'fitness' of the virus, the rate of disease progression and to treatment response rates (Enomoto et al., 2006). HBV genotype B is associated with slower disease progression compared with genotype C (Chan et al., 2004). Sustained response to pegylated interferon (PEG-IFN- α) is associated with genotype A, whereas patients with genotype D who receive PEG-IFN- α are less likely to acquire a sustained response to the treatment (Urban et al., 2021). The highest rates of HBeAg seroconversion in combination regimens of interferon plus nucleot(s)ide therapy have been reported in patients infected with genotype A (47%) (Janssen et al., 2005). However, most of these studies have compared Genotype A versus D or Genotype B versus C and further studies incorporating all genotypes equally for a head-to-head analysis would add greatly to our understanding of the importance of genotypes in treatment outcomes.

The reasons why genotypes differ in treatment outcomes is not well understood. It has however been suggested that may reflect alternations in viral sequences during interferon therapy and the host immune response (Yuen et al., 2007).

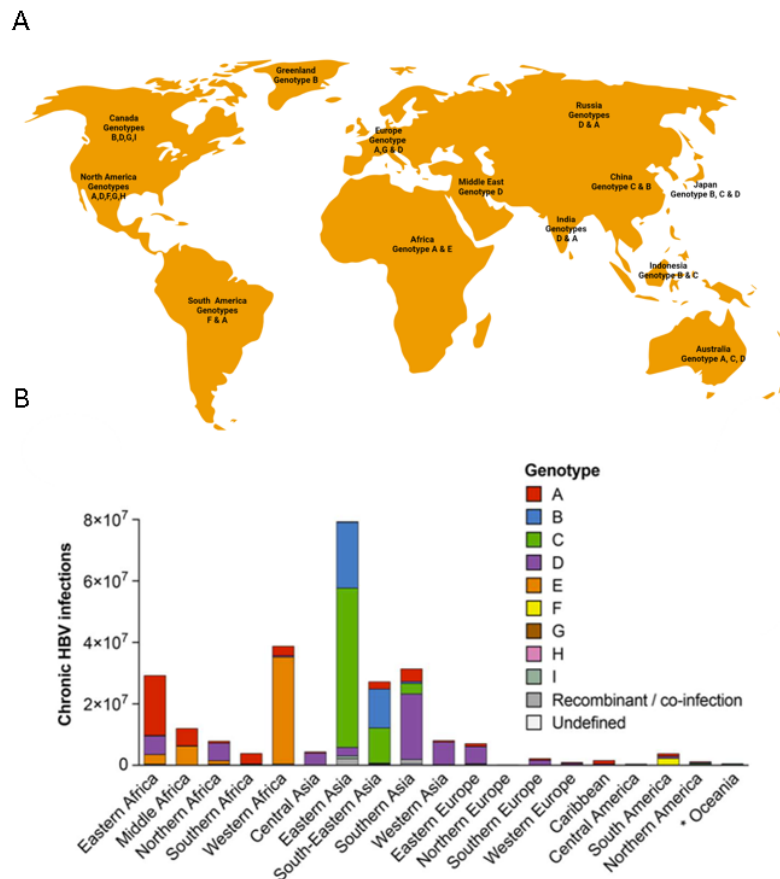


Figure 4: Global distribution of HBV genotypes. Panel A shows predominant genotypes in each area (This illustration was created in <http://biorender.com/>). Panel B represents approximate distribution of genotype to burden of CHB disease. Adapted from: (Sunbul et al., 2014).

1.6 Natural History of HBV infection

The varying clinical manifestations and outcomes of HBV infection are a reflection of the balance between viral and host factors. Some patients infected with HBV may develop clinical or subclinical acute hepatitis. In some cases, the infection may be self-limiting, and the patient resolves the infection without long-term clinical consequences. The antiviral immune response is robust in these patients and involves the cytolytic (clearance of infected hepatocytes) and non-cytolytic immune mechanisms against the virus. Whereas other patients fail to clear the virus and develop a persistent infection which can lead to cirrhosis and HCC. In immunocompetent adults' exposure to HBV results in a self-limited, transient liver disease with viral clearance in approximately 95% of patients who subsequently develop a lasting immunity. In contrast,

more than 90% of neonates exposed to HBV at birth become persistently infected. The distinction between the two outcomes after exposure to HBV - sustained control of infection or establishment of a persistent infection - may be rooted in the difference in the level of maturity of the host's immune system. Infected new-borns tend to tolerate virus replication without the induction of a vigorous immune response, a situation that invariably leads to chronic infection. In most immunocompetent adults, the immune response is robust, causing a more symptomatic acute infection resulting in spontaneous resolution. However once established, CHB is a complex and dynamic disease, and it is widely accepted that the interplay between HBV and the host immune responses determines the outcome of infection (Yuen et al., 2007).

1.6.1 Acute Hepatitis B

In acute infection there is an initial incubation phase which lasts 2 to 6 weeks, followed by acute hepatitis with raised serum aminotransferases for up to 6 months.

Symptoms can be varied in patients and can include loss of appetite, nausea, vomiting, malaise, fatigue, jaundice, and upper right quadrant abdominal pain. During the clearance of infected hepatocytes, serum alanine aminotransferase (ALT) levels may be highly increased to more than 100 times upper level of normal. Between 50 and 70% of patients, however, have an asymptomatic disease (Hann et al., 2008).

Self-limiting acute hepatitis with spontaneous resolution is characterised loss of HBsAg and HBeAg and presence of antibodies to HBsAg (anti-HBs) and HBeAg (anti-HBe) and the expansion of HBV-specific T-cells (Figure 5). These markers of infection will be discussed in greater detail in Section 1.9. Although patients who recover have clearance of HBsAg, they do not completely eradicate the virus, low-level viral replication due to the presence of covalently closed circular DNA (cccDNA) in hepatocytes may be lifelong even after HBsAg clearance. This dormant HBV infection can reactivate if for example, the subject is

immunosuppressed (B. Wang et al., 2019). A small proportion of patients with acute HBV infection will develop fulminant hepatitis (<1%) with massive immune-mediated destruction of infected hepatocytes (Djokic et al., 2003).

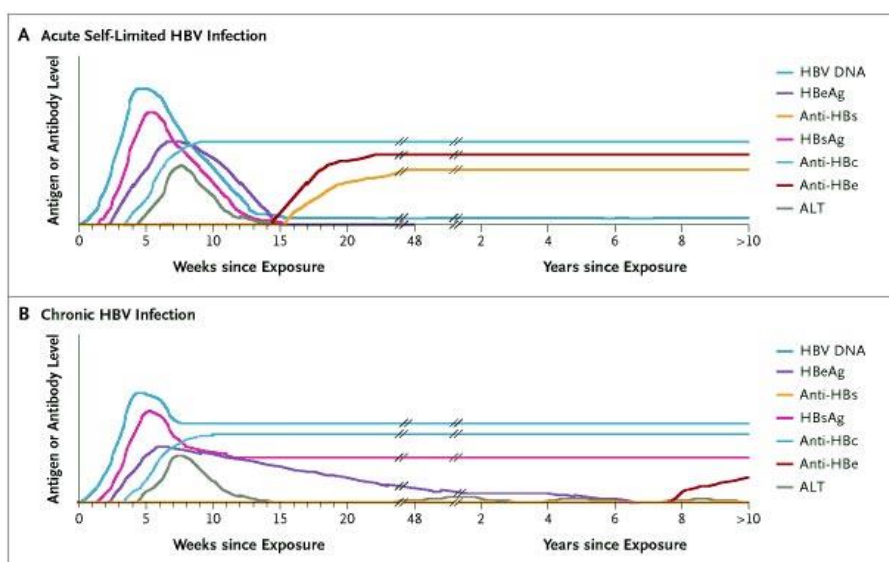


Figure 5: Natural history and clinical consequences of HBV infection. In both acute self-limited HBV infections (Panel A) and infections that progress into chronic infections, normal levels of alanine aminotransferase (ALT), HBV DNA, hepatitis B s and e antigens (HBsAg and HBeAg), and anti-HBc, anti-HBe, and anti-HBs antibodies are seen (Panel B). Diagrammatic representation of the intensity of the responses as a function of time after infection. Long after the resolution of an acute, self-limited infection, HBV DNA may still be present. Adapted from: (Ganem D et al., 2004)

1.6.2 Chronic Hepatitis B

CHB virus infection is defined by the presence of HBsAg in the serum of a patient for more than 6 months following exposure to the virus (see Figure 6 panel B). The majority of chronically infected patients remain asymptomatic for many years. During chronic infection, there is persistent injury due to immune mediated inflammation and killing of infected hepatocytes. This causes scarring (fibrosis) can progress to life-threatening cirrhosis, if the virus is not controlled (Suhail, 2014). Persistent viral replication coupled with liver inflammation and development of cirrhosis significantly increases the risk of developing HCC (Han & Kim, 2008). HBV is an oncogenic virus and there is a striking

overlap of HBV and HCC incidence geographically. The yearly risk of HBV related HCC in patients without cirrhosis, is between 0.02-0.6% depending on ethnicity, which significantly rises to 2.2% and 3.7% in patients with cirrhosis, in Caucasians and Asians respectively (Michielsen & Ho, 2011).

The natural history of chronic HBV and phases of infection are still not fully elucidated. It has long been recognized that phases of chronic HBV disease consist of an immunotolerant phase (or high-replication, low-inflammation phase), an immunoactive phase, an inactive carrier state (low replication levels and normal/nearly normal serum aminotransferase levels), and reactivated disease. These have been recently updated based on disease markers as assessed by diagnostic assay results and include HBeAg-positive infection, HBeAg-positive hepatitis, as well as HBeAg-negative infection and HBeAg-negative hepatitis. This is described in Figure 6.

Briefly, in children, the high viral replication, low-inflammation phase is associated is defined by serum HBV-DNA, HBsAg and HBeAg concentrations with mildly increases in transaminases and minimal liver disease. This phase may be followed by (1) an active inflammatory stage (also referred to as HBeAg hepatitis, or the immunoactive phase) or (2) HBeAg seroconversion and remission (inactive carriers). HBeAg-negative anti-HBe-positive disease (reactivation phase) is associated with progressive disease. HBeAg is not detectable in this phase because of mutant precore HBV, however hepatic HBcAg is detectable.

Characteristics Phases of Chronic Hepatitis B Note: CHB follows a nonlinear clinical course so not all patients go through each phase				
Immune-tolerant phase	Immune activation	Low replication	Reactivation	Remission
Occurs in patients with perinatally acquired infection	High or fluctuating HBV DNA levels	Low or nondetectable HBV DNA levels	Fluctuating ALT and HBV DNA levels	After many years, some patients may enter a remission phase
Minimal liver inflammation	High or fluctuating ALT levels	Normal ALT levels	Usually older patients with more advanced liver disease	Not considered a cure because trace HBV DNA is still present
May last from 1 year to decades	Active inflammation and liver damage	Mild hepatitis and minimal fibrosis	Chronic hepatitis	
Can lead to chronic infection	Can lead to chronic infection	Cirrhosis may be present due to previous liver damage	HBsAg positive	
ALT levels normal	HBeAg positive → HBeAg negative and anti-HBe positive	Chronic infection	HBeAg negative, anti-HBe positive	
HBV DNA levels high	HBsAg positive	HBsAg positive		HBeAg negative, anti-HBe positive
HBeAg positive		HBeAg negative, anti-HBe positive		HBsAg negative
HBsAg positive				

Figure 6: Characteristics of the clinical presentation of each phase of chronic HBV infection. Adapted from: (Nguyen et al., 2020).

1.6.3 Disease Progression

Male sex, age and high viraemia levels are associated with an increased incidence of fibrosis, cirrhosis, and HCC (El-Serag, 2012). The continuous damage incurred by infected hepatocytes as a consequence of the host immune response stimulates complex cellular repair mechanisms, which can induce the development of liver fibrosis (Dhar et al., 2020). Progressive fibrotic damage can over time result in the development of cirrhosis with severe architectural distortion causing the obstruction of blood flow through the liver and consequent disruption of liver function. Once cirrhosis has manifested, there is high risk of serious complications including portal hypertension, liver failure and liver cancer (Xu et al., 2012). The rate of liver cirrhosis development is dependent on a number of factors including the phase of infection or the type of CHB infection. Patients with HBeAg-negative hepatitis B have higher rates of cirrhosis (El-Serag, 2012) whereas the inactive carrier state is associated with lower incidence (Sharma et al., 2005).

The precise mechanisms that lead to the development of HCC are unknown. However, it is believed that the constant cycle of liver damage followed by repair, during fibrosis and cirrhosis, may contribute to the incorporation of genomic mutations during cellular regeneration leading to carcinogenesis (An et al., 2018).

1.7 Viral lifecycle

The HBV life cycle is shown in Figure 7 (Rehermann & Nascimbeni, 2005). The life cycle of HBV is initiated when the virus attaches itself to the host cell membrane via its envelope protein. It has been proposed that, as the first step in HBV infection, HBV binds to receptors on the plasma membrane expressed primarily on human hepatocytes through the pre-S1 domain of the large envelope protein (Beck, 2007). However, the nature of the receptor remains controversial. Upon attachment, the viral membrane fuses with host cell membrane and the HBV genome is released into the cell. The rate of HBV replication can be regulated by a number of factors, including hormones, growth factors, inflammasome

activation and cytokines. After the viral genome reaches the nucleus, the viral polymerase converts part of the dsDNA genome (double-stranded DNA) into cccDNA (covalently closed circular DNA) (Nassal, 1999). The cccDNA serves as a template for the transcription of viral RNA by the host cell's RNA polymerase II enzyme. The resulting RNA transcripts are used as a template for further propagation of pre-genomic and sub-genomic RNAs. Pre-genomic RNAs have dual functions, serving both as a template for viral DNA synthesis and as a messenger for the translation of pre-C, C, and P. Sub-genomic RNA is specialized for translation of the envelope and X protein (Seeger & Mason, 2000). All viral RNA is transported to the cytoplasm, where its translation produces viral envelope, core, and polymerase proteins, as well as HBx and HBcAg. HBV core particles are assembled in the cytosol, during which a single molecule of pre-genomic RNA is integrated into the assembled viral core. Once the viral RNA is encapsidated, reverse transcription begins. The sequence of synthesis of the two viral DNA strands occurs (Summers & Mason, 1982). The first strand of DNA is made from the encapsidated RNA template; during or after the synthesis of this strand, the RNA template is degraded, and the synthesis of the second DNA strand continues using the newly generated first DNA strand as a template. Some cores carrying mature genome are transported back into the nucleus, where their newly imprinted DNA genomes can be converted to cccDNA to maintain a stable pool of transcriptional templates (G.-H. Wang & Seeger, 1992). HBV surface protein (HBsAg) is first synthesized and polymerized in the rough endoplasmic reticulum (RER). These proteins are trafficked to the post endoplasmic reticulum (ER) and pre-Golgi compartments where budding of the nucleocapsid occurs. As a final step in the lifecycle, assembled HBV virion and sub-viral particles are transported to the Golgi for final glycan modifications of HBsAg proteins, and secreted from the cell (Chua et al., 2005).

HBV has a lack of proofreading and a very high daily virus production, together this results in a significant error rate (Steinhauer et al., 1992), allowing the existence of many viral variants within the same host at any

given time. The genetically different viral strains concomitantly present in a single cell or individual are termed the viral quasispecies (Lucifora et al., 2014).

It is important to also note that selection pressures from the host immune system (endogenous) and antiviral therapies (exogenous) are key in shaping the nature of the predominant HBV quasispecies that exist in an infected individual at any given time point. This quasispecies will include a mixed population of Hepatitis B variants with differing levels of viral fitness both at a cellular level (e.g., viruses which replicate most efficiently will predominate) and at an extra-cellular level (viruses that avoid immune elimination or develop resistance to anti-viral drugs when a patient is on therapy, will become dominant) (Xia et al., 2016).

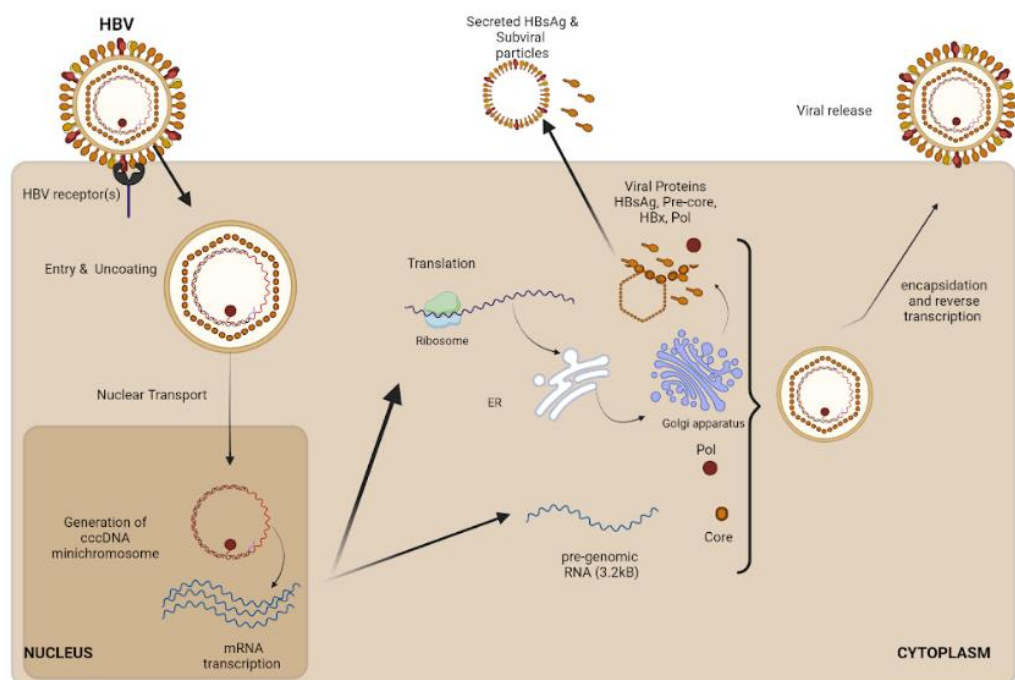


Figure 7: Viral lifecycle. The genomic HBV DNA is carried by hepatitis B virus (HBV) nucleocapsids into the cell after entry, where the relaxed circular DNA is transformed into covalently closed circular (ccc) DNA. The cccDNA serves as a template for the transcription of four viral RNAs that are exported to the cytoplasm and employed as mRNAs to translate the HBV proteins. These RNAs are of the following sizes: 0.7 kilobases (kb), 2.1 kb, 2.4 kb, and 3.5 kb. The pre-genomic RNA, which is the longest, acts as a template for replication, which takes place inside

nucleocapsids in the cytoplasm. When nucleocapsids transit through the endoplasmic reticulum (ER) and/or Golgi complex, they are encased and are later excreted by the cell. Illustration created in <http://biorender.com/>.

1.8 Treatment

Treatment of hepatitis B remains a clinical challenge. Once chronic HBV has been established, the HBV genome persists for life, either integrated into the host genome and/or as episomal cccDNA. The latter is the source of renewed viral replication in patients with HBV reactivation during immunosuppression or after antiviral drug withdrawal (Shi & Zheng, 2020). The presence of cccDNA during the replication cycle makes viral eradication extremely difficult and therefore the primary goal of treatment is not the elimination of virus but permanent suppression of HBV replication, in order to stop the progression of chronic liver inflammation leading to cirrhosis, HCC and death (Kapoor & Kottlilil, 2014). It is important to note that reduction of HBsAg is the key marker for successful clearance.

1.8.1 Interferon therapy

Interferon- α is a member of the Type I Interferon family and imparts its antiviral activity in two ways: (1) a directly antiviral effect achieved through inhibiting the synthesis of viral DNA and by activating antiviral enzymes, and (2) by augmenting the virus-specific cellular immune response by increasing the expression of MHC class I antigens on infected hepatocytes and stimulating the activity of CD4+ and CD8+ T-cells (Fung et al., 2011). Interferon- α has been a mainstay of therapy for CHB. Several studies have shown its efficacy, with 25-40% of patients clearing HBV-DNA and normalising transaminases on therapy (Thomas et al., 2003) with significantly higher rates of HBsAg seroconversion compared to untreated patients (S. Li et al., 2020).

The efficacy of IFN α has been improved by its conjugation with polyethylene glycol, PEG-IFN α . This new form has a reduced renal clearance rate prolonging the half-life of the molecule, resulting in more

stable plasma concentrations, and has allowed the number of injections to be reduced from three to once per week. The first study comparing standard vs. PEG-IFN α showed that the pegylated formulation had a superior efficacy with up to 37% patients receiving PEG-IFN α -2a clearing HBeAg compared with 25% of patients on conventional IFN α -2a. Furthermore, the combined response (HBeAg loss, HBV DNA suppression, and ALT normalization) of PEG-IFN α -2a (40 kDa) was twice that achieved with conventional IFN α -2a (24% vs 12%; P = 0.036) (Cooksley et al., 2003).

In terms of HBsAg seroconversion, PEG-IFN α is associated with a more durable response and even post-treatment a higher steady rate of patients (6% per year) continues to undergo HBsAg seroconversion after cessation of interferon therapy in comparison to the low rate of HBsAg seroconversion observed in patients treated with nucleos(t)ide analogues (1.6% per year) (Dogan et al., 2013). These data show that suppression of viral replication is a condition necessary but insufficient to achieve HBsAg seroconversion. While the mechanisms have not been fully elucidated, this is likely to be due to the immune modulating effects of IFN. HBsAg levels also seem to predict outcome of PEG-IFN α therapy. In HBeAg(+) patients low pre-treatment HBsAg levels was found to be better than HBV DNA in predicting good response to PEG-IFN α (Tangkijvanich et al., 2009). This has also been shown in a group HBeAg(-)patients, where an on-treatment decline of >1log₁₀ IU/mL could predict sustained HBsAg clearance 3 years after PEG-IFN α (Barone, 2014). Highlighting the importance of quantitative HBsAg assessments in guiding treatment regimens. Quantitative HBeAg assessment have also shown to be a useful utility in guiding treatment decisions in patients treated with PEG-IFN α . High levels of HBeAg had a greater negative predictive value (>96%) of sustained virological response (SVR) than that obtained for HBV-DNA (86%) (Tangkijvanich et al., 2009).

In summary there are several factors that favour the use of PEG-IFN α over oral nucleos(t)ide analogues including a known and fixed duration

of therapy, higher rates of HBeAg and HBsAg seroconversion, the absence of the development of drug-resistant HBV strains and superior durable serological responses off-treatment probably as a consequence of its immunomodulatory activity (Sonneveld & Janssen, 2010).

However, IFN-based therapy is not without problems. It can be poorly tolerated, mainly because of the high incidence of flu-like symptoms and the need to give the medication as a subcutaneous injection. Additionally, therapy can be associated with more serious adverse effects, including myelo-suppression, endocrine abnormalities, psychiatric disturbances, and the development of autoimmune diseases (Sonneveld & Janssen, 2010).

1.8.2 Nucleos(t)ide Analogue therapy.

Nucleos(t)ide analogues signalled a new era in the treatment of CHB, by providing safe, efficacious, and well-tolerated alternatives to IFN-based therapies. Nucleos(t)ide analogues interfere with the elongation of viral DNA chains through competitive inhibition of the viral polymerase, by targeting three main steps of viral genome replication. They can be classified as anti-priming (e.g., Adefovir), anti-minus strand DNA synthesis (e.g., Lamivudine) and anti-plus strand DNA synthesis (Chien & Liaw, 2008). These oral antiviral drugs are effective in suppression of viral replication which is associated with reduction in viral load, antigenemia and improvements in serological markers of liver damage.

A consequence of long-term use of these therapies is the emergence of HBV strains with mutations conferring drug resistance, as indicated by virological and biochemical breakthrough, which can result in major life-threatening complications such as hepatic decompensation and death (Zoulim & Locarnini, 2009). Lamivudine or Adefovir monotherapy, which until recently have represented the standard of care for CHB have high levels of resistance, with 5-year viral resistance rates of >50% and 20% respectively (Ijaz et al., 2008; Lee et al., 2006). More alarming are reports of multi-drug-resistant Hepatitis B virus (MDR-HBV) strains. These

studies report MDR-HBV strains that are replication competent and harbour multiple polymerase mutations conferring strong resistance to more than one nucleos(t)ide analogue (Pastor et al., 2009). The inability of chemotherapy to purge the infected hepatocytes of the viral replication template (episomal ccc HBV-DNA), the lack of proof reading and low fidelity during replication are all contributing factors to the emergence of drug-resistant-HBV. However, experience with the use of established oral antivirals (e.g., Lamivudine) has confirmed that the immense natural selection pressure exerted by continuous long-term monotherapy directed at a single viral target is the dominant factor for breeding drug-resistant-HBV strains which quickly acquire compensatory mutations resulting in increased replication fitness allowing them to dominate the viral quasispecies within the host (Zoulim, 2011).

Prediction of response is important in patients treated with nucleos(t)ide analogues to assist the selection of patients: 1) most likely to respond 2) whom nucleos(t)ide analogue therapy can be stopped after a finite period and 3) at high risk to develop drug resistance. Pre-treatment factors predictive of HBe seroconversion are low viral load (HBV DNA below 10^7 IU/ml or $7 \log_{10}$ IU/ml), high serum ALT levels (above 3 times the upper limit of normal), high activity scores on liver biopsy. On treatment with lamivudine, adefovir or telbivudine, a virological response at 24 or 48 weeks was associated with a lower incidence of resistance in HBeAg(+) patients ("EASL Clinical Practice Guidelines: Management of Chronic Hepatitis B," 2009).

1.8.3 Nucleos(t)ide Analogue and Interferon combination therapy.

Evidence for the benefit of combination therapy with IFN and nucleos(t)ide analogues has been slow to emerge. A study of the likelihood of an additive effect of interferon-lamivudine combination therapy in patients with HBeAg(+) compared with IFN α or lamivudine monotherapy showed increased efficacy of combination therapy compared to monotherapy. Studies have demonstrated that at week 52, HBeAg seroconversion rates were 29% for combination therapy, 19% for interferon monotherapy, and 18% for lamivudine monotherapy (Schalm,

2000). A follow-up analysis study evaluated the efficacy of IFN α and lamivudine combination therapy versus lamivudine monotherapy in 150 HBeAg(+) CHB patients. They found that 33% of patients receiving combination therapy had persistent HBeAg seroconversion with undetectable serum HBV DNA levels, compared with 15% of patients on monotherapy (Barbaro et al., 2001). A large study evaluated the long-term sustainability of responses in patients with HBeAg(+) CHB treated with PEG-IFN α alone or in combination with lamivudine. They found that HBeAg loss was the same in patients who received PEG-IFN α alone and those who received lamivudine supplementation. However, patients in the combination arm were more likely to have undetectable HBV DNA than patients receiving PEG-IFN α monotherapy during long-term follow-up (Buster et al., 2008). Post-treatment response rates were also higher in HBeAg(-) patients who received the combination of PEG-IFN α , and lamivudine compared with those who received lamivudine alone (Bonino et al., 2007). These studies suggest that combination therapy may be more effective than monotherapy, but research is needed on other regimens that address issues such as the duration and effectiveness of nucleoside analogues.

1.8.4 Experimental Therapies

Significant research has been undertaken recently to develop new therapies for HBV that can achieve sustained suppression and HBsAg loss. There are multiple strategies being employed from inhibiting viral entry, targeting ccc-DNA and boosting host immunity to HBV, these are illustrated in Figure 8.

There is particular interest in identifying peptides and small compounds that inhibit viral fusion with host cells and thereby preventing HBV from entering hepatocytes. Entry inhibitors are a type of inhibitor that is particularly effective at lowering viral infection since virus entry is considered the first phase of infection. Nonetheless, like antibody-based vaccinations, these inhibitors are extremely selective for a specific virus.

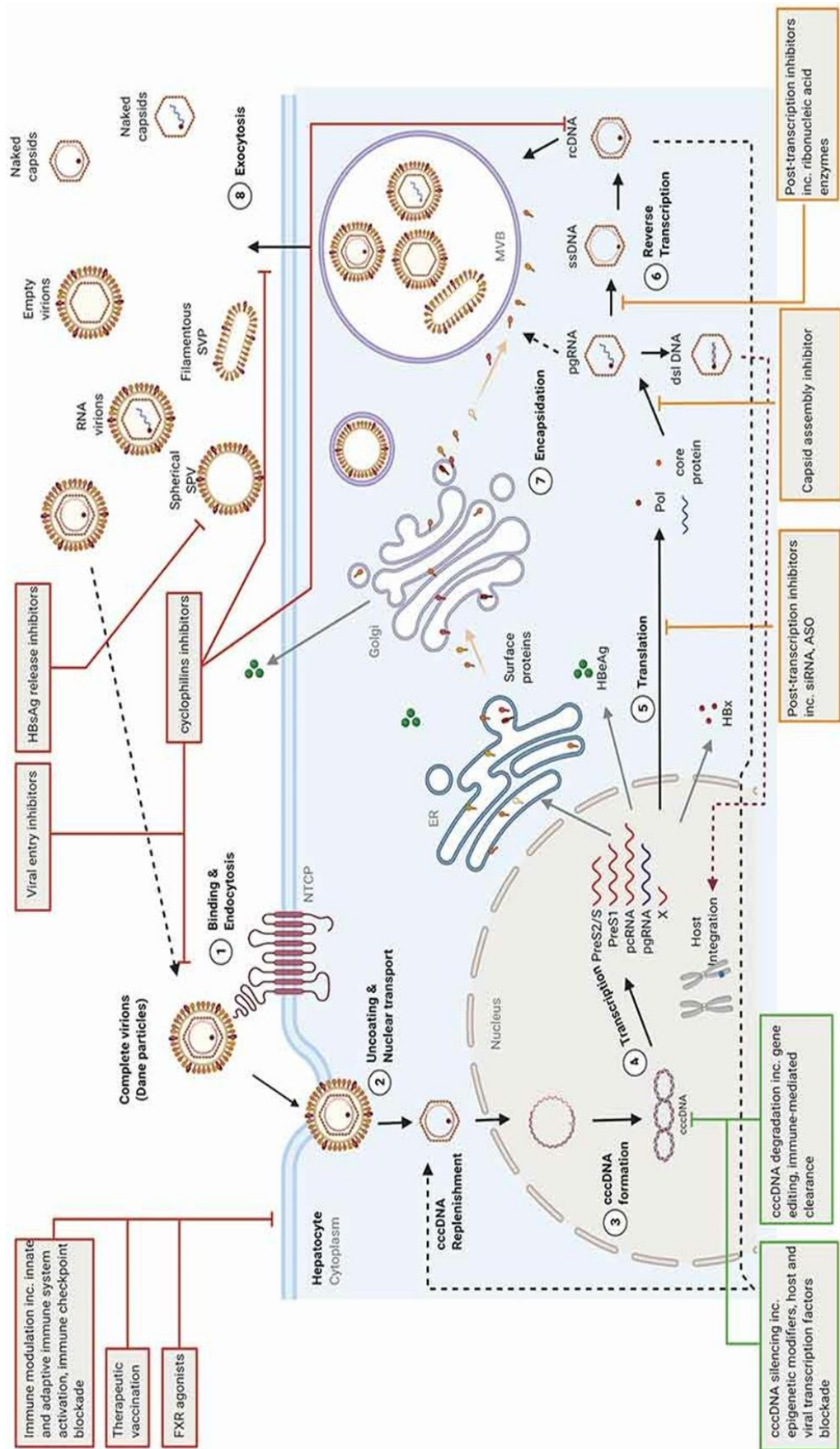


Figure 8: Experimental therapies in development for CHB. Illustrating novel pre-clinical therapeutic targets in development for chronic hepatitis B. In **orange** are the Directly acting antiviral (DAA) targets. In **red** are host-targeted therapeutics and in **green** are those agents directed toward the episomal cccDNA. Abbreviations include: ASO: antisense oligonucleotides; cccDNA: covalently closed circular DNA; ER: endoplasmic reticulum; dsDNA: double-stranded linear DNA; FXR: Farnesoid X receptor; NTCP: sodium-taurocholate co-transporting polypeptide; MVB: multivesicular bodies pcRNA: precore RNA; pgRNA: pregenomic RNA; Pol: polymerase; rcDNA: relaxed circular DNA, siRNA: small interfering RNA; SVP: subviral particles. Source: (Phillips et al., 2022).

1.9 Diagnosis

1.9.1 Hepatitis B virus serology

Diagnostic clarification of HBV infection involves measurement of several HBV-specific antigens and antibodies in the serum of infected patients. Various serological biomarkers are used to diagnose the stages of HBV infection and to determine if (1) a patient has acute or chronic HBV infection; (2) is immune to HBV due to previous infection or vaccination; or (3) has susceptibility to HBV infection. Table 2 illustrates the interpretation of serological markers used to diagnose hepatitis B infection (Mast et al., 2005). Various serological markers include:

1.9.2 Hepatitis B surface antigen (HBsAg)

This antigen forms the outer spherical envelope of the virus and is produced in large quantities during viral replication. High levels can be detected in serum during acute or chronic HBV infection, either associated with viral particles or as aggregates. Antibodies against HBsAg (anti-HBs) indicate prior infection and immunity, this state is usually acquired through spontaneous resolution of acute infection and

is the goal for clinical cure of CHB. Recombinant HBsAg is the protein used in the hepatitis B vaccine (Lada et al., 2006).

1.9.3 Hepatitis B core and e antigen (HBcAg; HBeAg)

HBcAg are viral nucleocapsids and are highly immunogenic in nature. It is present in infected liver cells and is a marker of active viral replication. HBcAg (anti-HBc) antibodies appear in serum after the onset of clinical symptoms and persist throughout the natural course of infection (Zheng et al., 2004). In addition, anti-HBc can still be detected years after anti-HBs seroconversion (Holtkamp et al., 2022). IgM antibodies to HBcAg (IgM anti-HBc) were the first to emerge, indicating recent infection; however, fluctuations in IgM anti-HBc were also observed during ALT exacerbations, followed by IgG anti-HBc HBcAg (Puri, 2013). The presence of anti-HBc IgM and anti-HBs allows for the discrimination between acute and chronic infection (Park, 2015).

HBeAg is a secreted protein produced by post-translational modification of pre-core protein and is routinely found in serum. It does not form part of the viral structure and is not required for replication but is thought to play a role in promoting immune tolerance. The presence of HBeAg indicated a high rate of viral replication in HBeAg + CHB, and the persistence of HBeAg was associated with chronicity in this cohort. The formation of anti-HBeAg (anti-HBe) antibodies is often a favourable process that marks the transition to the inactive carrier stage (Kramvis et al., 2018). However, in some patients, selective pressure on the virus can lead to the production and selection of pre-core mutant viruses that do not produce HBeAg but are replication competent. The most common mutation is a change from guanine (G) to adenine (A) at nucleotide 1896 (G1896A). These patients with HBeAg-negative disease may have an underlying severe and progressive disease course (Saikia et al., 2007).

Table 2: Interpretation of Hepatitis B serology. Measurements of antibodies and antigens that are specific to the hepatitis B virus (HBV). If a patient has acute or chronic HBV infection, is immune to HBV as a

result of a prior infection or vaccination, or is vulnerable to infection, these serological "markers" or combinations of markers are used to identify the various phases of HBV infection. Adapted from: (Mast, 2005).

Interpretation of Hepatitis B Serologic Test Results		
Tests	Results	Interpretation
HBsAg anti-HBc anti-HBs	negative negative negative	Susceptible
HBsAg anti-HBc anti-HBs	negative positive positive	Immune due to natural infection
HBsAg anti-HBc anti-HBs	negative negative positive	Immune due to Hepatitis B vaccination
HBsAg anti-HBc IgM anti-HBc anti-HBs	positive positive positive negative	Acutely infected
HBsAg anti-HBc IgM anti-HBc anti-HBs	positive positive negative negative	Chronically infected
HBsAg anti-HBc anti-HBs	negative positive negative	1. Interpretation unclear; four possibilities: Resolved infection (most common) 2. False-positive anti-HBc, thus susceptible 3. "Low level" chronic infection 4. Resolving acute infection

1.9.4 Molecular assays for HBV-DNA quantitation and genotyping.

In addition to serological indicators, viral quantification, viral genotyping, and resistance testing are utilised to monitor individuals with CHB.

1.9.4.1 HBV-DNA quantitation.

The occurrence of HBV DNA in peripheral blood, a robust marker of active viral replication, can be detected within a few days of infection, peaks with acute hepatitis, and progressively decreases and disappears with spontaneous resolution of infection. This is due to the reduction in HBV DNA. In patients with chronic infection, HBV DNA levels are not stable over time and depend on the stage of infection (C.-J. Chen et al., 2009). Detection and quantification of HBV DNA can be performed using signal amplification or target amplification (including polymerase chain reaction, PCR and transcription-mediated amplification, TMA) following molecular hybridization (including hybrid capture and branched DNA methods) (Yates et al., 2001). Detection of HBV DNA plays a key role in

diagnosing infection, making treatment decisions, and assessing treatment response (Jardi et al., 2001).

1.9.4.2 HBV genotyping

HBV genotypes are clinically relevant for the management of patients with acute and CHB (Lin & Kao, 2011). Studies have demonstrated that genotyping can be used to identify patients with a higher probability of responding to a finite course of treatment with peginterferon (Brouwer et al., 2019). In addition to this, genotype C is more strongly associated with poor prognosis and a more aggressive clinical phenotype (Chan, 2004), whereas genotype B is associated with early HBeAg seroconversion, potentially contributing to a lower prevalence of HBV-related cirrhosis (Chu et al., 2002). Core promoter and lamivudine resistance mutations were also observed more frequently in genotypes C and A, while pre-core termination mutations were more frequently observed in genotypes B and D (Shen, 2014; Sitnik et al., 2004). Therefore, HBV genotyping is a useful tool for treatment decisions.

Several genotyping technologies are currently available, each with different performance characteristics (Guirgis et al., 2010). Because of its relative stability, ubiquity, sensitivity, and specificity, most approaches target viral DNA.

The gold standard for HBV genotyping is whole genome sequencing followed by phylogenetic analysis, which detects prevalent, new, and recombinant genotypes (Martínez et al., 2015).

Inno-LiPA is based on the reverse hybridization principle and is intended to detect known, well-characterized genotypes as well as mixed genotypes. This assay's analytical sensitivity varies depending on viral load (Osiowy & Giles, 2003). The reverse dot blot assay is fast, low-cost, and accurate (EL-Fadaly et al., 2016). Another method utilised is the oligonucleotide microarray, which is more expensive than sequencing and quantitative polymerase chain reaction (PCR) methods (Pas et al., 2008). However, it is a sensitive assay that may detect mixed genotypes.

PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) is a cost-effective approach for detecting genetic variations and mutations in large populations. It can, however, produce approximately 6% indeterminate outcomes (Hashim & Al-Shuhaib, 2019a). The restriction fragment mass polymorphism (RFMP) assay is particularly sensitive, with a detection limit of 100 copies/mL. It can also detect drug-resistant mutations (YMDD) in the HBV wild-type strain's background. However, it necessitates the use of MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight), which is both costly and technically challenging. The presence of single nucleotide polymorphisms (SNPs) at restriction sites can result in a false band pattern, lowering the assay's sensitivity (Guirgis et al., 2010). In addition to this, another limitation this approach is that it can only detect known mutations, leaving unknown mutations elusive (Hashim & Al-Shuhaib, 2019).

1.10 HBV receptors and receptor entry

Initially, the only way to study the HBV life cycle in vitro, especially the early stages of infection (attachment, binding, and membrane fusion), was to utilise primary human hepatocytes (PHH). However, due to accessibility issues and wide variability in HBV infection susceptibility, the use of PHH within studies has been restrictive. The development of human hepatoma cell lines, such as Huh7 and HepG2, resolved this problem and allowed for the facilitation of experimental analysis for initial stages of HBV infection (Schulze et al., 2007).

1.10.1 Heparan sulfate proteoglycans (HSPGs)

There are multiple steps involved in the process of HBV entry via the hepatitis B surface antigen (HBsAg). HBV infection requires attachment receptors on the carbohydrate side chains of hepatocyte-associated heparan sulphate proteoglycans (HSPGs). In addition to this, multiple domains within the HBV L protein have been demonstrated to have key roles in HBV and hepatocyte interactions. Furthermore, recent studies have shown that the AGL HS binding site, which consists of only two positively charged residues (R122 and K141), is responsible for the

attachment of HBV to HSPGs (Sureau & Salisse, 2013). Individual HBV envelope proteins bind with low affinity for heparan sulfate, but once bound to the viral membrane, they would have enough avidity for sustained interaction between virus and cell surface HSPGs. Early HBV literature has also shown that 10-36 amino acid residues within the pre-S1 region, were a necessary component of hepatocyte recognition (Neurath et al., 1986).

1.10.2 Sodium Taurocholate Co-Transporting Polypeptide (NTCP)

HBV binds to the high-affinity cell receptor, sodium taurocholate co-transporting polypeptide (NTCP), via the N-terminal myristoylated peptide, which corresponds to the preS1/2–47 of HBs-L protein (Yato et al., 2021). HBV is then internalised by the process of endocytosis. The discovery of sodium taurocholate co-transporting polypeptide (NTCP) as a key entry receptor for HBV and its satellite virus, hepatitis delta virus (HDV), has significantly aided the understanding of the viral life cycle of HBV, particularly the primary stages of infection (Yan et al., 2015). This discovery was demonstrated by a combination of affinity purification and spectrometry analysis utilizing an HBV preS1-derived lipopeptide. It was identified that *Tupaia belangeri* NTCP (tsNTCP) was interacting with this lipopeptide. NTCP, found in the basolateral membrane of hepatocytes, is involved in the absorption of primarily conjugated bile salts in the liver. The lipopeptide was shown to bind human NTCP (hNTCP), as well as tsNTCP, but not crab-eating monkey NTCP (mkNTCP), which corresponded to the species specificity of HBV infection: Humans and *Tupaia* are both susceptible to HBV infection, while crab-eating monkeys are not (Watashi et al., 2014). Additionally, in primary human hepatocytes (PHH), primary *Tupaia* hepatocytes, and differentiated HepaRG cells, siRNA-mediated knockdown of NTCP decreased HBV and HDV infection, but an ectopic expression of NTCP enhanced HBV susceptibility in HepG2 cells, which had previously been resistant to infection (Yan et al., 2012). The transduction of NTCP into hepatoma cell lines Huh-7 and undifferentiated HepaRG cells showed HBV infection to

a certain extent as well (Ni et al., 2014). With all the published literature, it is confirmed that NTCP is a primary entry receptor for HBV.

1.10.3 Myrcludex B as an anti-HBV entry inhibitor

The myristoylated preS1-domain of the HBV envelope L-protein9 mediates HBV binding to the NTCP protein on the host cell. In well-established cell culture models of HBV/HDV infection, Myrcludex B, a imitates the HBV-specific NTCP-binding domain 10, which substantially impairs viral docking and hence eliminates HBV infection. Myrcludex B has been clinically being investigated as a new inhibitor of HBV entry. In a clinical study, patients with chronic hepatitis delta (with co-infects with HBV) patients treated for 24 weeks with Myrcludex B, a significant reduction of HBV DNA serum levels was observed (Bogomolov et al., 2016).

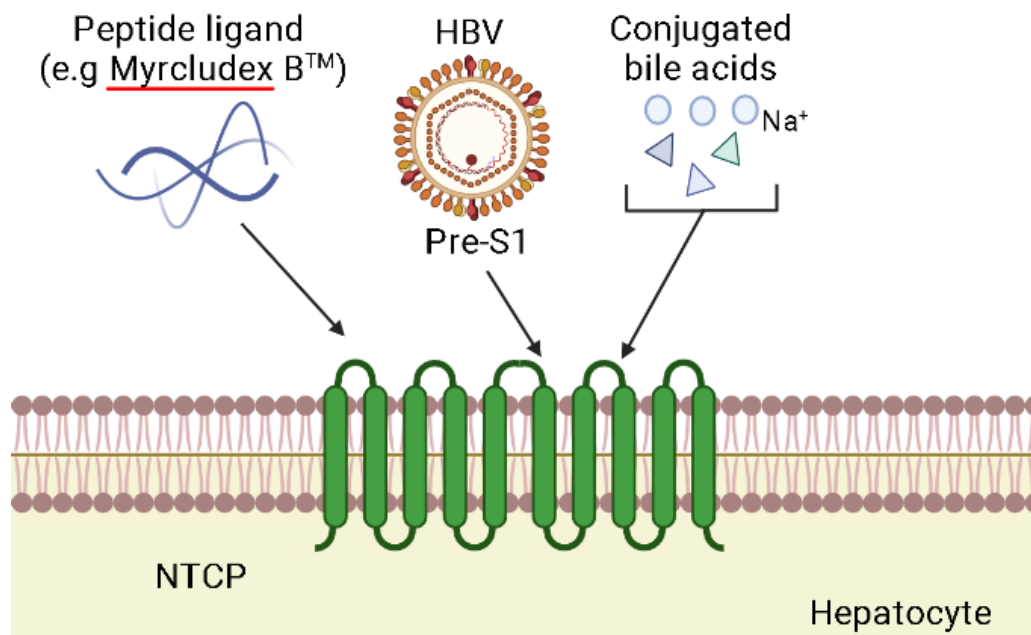


Figure 9: Inhibition of HBV entry using Myrcludex. Myrcludex competes with HBV by imitating the HBV-specific NTCP-binding domain 10, substantially impairing viral docking and inhibits HBV entry. This illustration was created in <http://biorender.com/>.

1.11 Models of HBV entry:

1.11.1 Pseudotypes:

The process known as pseudotyping is a technique utilised to produce a 'customized' virus with envelope glycoproteins that are not encoded by viral genome that is within. As a result, a virus that has its genome and capsid coated in foreign surface glycoproteins is known as a pseudotyped virus, also known as a pseudotype (Bentley et al., 2015). The advantage of using this technique is that once the pseudotype enters a susceptible cell and promotes nucleic acid replication, it is unable to produce infectious particles (King et al., 2016). This indicates that it can only cause one infection.

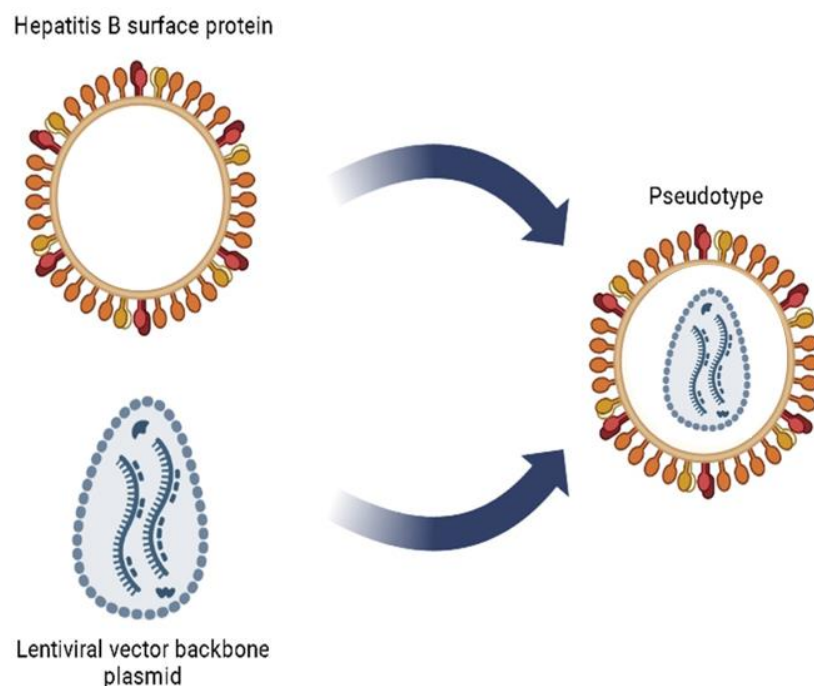


Figure 10: Pseudotype production. This illustration shows how pseudotypes are made which includes the combination of an HBV surface glycoprotein and a lentiviral vector backbone plasmid. This illustration was created in <http://biorender.com/>.

Pseudotypes can be utilized in a diverse array of clinical and experimental manners, allowing for in-depth study of viral mechanisms. This can include viral tropism, viral glycoproteins, and cell receptor interactions. As a result, this technique can be used as a tool to identify

novel entry receptors and other factors involved in viral attachment. Furthermore, pseudotyping makes genetic modification of viral genes simple, enabling the introduction and examination of mutations within the envelope gene in order to grasp how these changes alter the steps involved in virus entry (Gutierrez-Guerrero et al., 2020). Due to the fact that pseudotypes do not contain a full viral genome, they pose less biosafety risk and can therefore be carried out in a lower biosafety level (BSL) to cultured HBV, which requires CL3 laboratories. This enables the technique to be more accessible to institutions that do not have access to higher biosafety levels (Bentley et al., 2015).

Ultimately, a pseudotype is a viral particle that contains the envelope protein of one virus and the genetic material of another virus. In the case of HBV and HIV, a pseudotype virus can be created by incorporating the envelope protein of HBV onto the surface of an HIV virus particle. To produce pseudotypes, a viral vector backbone plasmid containing the core and nucleic acid is first constructed. This plasmid, along with the reporter plasmid and envelope protein, is then transfected into the host producer cell, typically HEK293T cells. The viral genome consisting of the viral envelope proteins is stably integrated into the host's genome through the process of transfection (Merten et al., 2016). Currently, the most commonly used retroviral vector system is a human immunodeficiency virus type-1 (HIV-1) lentiviral vector due to their nuclear membrane translocation capabilities and transduction of cells whether they are dividing or non-dividing. In addition to this, lentiviral vectors are able to be produced at fairly high titres (Liu & Berkhout, 2014). However, other systems have been previously used such as gammaretroviral vectors such as murine leukemia virus (MLV). There are advantages to using different vectors depending on which viral glycoprotein being pseudotyped.

The introduction of the SV40 simian virus T antigen into the HEK293T human embryonic kidney cell line improves lentiviral vector production, making it the cell line of choice for pseudotyping. The SV40 origin of replication is primarily present in lentiviral vectors; therefore, binding of

the SV40 T antigen from the SV40 origin of replication facilitates replication of the lentiviral genome. In addition, the SV40 T antigen inhibits tumour suppressor proteins, increasing cell proliferation and transfection efficiency (Bae et al., 2020; Lin et al., 2014). To generate pseudotypes, the lentiviral vector and the desired envelope plasmid were co-transfected into HEK293T cells. An internal promoter controls the production of both plasmids.

The glycoproteins of interest are expressed on the exterior of the lentiviral nucleocapsid that is within, as the virus buds out of the host cell. Once budded from the host cells (Garoff et al., 1998), the pseudotypes can be harvested from media supernatant and are ready for experimental use. The lentiviral vector plasmid typically contains a reporter gene. In this case, luciferase was utilized, however, green fluorescent protein (GFP) can be cloned into the vector too. This is necessary for assessing viral infection, replication, and expression of viral pseudotype genes (Lutz et al., 2005). Due to the safety advantages of pseudotyping, crucial clinical research has been allowed for the study of BSL 3 and 4 organisms in as low as BSL 1 labs.

A limitation of using full length replicating hepatitis B genomes in culture to assess the consequences of a specific mutant or polymorphism is that you cannot necessarily unpick phenotypic consequences on the virus from the surface antigen and the polymerase, given that they are overlapping ORFs (S. Li et al., 2017). In the pseudotype system for HBV, the polymerase ORF is not expressed, and as a result, only the phenotype of the surface antigen is being assessed (Gutierrez-Guerrero et al., 2020). Therefore, any polymorphism or mutation in the surface antigen gene can be directly matched to the phenotype of the entry cascade without any influence of the polymerase. If there is a mutation in the surface antigen, it may result in a mutation in the polymerase, which can lead to a lack of viral production. However, it can be difficult to distinguish whether the lack of viral production is due to the virus not entering the cell or the polymerase not being functional.

1.11.2 Animal models

The use of animal models in clinical studies are a valuable tool for investigating novel therapeutic strategies and understanding viruses on a more representative level than *in vitro*. Due to the ineffectiveness of HBV removal in the majority of chronic HBV patients by current treatments such as interferon and nucleoside (acid) analogues, there is a call for further exploration into new drugs to combat HBV (Ye & Chen, 2021). However, due to the narrow host range of HBV, the battle is ongoing (Winer & Ploss, 2015).

The hepadnaviridae family include HBV, woodchuck hepatitis virus and duck hepatitis B virus. Currently, the only known non-human primate that is completely susceptible to HBV is the chimpanzee (W.-N. Guo et al., 2018). However, a disadvantage to this model is that HBV research in chimpanzees is heavily restricted (Wieland, 2015). There are various non-primate species that are able to be infected with HBV, one of these species is the tree shrew (*Tupaia belangeri*) which is phylogenetically related to *scandentia*. Clinical HBV studies of primary Tupaia hepatocytes have led to the discovery of novel HBV receptor NTCP in 2012 (Yan et al., 2012). In addition to this, non-HBV hepadnaviral infection in animals had been common practice for a long time.

1.11.2.1 Chimpanzees

The only immune-competent animal completely susceptible to HBV infection is the chimpanzee. Chimpanzees can acquire acute and chronic HBV infections following serum from HBV patients' inoculations, along with liver inflammation and cellular immune responses, which closely resembles the course of HBV infection in humans (Maynard et al., 1972).

Chimpanzee models have been utilised to evaluate HBV therapy approaches as well as the efficacy and safety of HBV vaccinations. Recent research has shown that the oral toll-like receptor-7 agonist GS-9620 can significantly lower chimpanzee HBV titres by activating NK cell and virus-specific T cell responses (Lanford et al., 2013). It has also been performed on chimpanzees to investigate the pathophysiology of HBV

infection. Using this model, it was found that CD4+ T cell priming was essential in the early stages of HBV infection and that a robust and polyclonal CD8+ T - cells response against HBV was a major element in antiviral therapy through cytopathic and non-cytopathic routes (Asabe et al., 2009).

Despite the advantages to chimpanzee models, they are not frequently utilized in HBV studies as there are ethical and resource constraints (Prince & Brotman, 2001). There have been ongoing efforts to infect smaller non-human primates with HBV. Recently, naturally occurring chronic HBV infection in cynomolgus monkeys from Mauritius was discovered, and rhesus macaques can develop HBV infection following viral vector-mediated *in vivo* expression of human NTCP in its hepatocytes (Bukh et al., 2013).

1.11.2.2 *Tupaia belangeri*

Phylogenetically, *Tupaia belangeri* is closely related to primates. Recently, the Chinese tree shrew's entire genome sequence suggested that its neurological, immunological, and metabolic systems resembled those of humans. *Tupaia belangeri* seems to be the only non-primate animal that has been discovered to be prone to HBV infection so far (Xiao et al., 2017). Primary *Tupaia* hepatocytes have been employed in HBV research for a long time because they are more readily available than primary human hepatocytes (Köck et al., 2001). In the last decade, it was discovered in the *Tupaia* model that the cellular receptor responsible for HBV entrance is called NTCP. The *in vivo* infection effectiveness in *Tupaia* needs to be improved, despite the fact that HBV infection in neonatal tree shrews can cause chronicity and pathological alterations, including fibrosis (Tsukiyama-Kohara & Kohara, 2014). In more recent years, the A2 HBV genotype has been discovered in *Tupaia belangeri* in Japan. This genotype has been found to have higher rates of replication and chronicity. Furthermore, the production of interferons in response to viral infection was found to be significantly impaired (Kayesh et al., 2017).

1.11.2.3 Human chimeric mice

Mice induced with the gene uPA, which causes necrosis of hepatocytes, was utilised to induce subacute liver failure (Tesfaye et al., 2013). This allowed for the transplantation of human hepatocytes into the liver of the mice. These mice are known as human chimeric mice. The result of this transplantation lead to a humanized liver model with an increased human hepatocyte reconstruction rate and that was able to be infected with HBV and HCV (Dandri, 2001; Tsuge et al., 2005).

The induction of liver failure in the mouse models was induced by causing a deficiency in fumarylacetoacetate hydrolase (Fah) and the knockout of Rag 2 and the IL-2 receptor gamma chain. This allowed for necessary repopulation of human liver cells in mice (Azuma et al., 2007).

An advantage of using chimeric mouse models in order to investigate the infections of HBV is that naturally occurring variants and drug resistant mutants in combination with HBV genotypes are able to be studied. Furthermore, this allows for host interactions and anti-viral treatment studies such as monoclonal anti-body therapy (Zhang et al., 2016). However, a disadvantage to utilising these models is that the interactions between the mouse and the humanized liver is not representative of human model. In addition to this, chimeric animal models are genetically predisposed to immunodeficiency and are therefore inapt to carry out studies on immunotherapy therapies and the adaptive immunity.

1.11.3 Animal models with non-HBV hepadnaviral infection

Hepatocytes *in vivo* and hepatoma cell lines in culture both support hepadnavirus replication. There can be an occurrence of HBV replication in non-hepatoma cell lines when there is the activation of pregenomic RNA synthesis from the viral DNA. This activation occurs due to the expression of nuclear hormone receptors such as hepatocyte factor 4 (HNF4), peroxisome proliferator activated receptor alpha (PPAR alpha) and retinoid X receptor alpha (RXR alpha). The hepatocyte nuclear factor 3 (HNF3) inhibits nuclear hormone receptor dependent replication in HBV. Interestingly, HNF3 and HNF4 promote the replication of duck

hepatitis B virus (DHBV) in non-hepatoma cell lines, whereas the RXR alpha-PPAR alpha heterodimer prevents HNF4-dependent DHBV replication. Synergistic activation of DHBV pregenomic RNA synthesis and viral replication is caused by HNF3 and HNF4. Woodchuck hepatitis virus cannot be replicated under the same circumstances as HBV or DHBV in non-hepatoma cells. These findings suggest that the transcription factors needed by avian and mammalian hepadnaviruses are different (Tang & McLachlan, 2002).

1.11.3.1 Duck model

Duck hepatitis B virus, found in the serum of ducks which comes under the classification of Avihepadnavirus. The outcome of the infection, similarly to humans, is dependent on the age of the duck and dose of the infection. Ducks as models for HBV infection are commonly utilised to assess the efficacy of anti-viral therapeutic strategies such as nucleocapsid assembly inhibitors, nucleot(s)ide analogues and combined immunotherapies (Ji et al., 2020).

Ducks as a model to investigate HBV infection pathogenesis are useful due to the absence of innate immunity within the first 5 days of DHBV infection, allowing for persistence of DHBV in ducks that are newly hatched. This provides a clear window to study DHBV infection (Tohidi-Esfahani et al., 2010).

1.11.3.2 Woodchuck model

Woodchuck hepatitis virus (WHV) was identified in a group of marmots in an American forest. The colony exhibited significant levels of chronic hepatitis and HCC. This type of hepatitis is classified as Orthohepadnavirus. There are many similarities between WHV and HBV in terms of the characteristics the viruses have (Summers et al., 1978). In addition to this, evolution, and the immune response to the WHV infection are similar. As a result of this, this model is also frequently used to study HBV pathogenesis to test the effectiveness of novel therapeutic strategies, prophylactic vaccines and anti-viral treatments (Galibert et al., 1981). A close relative of the American woodchuck is the Chinese

woodchuck which has been used in various literature as another model for HBV infection (Zhu et al., 2018). This is significant as it broadens the range of HBV models and increases its availability to be studied.

1.11.3.3 Cell lines harbouring HBV infection in in vitro culture

There are several in vitro models available, and these are described in Table 3 below.

Table 3: Cell lines supportive of HBV infection. Advantages and disadvantages of different cell lines. Adapted from (Hu et al., 2019).

Cell line models	Pro's	Cons
Human hepatoma cell lines (HepG2, Huh7)	<ul style="list-style-type: none"> • Cheap • Easy to use • Supports some of the lifecycle 	<ul style="list-style-type: none"> • Is not infected by HBV • Cancer origin
HepaRG	<ul style="list-style-type: none"> • Supports infection • Recapitulates normal hepatocytes better than other hepatoma cell lines 	<ul style="list-style-type: none"> • Low infection rates • Difficult differentiation
HepG2-NTCP	<ul style="list-style-type: none"> • Easily available 	<ul style="list-style-type: none"> • Cancer origin
Primary human hepatocytes	<ul style="list-style-type: none"> • Harbours full HBV lifecycle 	<ul style="list-style-type: none"> • Low infection rates • Expensive • Short lifespan in culture
Induced pluripotent derived hepatocytes	<ul style="list-style-type: none"> • Unlimited supply • Support full HBV life cycle • Not cancer origin 	<ul style="list-style-type: none"> • Difficult and expensive • Lack of mature hepatocyte phenotype • Dedifferentiation in culture a problems

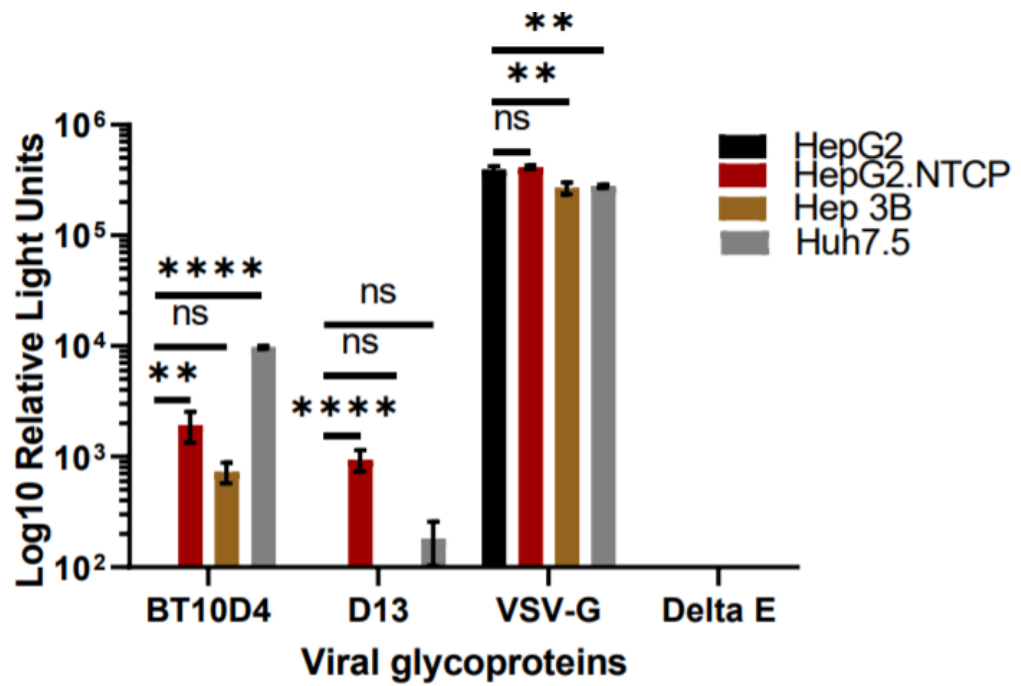
2 Previous work leading to this research project

2.1 HBV clinical isolate BT10D4

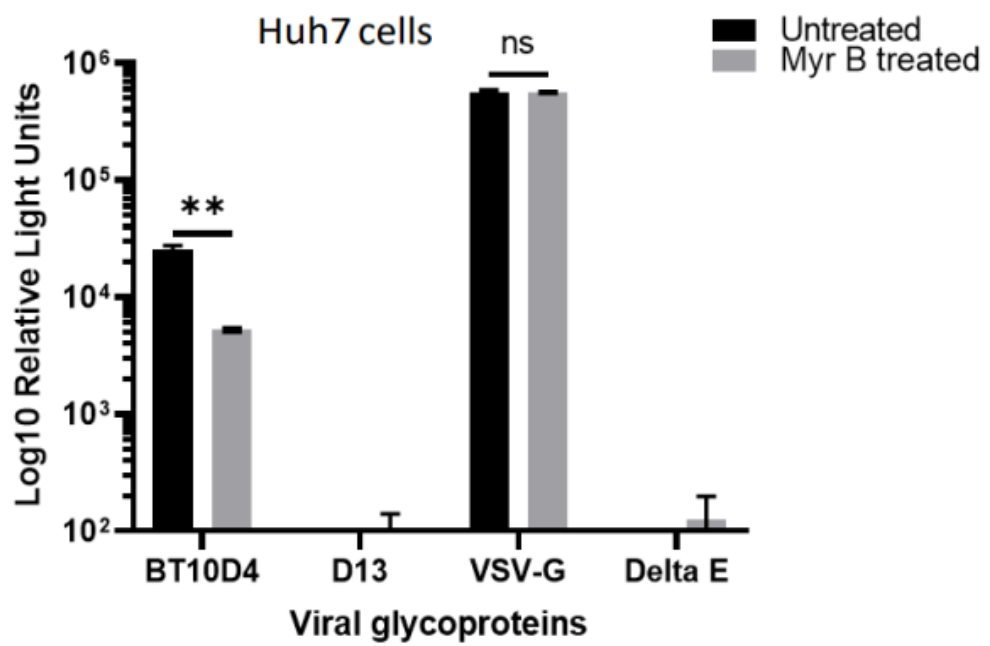
Pseudotyped HBV particles (see section 3.4) were examined for entry into Huh7 hepatoma cell line that had been transduced to express NTCP receptor. This study yielded some unusual data, with a particular focus on the entry phenotype of the HBsAg clone BT10D4 and its ability to infect Huh7 cells independently of NTCP receptor. Two rare amino acid changes at positions s69 and s96 in this isolate's sequence were discovered; it is possible that they interact with one another or with one or more other amino acid sites to produce the atypical entry phenotype. In order to investigate BT10D4, the variant was isolated from the patient and the HBV DNA of the BT10D4 genotype was extracted, amplified, and cloned into various pcDNA3.1(+) and pL18 vectors (ThermoFisher Scientific) by a previous member of the laboratory.

The cell lines in Figure 11A were infected with pseudotypes prior to a 72-h incubation. These cells were then lysed, and infectivity was measured via a luciferase assay. BT10D4 infected HepG2.NTCP significantly more than HepG2 cells ($p=0.0051$) (Figure 11) (Chidinma Raymond, 2021). In addition, infection of Huh7.5 compared to both HepG2 and Hep3B cell lines was significantly greater ($p<0.0001$). The BT10D4 infection differences between the cell lines Hep3B and HepG2 was non-significant. Unpublished data from our laboratory has shown that BT10D4 utilizes the HBsAg PreS2-48 amino acid, epidermal growth factor receptor (EGFR) and clathrin-dependent endocytosis for entry and internalization (Chidinma Raymond, 2021).

A



B



C

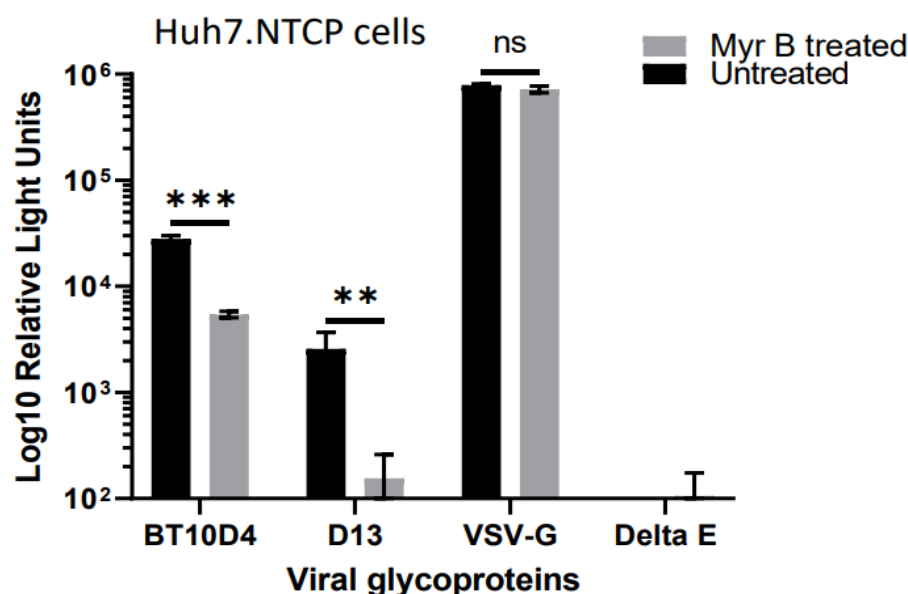


Figure 11: A. HBV infection in hepatic and non-hepatic cell lines (HepG2, HepG2.NTCP, Hep3B and Huh7.5). These cell lines were subjected to BT10D4 and gtD13 infection which were incubated at 37 °C and 5% CO₂ for 72 h. VSV-G and ΔE were used as positive and negative controls. The infection levels are presented as mean relative light units ± SD. Statistical analysis was carried out utilizing an ordinary one-way ANOVA. **** indicates $p < 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, ns indicates $p > 0.05$. (Courtesy of Dr Chidinma Raymond, Tarr Laboratory). **B. Myrcludex-B inhibition of HBV entry in Huh7 and C. Huh7.NTCP cells** were pre-treated with 1 μM of MyrB. Cell lines infected with the HBV pseudotypes BT10D4, D13 and incubated at 37 °C and 5% CO₂ for 72 h. VSV-G and PNL4.3 (Delta E) were used as positive and negative controls. Data are shown as means relative light units ± SD. Statistical significance was analysed with an unpaired t-test set at a 95% confidence interval. *** indicates $p < 0.001$, ** indicates $p < 0.01$, ns indicates $p > 0.05$. (Courtesy of Dr Chidinma Raymond, Tarr Laboratory)

These data revealed that whilst BT10D4 was unable to infect HepG2 hepatoma cells in the absence of NTCP, infection was apparent in Huh7 cells in both the presence and absence of NTCP (Figure 11). This data suggests the variant BT10D4 is entering Huh7 via an alternative receptor pathway to NTCP.

More data generated from our group demonstrated that, pre-treatment of Huh7 and NTCP expressing-Huh7 (Huh7.NTCP) hepatoma cell lines with Myrcludex B (MyrB) significantly decreased the infectivity of variant

BT10D4 in Huh7 ($p=0.0011$) and Huh7.NTCP ($p=0.0001$) cells (Figure 11B and C). Additionally, pre-treating Huh7.NTCP with MyrB prior to infection with D13 reduced its ability to infect Huh7.NTCP cells ($p=0.0013$). The infectivity of the VSV-G (cell viability control) was not significantly reduced by MyrB in Huh7 ($p=0.9847$) or Huh7.NTCP ($p=0.3079$) cells (Chidinma Raymond, 2021).

3 Materials and Methods

3.1 Cloning, Plasmid Preparation and PCR

Cloning of HBV and HCV DNA pcDNA3.1(+) and pl.18 vectors (ThermoFisher, 2022) was previously performed by previous members of the team. These included: HBV-Genotype A (clone BT10D4), HBV-Genotype B (clone gtD13) and HCV-Genotype C (clone H77). Each clone was transformed into Stellar Competent Cells (Takara Bio), which are an *E. coli* HST08 strain. Transformed bacteria were streaked on to LB agar plates containing 100 µg/ml ampicillin, from glycerol stocks. Subsequently a bacterial colony was grown in selective lysogeny broth (LB) containing 100 µg/ml ampicillin and incubated on a shaker at 250 revolutions per minute (rpm) at 37°C overnight. Plasmids were then prepared from these cultures using the GenElute Plasmid Miniprep and Midi Kit (Sigma-Aldrich) following the manufacturer's protocol. Isolated plasmids were quantified using the Nanodrop 1000 (ThermoFisher).

Polymerase chain reaction (PCR) was then performed to confirm the insert of interest was cloned successfully. This was done with standard T7 forward and bGH reverse primers. The PCR was set up following the HotStart Taq DNA Polymerase (Qiagen) protocol. In brief, 2.5 µL of template DNA from the DNA extract was added to the PCR mix. PCR thermocycling condition were as follows:

Initial denaturation at 95°C for 15 min; 25 cycles of [denaturation at 95°C for 30 seconds; annealing at 60 °C for 20 seconds; extension at 72°C for 90 seconds]; 1 x final extension at 72°C for 2 minutes. Following PCR, the amplicons were analysed on an 2% agarose gel (Thermo Fisher Scientific) containing 0.5 µg/mL ethidium bromide (Thermo Fisher Scientific). 2 µL of DNA gel loading dye (Thermo Fisher Scientific) was mixed with 5 µL of the PCR product and loaded on to the gel alongside a DNA ladder (Thermo Fisher Scientific). Gel electrophoresis was performed in Tris-Acetate-EDTA (TAE) (60 mM Tris-acetate 50 mM EDTA, pH 7.8) buffer and was run at 90 volts (V) for 36 min. Finally, DNA bands were visualised by UV transillumination.

3.2 Cell culture

Cell lines included:

1. Human embryonic kidney cells HEK293T
2. Human hepatoma Huh7 cells
3. In-house derived NTCP-expressing Huh7.NTCP cells (see below)

Using a Class II tissue culture hood, cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher Scientific), supplemented with 10 % Foetal Bovine Serum (ThermoFisher Scientific) and 1 % Non-Essential Amino Acids (NEAA) (ThermoFisher Scientific) and maintained in a humidified atmosphere at 37 °C and 5% CO₂ in a tissue culture incubator.

3.3 Cell passaging

HEK293T

HEK293T cells were harvested from a T75 flask and 2 mL trypsin was added which was incubate at room temperature for 2 minutes. Next, 10 mL of complete DMEM was added to neutralize the effect of the trypsin and recovered into a 50 mL Falcon and centrifuged at 300g for 7 minutes, with acceleration and deceleration set to 7 on the Eppendorf Megafuge 16 centrifuge. The cells were then resuspended in 10 mL complete DMEM and counted using a haemocytometer under a microscope at 200X magnification. For each T75 flask, 1.5×10^6 HEK293T cells were seeded in 10 mL of complete DMEM and left incubating for 4 days for 37°C and 5% CO₂.

Huh7

Huh7 (or Huh7 transduced with the NTCP-encoding SLC10A1 gene) were harvested from a T75 flask and 4 mL trypsin was added which was incubate at room temperature for 10 minutes. Next, 10 mL of PBS was added to wash the cells and after the PBS was poured out, 10 mL of complete DMEM was added and recovered with the cells into a 50 mL Falcon. The cells were centrifuged using the same settings as the

HEK293T cells and were then resuspended in 10 mL complete DMEM and counted using a haemocytometer under a microscope at 200X magnification. For each T75 flask, 2×10^6 Huh7 cells were seeded in 10 mL of complete DMEM and left incubating for 4 days for 37°C and 5% CO₂.

3.4 Production of Pseudotypes

HEK293T cells were seeded at 1.5 million in Primaria-coated cell culture dishes of 8.5 cm diameter (Corning) in 10 mL of DMEM and incubated overnight at 37 °C and 5% CO₂ in a humidified tissue culture incubator.

Then, in tube (1) - 2 µg of envelope plasmids cloned into pcDNA3.1(+) were added to 2 µg of the lentiviral vector pNL4-3.Luc.R-E- with firefly luciferase (NIH AIDS) in Opti-MeM to a final volume of 300 µL.

In tandem, in tube (2)- 24 µL 1mg/mL 1-polyethyleneimine (PEI) (Polysciences) was mixed with 300 µL of Opti-MeM.

The solutions in Tubes (1 and 2) were combined, mixed and incubated for 1 h at room temperature. This was then added to HEK293T cells in 7 mL of fresh Opti-MeM medium and incubated for 6h at 37 °C and 5% CO₂ in a humidified tissue culture incubator. Subsequently, the media was replaced with 10 mL fresh DMEM and cells were incubated for 72 h at 37 °C and 5% CO₂. After this period, the pseudotypes were harvested by filtering the cell culture supernatant using a 0.45µm PVDF filter (Millipore) and the clarified supernatant containing the pseudotypes were stored at 4°C for up to 2 weeks.

Virus	Viral glycoprotein	Lentiviral vector
	BT10D4	PNL4.3
Hepatitis B	gtD13	
	Btmut	
Hepatitis C	J6.4	
	H77	
Vesicular stomatitis	VSV-G	

Table 4: A breakdown of the pseudotypes constructed. The virus, glycoprotein and vector of each pseudotype are shown above.

3.5 HBV pseudoparticle Infection assay

Huh7 cells and in-house derived Huh7.NTCP cells were seeded at 15,000 cells/well in sterile flat-bottom 96-well plates and cultured in DMEM at 37 °C and 5% CO₂ in a humidified incubator overnight. Subsequently, 100µL of HBV pseudotype preparation and control was added to the cells in triplicate for 6 h. Initially, to assess transfection efficiency and infection vesicular stomatitis virus glycoprotein (VSV-G) was used as a positive control based on its broad cell tropism and high titre pseudotype generation. The negative control used was the HIV-1 backbone plasmid, in the absence of envelope glycoprotein (ΔE). Then, 72 hours post-infection, 50 µL of cell lysis buffer was added to plates and placed on a shaker at room temperature for 15 min followed by 15 seconds of vigorous vortexing. The pseudotypes included a luciferase gene in the lentivirus backbone and viral titre was therefore detected by assessing fluorescence using a FLUOstar Omega chemiluminescence microplate reader (BMG LABTECH) after injection with 50 µL luciferase substrate (Promega). Chemiluminescence was measured at a machine gain of 3600, reporting chemiluminescence values as relative light units (RLU).

3.6 Neutralisation and inhibition infection assay

Huh7 and Huh7.NTCP cell lines were seeded at 15,000 cells/well in a sterile flat-bottom 96-well plate (Costar-3879) and grown in DMEM at 37 °C and 5% CO₂ in a humidified incubator overnight. Five-fold serial dilutions of anti-annexin A2 in DMEM (0 µg, 0.4 µg, 2 µg and 10 µg) and 30 µL of each anti-annexin (or soluble annexin A2) dilution were prepared and added to each well of the plate and incubated at 37 °C and 5% CO₂ in a humidified incubator. In parallel, the experiment was repeated with 20 µg of anti-annexin A2 (and with 20ug of soluble annexin A2). Subsequently, cells were incubated with 100 µL of the HBV pseudotype preparations and controls in triplicates for 6 h 37 °C and 5% CO₂ in a humidified incubator. The HIV-1 backbone plasmid, in the absence of

envelope glycoprotein (ΔE) served as a negative control. After 72 hours post-infection, 50 μL of cell lysis buffer was added to each well and the plate placed on a shaker at room temperature for 15 min followed by vortexing for 15 sec. As in the section above (HBVpp Infection Assay), plates were read using a FLUOstar Omega chemiluminescence microplate reader (BMG LABTECH).

3.7 Immunoprecipitation pull-down assay

Protein A dynabeads were resuspended by vortexing for 30 seconds and 20 μL transferred to a 1.7mL eppendorf tube, this was placed on a Promega magnetic rack to separate dynabeads from solution. 1 $\mu\text{g}/\text{ml}$ of anti-annexin A2 was then diluted in 100 μL of 0.05% PBS-T, in parallel with anti-CD81. This mixture was then incubated for 10 minutes at room temperature in a rotating holder. During this incubation, the soluble form of annexin A2 (1 $\mu\text{g}/\text{ml}$) was incubated with the viral pseudotypes BT10D4, gtD13 and H77 for 10 minutes at room temperature. This was performed in parallel with the soluble form of CD81 as a control for the HCV sample. The antibody/dynabead mixture was placed back into the magnetic rack and the supernatant was removed. The samples containing the Ag/pseudotype were then added to the antibody/dynabead mixtures and incubated at room temperature to allow the Ag to bind to the dynabead-antibody complex. Post incubation, the tubes containing the mixture were placed onto the magnetic rack and the supernatant was removed. The complex was then washed with 200 μL of washing buffer (0.05% PBS-T) for each wash. The wash was carried out 4 times and the complexes were removed from the magnetic rack with each wash. The bead complex was then resuspended in 30 μL of washing buffer (0.05% PBS-T) and transferred into 0.2 ml PCR eppendorfs. The tubes were placed in a heat block and incubated at 95°C for 5 minutes and the beads were removed. A PCR master mixture was set up for 7 reactions including a blank. This master mixture contained 43.75 μL of PCR BIO One- Step Mix, 3.5 μL forward and 3.5 μL reverse luciferase primers diluted in H_2O (1/10), 4.38 μL RTase and 25.38 μL H_2O . The master mix was aliquoted into 7 tubes of 11.5 μL . 1 μL of the RNA templates were

extracted from the heated solution and added to the master mix aliquots, 1 μ L of H₂O was used as a blank.

PCR thermocycling conditions were as follows:

1 cycle of 10 minutes reverse transcription at 53 °C; 1 cycle of polymerase activation for 2 minutes 40 cycles of: [Denaturation at 95 °C for 10 sec; annealing at 60 °C for 10 sec; extension at 72 °C for 15 sec]. Amplicons were then visualised by gel electrophoresis as described above.

3.8 Annexin A2 binding ELISA

To perform the ELISA, pseudotypes were diluted 1:1 in carbonate bicarbonate buffer (Sigma C3041) and 150 μ L of the diluted prep was incubated overnight at 4°C. Excess liquid was removed from the wells by tapping the plate. Next, 250 μ L of blocking solution PBS-TM (PBS-0.05% Tween- 5% Milk powder) was added and incubated at room temperature for 1 hour. Excess liquid was removed by tapping and 50 μ L of annexin A2 protein sample at 1 μ g.mL in PBST was added.

The plate was incubated for 1 hour at room temperature in a humid box. The plate was washed 4 times with PBST wash buffer using 300 μ L of PBS-T per well, discarding wash buffer down the sink and tapping on clean paper towel each time before adding the next wash. Next, 50 μ L of anti-annexin A2 diluted appropriately at 1 μ g.mL in PBS-TM was added and the plate was incubated at room temperature for 1 hour. The plate was washed a further 4 times with PBST.

Then, 50 μ L of anti-mouse IgG conjugate (diluted 1:2000 in PBS-T) was added and left for one hour at room temperature. The plate was washed 4 times with PBS-T and 100 μ L of TMB (Thermo) substrate was added. The plate was read after 20 minutes at 620 nm on a plate reader.

3.9 Western Blotting

Cell lysates and/or cell suspension preparations were used to perform the western blot. To do this, protein (30 μ g) was resuspended in 10 μ L of 5x loading buffer (2.5 mL of 0.5 mol/L Tris HCl pH 6.8, 0.39 g Dithiothreitol

(DTT), 0.5 g Sodium Dodecyl Sulfate (SDS), 0.025 g Bromophenol Blue, 2.5 mL Glycerine). This was then heated to 95 °C for 5 min on a heat block to denature the proteins. Proteins and a BR spectra ladder were added to the 10-12 % precast gel (Bio-Rad) and SDS running buffer (15.18 g Tris HCl, 93.85 g Glycine, 50 mL of 20 % SDS) at 150 V for 40 min. Blotting paper and Nitrocellulose membranes were then soaked in the transfer buffer (5.8 g Tris HCl, 2.9 g Glycine, 1.85 mL of 20 % SDS, 200 mL methanol). Resolved proteins were subsequently transferred to nitrocellulose membranes at 100 mA for 45 min using a Trans-Blot transfer system. Protein transfer was confirmed by gel stain with coomassie blue stain (Thermo Fisher Scientific). Nitrocellulose membranes were blocked overnight with freshly prepared PBS, 0.1 % Tween and 10 % skimmed milk (PBSTM) at 4 °C. The membranes were then washed five times with PBST (with 150 mM NaCl) for 5 min per wash whilst being gently rocked from side to side. The membranes were incubated with 1:1000 of the mouse monoclonal anti-HBsAg primary antibody Ma1694 (Abnova) and mouse 104 monoclonal anti-HIV1 p24 antibody ab9071 (Abcam) in PBSTM at 4°C overnight. After incubation membranes were washed and incubated with 1:2000 of rabbit anti-mouse Horseradish peroxidase (HRP) – the secondary antibody (Sigma) for 1 h at room temperature with rocking. Subsequently, the Radiance Plus Chemiluminescent HRP substrate reagent (Azure Biosystems) was added to the membrane for 5 min. The membrane was then put in a clean, transparent sheet and viewed with a G: BOX F3 gel doc system for imaging. The band sizes of the amplicons were compared to the Spectra Multicolour Broad Range Protein Ladder (Thermo Fisher Scientific).

3.10 Statistical Analysis

For the statistical analysis GraphPad Prism (version 8) was used to perform ordinary one-way ANOVA's, unpaired T-tests set at 95% confidence intervals, repeated measures one-way ANOVA with Geisser-Greenhouse correction and Dunnett's multiple comparison test. Statistical significance was set at two-tailed $p \leq 0.05$.

4 Results

4.1 Proteomics data analysis from publicly available datasets

From publicly available datasets (Proteomic Identification Database, 2022), the expression of host proteins in cells that either support, or do not support HBV infection by different viral strains, specifically clone BT10D4, was investigated. This included BT10D4-susceptible lines such as Huh7, Hep3B and cells lines refractive to infection i.e., HepG2.

Five genes had the highest differences in protein expression between HepG2 and the other cell lines (Table 5). The data was taken from online proteomics data sets (EMBL's European Bioinformatics Institute, 2022).

From the preliminary infection data (See section 2), it was identified that BT10D4 was unable to infect HepG2 hepatoma cells in the absence of NTCP whereas infection was observed in Huh7 independent of NTCP expression (Figure 11). This led to investigations to identify proteins expressed in Huh7 cells and not in HepG2 (Table 5).

Table 5: Identification of top 5 genes with the highest differences in proteomic expression between the different hepatoma cell lines and Human liver microsomes (HLM), data was normalised to the presence of a control bacteriophage MS2 peptide peak added to the sample set as a reference. Normalisation was performed using the 'Localisation' strategy as implemented in the Spectronaut software package.

Genes	HLM (Mean Average)	Hep3B (Mean Average)	HepG2 (Mean Average)	Huh7 (Mean Average)
ANXA2	1100.1	740.1	108.3	2728.4
LIN28B	NA	166.6	58.5	1106.3
CHDH	435.7	81.2	130.4	748.7
FGB	7840.4	58.3	98.2	710.1
ATL3	757.1	131.6	49.4	591.2

The five proteins identified to have differential expression included:

Annexin A2 (AnxA2)

The membrane-associated protein, annexin A2 (AnxA2), has a variety of intracellular functions and is a common host factor in viral infections. AnxA2 is located in various parts of the cell such as the nucleus and cytoplasm, as well as on the internal and external leaflets of the plasma membrane, where the protein is vesicle-bound. AnxA2 can be expressed in multiple forms, as either a monomer or in a heterotetramer with S100A10, known as AnxA2/S100A10 (A2t). The proteins, AnxA2 and A2t have been linked to a variety of cellular functions, including endocytosis, exocytosis, membrane domain assembly, and RNA binding-mediated translational regulation (Bharadwaj et al., 2013).

Lin-28 homolog B

In embryonic stem cells, developmental tissues, cancer, and pluripotency, Lin-28 homolog B (LIN28B) is an RNA-binding protein that is involved in the regulation of mRNA translation and miRNA let-7 maturation (Zhang et al., 2016). In cancer, LIN28B is activated and assists as a key oncogene, according to a variety of studies. However, the molecular processes underpinning LIN28B's role in tumorigenesis remain mainly unclear. Furthermore, LIN28B was found to be expressed in more than half of the epithelial ovarian cancer patients in a study (Lin et al., 2018).

Choline Dehydrogenase (CHDH)

Choline oxidase and its membrane-associated choline dehydrogenase synthesize glycine betaine, one of a small number of biocompatible solutes utilized by cells to adapt for excessive osmotic stress (Gadda, 2020). Microorganisms need to accumulate substantial amounts of glycine betaine in the cytoplasm since they regulate intracellular water content by altering intracellular solute levels. In hypersaline settings,

failure to do so might result in dehydration, osmotic shock, and plasmolysis (Lai et al., 1999).

FGB

The FGB gene is responsible for the expression of the protein known as fibrinogen B beta (B β) chain which is one component (subunit) of the protein fibrinogen. Fibrinogen plays a crucial role in the formation of blood clots (coagulation), which is a process essential for the prevention of excessive blood loss after sustaining an injury. The Beta chain binds to the alpha (A α) and gamma (γ) fibrinogen chain proteins to form fibrinogen. These proteins are all expressed by different genes (Ceznerová et al., 2022). Another protein called thrombin takes a component from the alpha and beta subunits of the fibrinogen protein to induce coagulation (the components are known as the A and B fibrinopeptides). Fibrinogen is converted to fibrin, the primary protein in blood clots, during this process (Kattula et al., 2017).

ATL3

The ALT3 gene encodes for a dynamin-like membrane GTPase, known as Atlastin-3. This expressed protein is essential for the optimal development of the ER network of linked tubules (Hu et al., 2009). The protein, Atlastin-3, has been demonstrated to also have a role in Golgi apparatus and ER morphogenesis. A study showed that the use of siRNA (small interfering RNA) to knock down atlastin-2 and -3 levels in HeLa cells causes Golgi morphology to be disrupted (Rismanchi et al., 2008).

4.2 Possible entry receptor candidate for BT10D4

The receptor which demonstrated the greatest likelihood of being the entry receptor for BT10D4 was AnxA2. This was because AnxA2 had 25-fold difference in expression between HepG2 and Huh7 cell lines, this was the greatest difference observed in the dataset (Table 5). Furthermore, of the proteins with the highest differences in expression between HepG2 and Huh7, AnxA2 is the only protein which has already been demonstrated to be a viral entry receptor, for Human papillomaviruses (HPV) (Woodham et al., 2012) and it is cell surface

expressed. For these reasons AnxA2 was an ideal candidate for investigation.

4.3 HCV pseudotype generation and infection

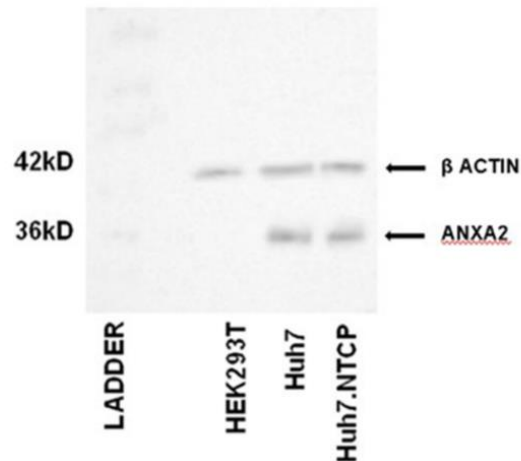
To ensure the experimental system was optimal, HCV pseudotypes were firstly used as reference model system to investigate whether the process of producing functional pseudotypes was possible. This experimental approach allowed confirmation that HEK293T cell lines (producer cells) were capable of producing infectious pseudotypes that could infect target Huh7 hepatoma cell lines.

The HCV J6.4 and H77 pseudotypes were successfully generated and infected Huh7 cells as expected, as shown by reporter gene expression (measured in RLU) (Figure 12). However, rates of infection were sub-optimal as compared to previously published data from our group (Urbanowicz et al., 2016). This may be due to a variety of reasons such as the 'health' of the producer (HEK293T) or target cells (Huh7). Overgrowth of these cells with lack of nutrients, impaired proliferation or growth can negatively affect their performance to produce pseudotypes (HEK293T) or become infected (Huh7 cell lines). Increased cell death may also be another factor. The positive control VSV-G infection did have high RLUs (surrogate for infectivity), (Figure 12), however it is important to note that VSV-G is highly infectious due to its highly efficient delivery of the retroviral cores to the target cells, as such high levels of infection maybe seen even if the cell line health was not maximal.

Before the pseudotype assays were performed, western blotting was adopted to separate and identify the proteins within the cell lines used in the study. The technique works by separating the mixture of proteins within the sample by its molecular weight which can then the type can be identified though gel electrophoresis. The bands illustrated in the images represent the membrane transferred proteins. The results are shown in Figure 12B, where the presence of annexin A2 in the different cell lines were visualized. The presence of annexin A2 (36kD) in both Huh7 and

Huh7.NTCP cells is clearly seen in these blots. In this experiment, the HEK293T cell line was the negative control, and the beta-actin (42kD) was the positive control.

A



B

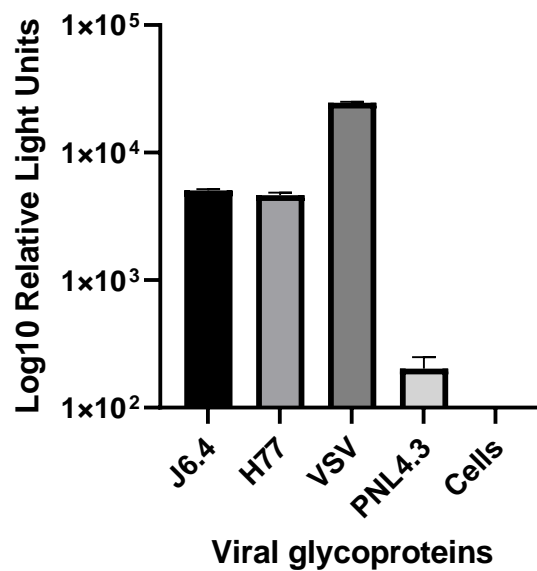


Figure 12: HCV pseudotype particle infection in Huh7 cells.

A Western blot analysis of annexin A2 expression in Huh7 and Huh7.NTCP. Lysate samples of Huh7, Huh7.NTCP and HEK293T were run by gel electrophoresis and then transferred to a nitrocellulose membrane. The nitrocellulose membrane was then incubated with a 1:1000 dilution of the mouse monoclonal primary antibody anti-annexin

A2 and anti-beta actin. The samples were then incubated with rabbit anti-mouse HRP secondary antibody (Sigma) and visualised using a G: BOX F3 gel doc system. A Spectra Multicolour Broad Range Protein Ladder was utilised as a molecular marker for comparison. B Huh7 cells seeded at 20,000 cells/well were infected with HCV pseudotypes (J6.4 and H77) and incubated at 37 °C and 5% CO₂ for 72 h. Infectivity was analysed by luciferase assay. VSV-G, and PNL4.3 (Delta E) were used as positive and negative controls. Reporter gene expression is presented as mean relative light units (RLU) \pm SD, this is an indication of Infection levels. J6.4 and H77 HCV pseudotypes successfully infected Huh7 cells.

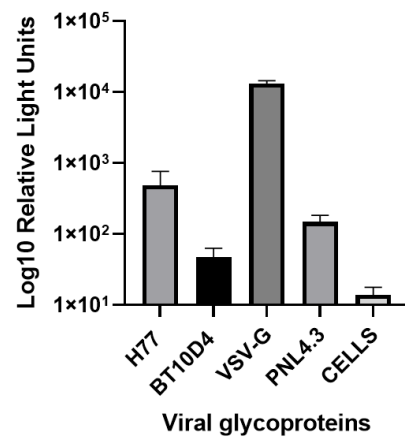
4.4 Optimisation of pseudotype generation

The viral glycoprotein inserts for BT10D4, gtD13 and H77 were all confirmed to be present in plasmids (Figure 13D) by PCR. A blank (no template control) was also included in the PCR reactions. Figure 13E confirms the plasmids in question are not contaminated, this was based on the size discrimination on the agarose gel and comparison with the ladder (last lane of gel). The image shows no other bands present within each lane, showing that there is no contamination. The correct insertion of the genes into the plasmids was further confirmed by Sanger sequencing (data not shown).

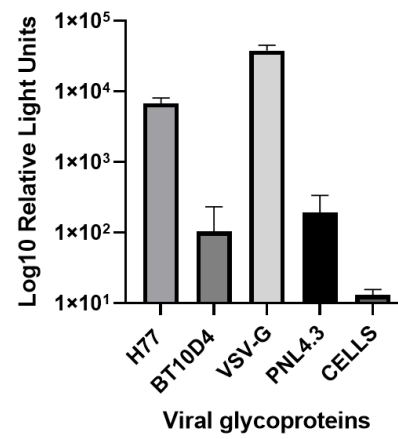
Earlier it was described that sub-optimal HCV infection was observed (Figure 12 and Figure 13A) however as seen in Figure 13B, a more robust infection was observed for H77 with RLU values of approximately 6000. However, this was not the case for HBV isolate BT10D4 (Figure 13A and B). Whilst HBV pseudotypes are known to have lower infectivity than HCV, the observed result was significantly lower than expected (Hiroko Omori, 2015). As mentioned earlier the 'health' of the cells may impact the infectivity. The morphology of HEK293T cells (Figure 13C) was star-like with processes, revealing a healthy phenotype however these did not support successful viral production, demonstrated by the significantly lower than expected relative light units (RLU) for the HBV pseudotype BT10D4 (Figure 13A and B). The lack of active infection of BT10D4, above background levels, was likely due to poor production of HBV pseudotypes rather than the clinical isolate BT10D4's inability to infect Huh7 cells in the absence of the receptor NTCP. As the investigation of HBV pseudotype continued, it was noticeable that HBV

pseudotypes were more sensitive to the precise conditions for production, and as a consequence more difficult produce than HCV pseudotypes.

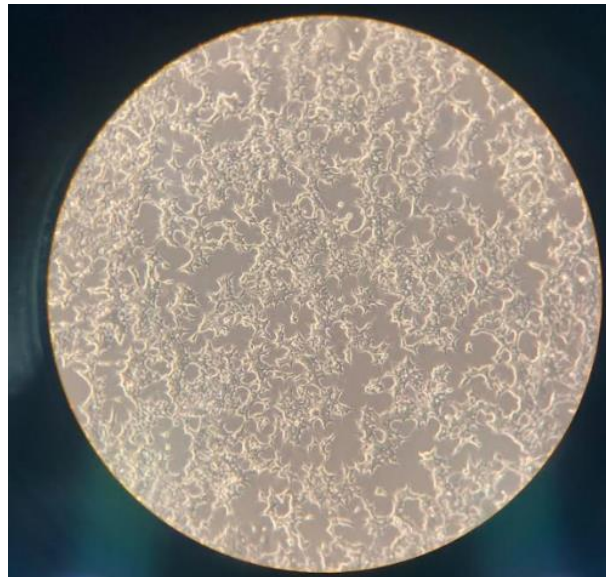
Panel A



Panel B



Panel C



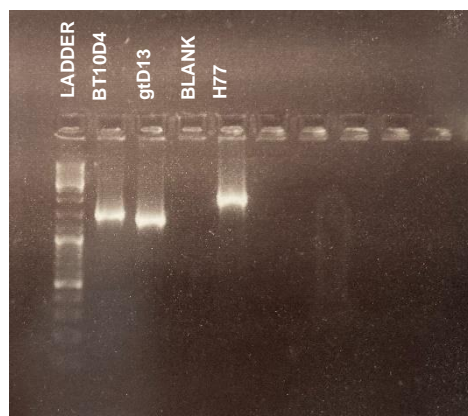
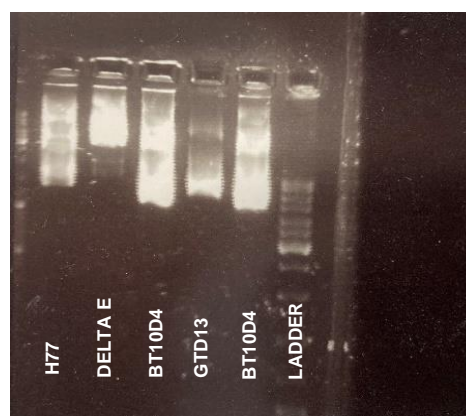
Panel D**Panel E**

Figure 13: HBV pseudotype infection in Huh7 cells. **Panel A:** Huh7 cells seeded at 20,000 cells/well were infected with HBV and HCV pseudotypes (BT10D4 and H77) and incubated at 37 °C and 5% CO₂ for 72 h. Infectivity was analysed by measuring the amount of luciferase expression in infected cells. VSV-G (1500 gain) and PNL4.3 (Delta E) were used as positive and negative controls respectively. Data are shown as log10 of mean data from 3 replicates of relative light units \pm SD. **Panel B:** HCV and HBV pseudotype infection in Huh7 cells. **Panel C:** Healthy post-transfected HEK293T cells (Day 3)- 200X magnification. **Panel D:** Viral templates were amplified using PCR and then run on an agarose Gel Electrophoresis showing the presence of the viral glycoprotein inserts in BT10D4 (1250bp), H77 (1700bp) and gtD13 (1200bp) plasmids. Thermo scientific Gene Ruler DNA Ladder was used as a molecular marker for comparison. DNA bands were visualised by UV transillumination. **Panel E:** Agarose Gel Electrophoresis used to examine contamination. Thermo scientific Gene Ruler DNA Ladder was used as a molecular marker for comparison. DNA bands were visualised by UV transillumination.

Repeat experiments of pseudotype infection in Huh7 cells were performed and clearly demonstrated BT10D4 infection in the absence of the receptor NTCP (Figure 14). However, in this experiment the infectivity values for the HCV H77 control were lower than previously observed (Figure 13B). To address this issue with the reproducibility of infection assays, an investigation of experimental factors contributing to the variation was undertaken, to identify the optimal conditions.

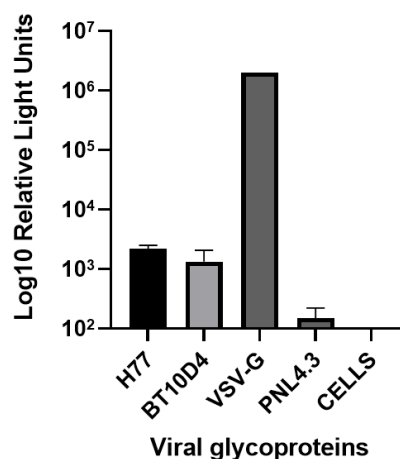


Figure 14: HBV pseudotype infection in Huh7 cells. Huh7 cells seeded at 20,000 cells/well were infected with HBV and HCV pseudotypes (BT10D4 and H77) and incubated at 37 °C and 5% CO₂ for 72 h. Infectivity was analysed by a luciferase assay. VSV-G (3600 gain), due to its broad tropism, and PNL4.3 (Delta E) were used as positive and negative controls. Data are shown as log10 mean relative light units \pm SD.

Increasing the ratio of packaging construct to glycoprotein did not improve production of viral particles (Figure 15). This experiment was repeated to confirm these findings. In addition, whether 14-day storage of the pseudotypes at 4°C affected ability to infect was explored. All pseudotypes were less infectious with a lower RLU at day 14 (Figure 15). However, due to the low RLU levels overall, it was difficult to establish statistically significant differences between day 1 and day 14.

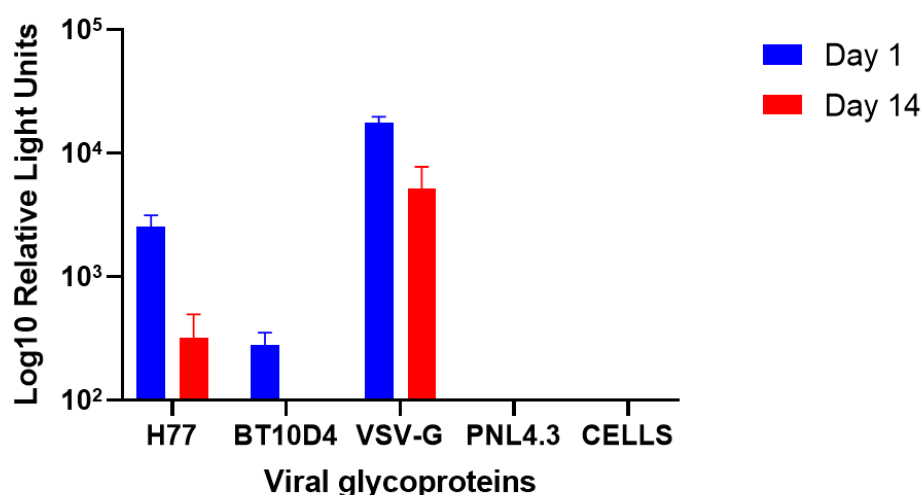


Figure 15: Pseudotype infection with a 2:1 packaging construct to HBsAg ratio at days 1 and 14. Huh7 cells seeded at 20,000 cells/well

were infected with HBV and HCV pseudotypes (BT10D4 and H77) that were constructed with 4 ug of Gag-expressing plasmid and 2 ug of viral glycoprotein expressing plasmid, a (2:1) ratio. These were incubated at 37°C and 5% CO₂ for 72 h. Infectivity was analysed by a luciferase assay. VSV-G (1500 gain), due to its broad tropism, and PNL4.3 (Delta E) were used as positive and negative controls. Data are shown log₁₀ of mean relative light units \pm SD. Statistical analysis was carried out utilizing an unpaired t-test set at a 95% confidence interval.

As use of samples after 14 days reduced the infectivity of the pseudotypes (Figure 15), infection was examined with pseudotypes at day 1 (the day of pseudotype harvest) and after 7 days of storage at 4°C. No significant difference in RLU was observed between viral particles harvested at day 1 and after storage for 7 days (Figure 16). This was true for HBV pseudotype BT10D4 ($p=0.2783$) and HCV pseudotype H77 ($p=0.5903$). Finally, the data shown in Figure 16 also confirmed previous findings demonstrating that increasing the ratio of packaging construct to viral glycoprotein, at ratio of 2:1 does not increase in viral particle production.

To finally verify the findings, an experiment was undertaken with glycoprotein to gag plasmid at 1:1 ratio from day 1 (plasmid harvest) and after 7 and 14 days of storage at 4°C.

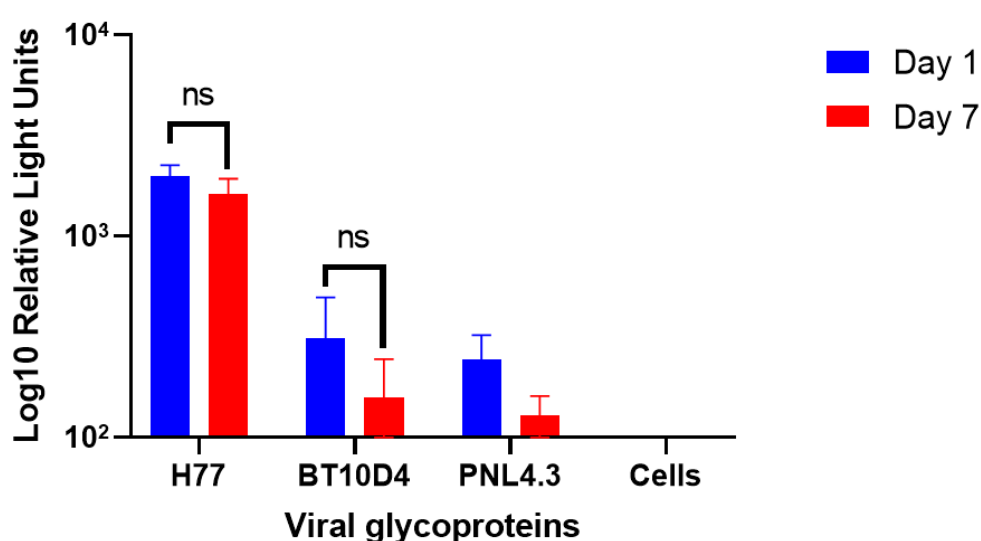
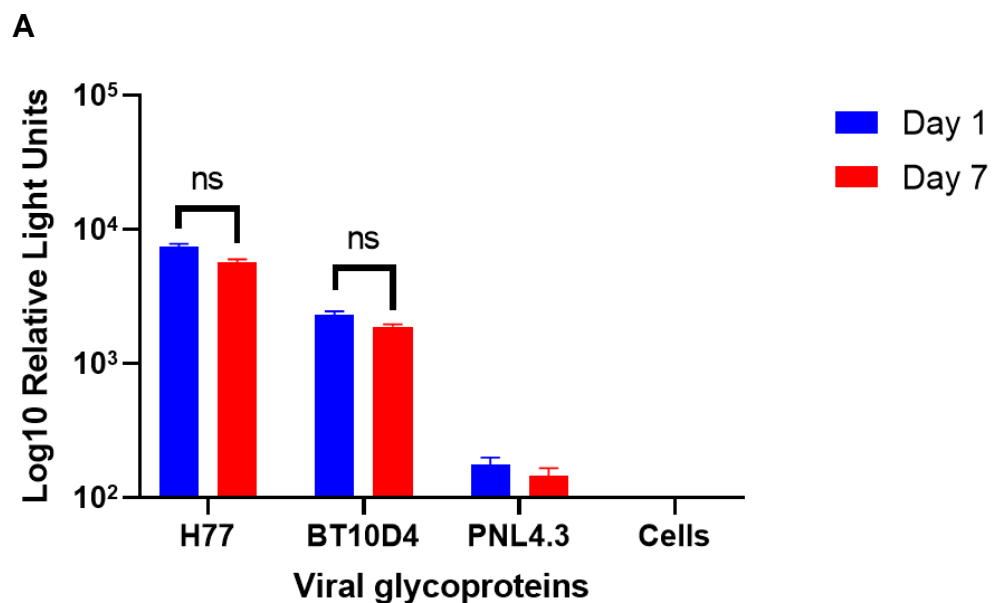


Figure 16. Days 1 & 7 Pseudotype infection with a 2:1 packaging construct to HBsAg ratio. Huh7 cells seeded at 20,000 cells/well were infected with HBV and HCV pseudotypes (BT10D4 and H77) that were

constructed with 4 ug of Gag plasmid and 2 ug of viral glycoprotein (2:1) packaging construct to viral glycoprotein and incubated at 37 °C and 5% CO₂ for 72 h. Infectivity was analysed by a luciferase assay. HCV and PNL4.3 (Delta E) were used as positive and negative controls. The data shows the comparison of particles used on day 1 and day 7, presented as log₁₀ of mean relative light units \pm SD. Statistical analysis was carried out utilizing an unpaired t-test set at a 95% confidence interval. **** indicates $p < 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, ns indicates $p > 0.05$.

Storage of pseudotypes for 14 days at 4°C was conclusively found to be detrimental to their infectivity (Figure 17B). There was a demonstrable reduction in infection between pseudotypes infected on day 1 compared to day 14. This was true for H77 ($p = 0.0002$) and BT10D4 ($p = 0.0035$). However, 7 days did not impair their infection ability particularly for H77 ($p = 0.0946$) and BT10D4 ($p = 0.2304$) (Figure 17A). However, it should be noted that there was a trend for luciferase signal to be lower on day 7 than day 1. The combined data (Figures 15, 16, and 17) also reveal that 2 μ g of gag plasmid and 2 ug of viral glycoprotein (1:1) results in best functional viral pseudotypes with highest RLU generated. These conditions were taken forward for the remaining experiments in this thesis.



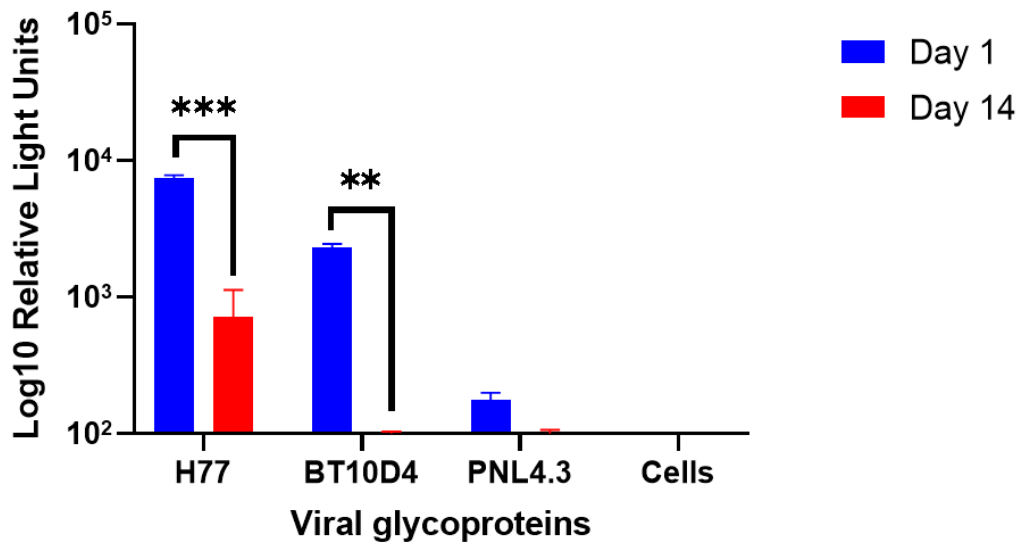
B

Figure 17 Comparison of pseudotype infection at day 1, 7 and 14 with a 1:1 packaging construct to HBsAg ratio. Huh7 cells seeded at 20,000 cells/well were infected with HBV and HCV pseudotypes (BT10D4 and H77) that were constructed with 2 ug of Gag plasmid and 2 ug of viral glycoprotein (1:1 packaging construct to viral glycoprotein) and incubated at 37 °C and 5% CO₂ for 72 h. Infectivity was analysed by a luciferase assay. HCV and PNL4.3 (Delta E) were used as positive and negative controls. **A** The data shows the comparison of particles used on day 1 and day 7. **B** The data shows the comparison of particles used on day 1 and day 14. Data presented as log₁₀ of mean relative light units ± SD. Statistical analysis was carried out utilizing an unpaired t-test set at a 95% confidence interval. **** indicates p < 0.0001, *** indicates p < 0.001, ** indicates p < 0.01, ns indicates p > 0.05.

4.5 NTCP dependent and independent HBV infection

The pseudotype assays carried out demonstrated that the HBV pseudotype BT10D4 was able to successfully infect the target Huh7 cells independently of the receptor NTCP. The next phase of the experiments was aimed to show that this was a specific phenomenon associated with BT10D4 and other HBV variants did not infect Huh7 cells in the absence of NTCP.

BT10D4 was found to infect Huh7 cells independently of NTCP, whereas the HBV variant Btmut, a BT10D4 variant with a C29R reversion mutation, was unable to infect these cells (Figure 18). A statistically significant difference of $p < 0.0001$ was found between BT10D4 and Btmut infection rates (RLUs). Subsequently, another variant of HBV was investigated to assess whether NTCP was required for infection. This was performed to consolidate the data surrounding the unusual phenotype of BT10D4.

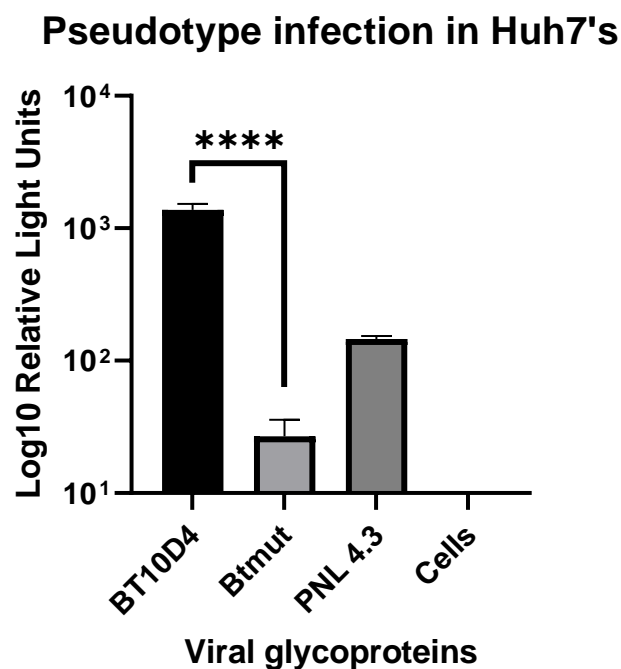
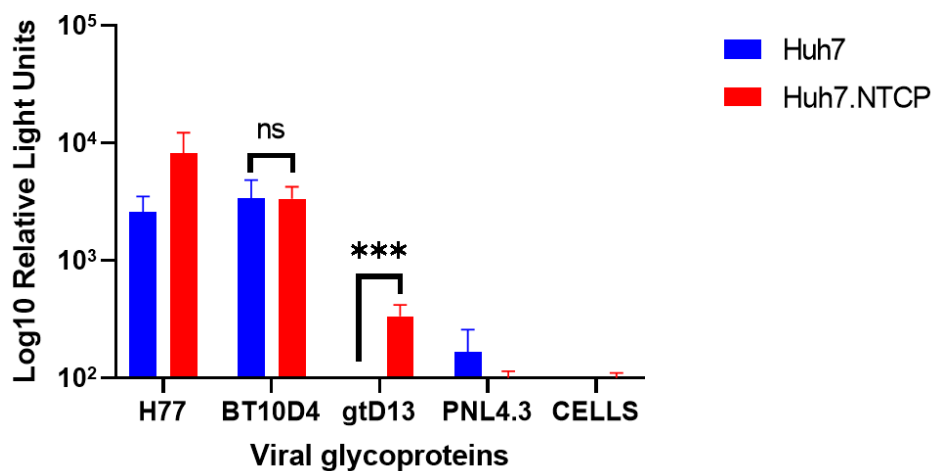


Figure 18: NTCP dependent and independent HBV pseudotype infection. Huh7 cells seeded at 20,000 cells/well were infected with HBV pseudotypes (BT10D4 and Btmut) and incubated at 37 °C and 5% CO₂ for 72 h. Infectivity was analysed by a luciferase assay. Btmut and PNL4.3 (Delta E) were used as positive and negative controls. Data presented as log₁₀ of mean relative light units ± SD. Statistical analysis was carried out utilizing an unpaired t-test set at a 95% confidence interval. **** indicates $p < 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, ns indicates $p > 0.05$.

HBV variant gtD13 was also found to be NTCP dependent (Figure 19). Here Huh7 and Huh7.NTCP cells were used to demonstrate that other variants of HBV are also dependant on NTCP for infection. In Figure 19A,

we can see that BT10D4 is equally infecting Huh7 cells in both the presence and absence of NTCP ($p=0.9582$). Whereas, for the HBV variant gtD13 there is only significant infection when NTCP is present ($p=0.0003$). The repeat of this experiment (Figure 19B) showed the same findings with an absence of significant difference in BT10D4 infection between Huh7 cells and Huh7.NTCP cells ($p=0.4716$). Similarly, to the first experiment, there was a difference in infection for gtD13 infection in Huh7 cells and Huh7.NTCP cells. However, there seemed to more of a difference between first gtD13 infection when compared to the gtD13 infection in second experiment. Due to the lower levels of infection in Huh7 cells and in Huh7.NTCP cells, this could potentially question the viability of the gtD13 pseudotype. To confirm these findings the experiment was repeated with new pseudotype particles to validate the results regarding the gtD13 infection. In Figure 19C, the representative RLU values for each pseudotype were achieved. This data confirmed results from the two previous experiments including no significant difference of BT10D4 infection between Huh7 cells and Huh7.NTCP ($p=0.9977$) and a significant difference in gtD13 infection between the two cell lines ($p=0.0023$). In this experiment it was clearer that pseudotype gtD13 were functional, due to the higher RLU levels in Huh7.NTCP infection.

A



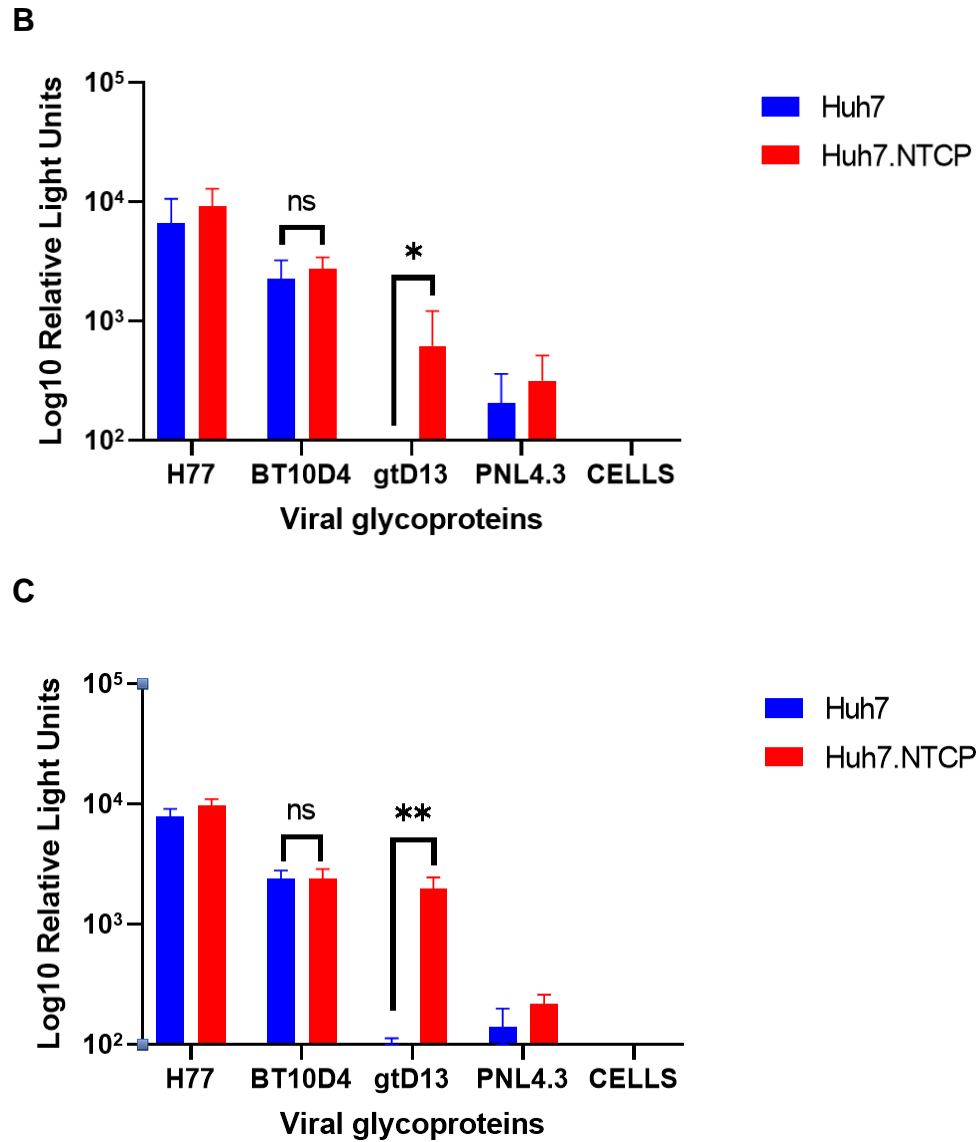


Figure 19. NTCP dependent and independent HBVpp infection in Huh7 cells and Huh7 cells expressing NTCP. **A** Huh7 and Huh7.NTCP cells seeded at 20,000 cells/well were infected with HBV pseudotypes (BT10D4 and gtD13) and incubated at 37 °C and 5% CO₂ for 72 h. Infectivity was analysed by a luciferase assay. Pseudotypes gtD13 and PNL4.3 (Delta E) were used as positive and negative controls. **B** Repeated experiment, performed in parallel using the same pseudotype particles. **C** Repeated experiment using new pseudotype particles. Data presented as log10 of mean relative light units \pm SD. Statistical analysis was carried out utilizing an unpaired t-test set at a 95% confidence interval. **** indicates $p < 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, ns indicates $p > 0.05$.

To further validate the findings, the complete experiment comparing BT10D4 and gtD13 NTCP-dependant infection was performed side-by-

side. No significant difference in BT10D4 infection between both cell lines with and without NTCP respectively, was observed ($p>0.9999$) (Figure 20). Functional gtD13 pseudotype particles clearly demonstrated a significant difference between its infection between Huh7 cells and Huh7.NTCP ($p= 0.0080$) (Figure 20). As expected, the statistical difference between the infection of BT10D4 and gtD13 was highly significant ($p= <0.0001$).

BT10D4 & gtD13 HBVpp assay

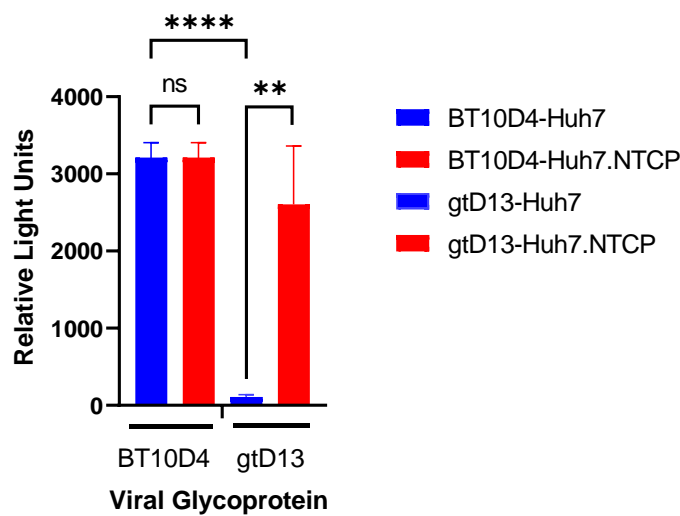


Figure 20 Direct comparison between BT10D4 and gtD13 infection in Huh7 cells and Huh7.NTCP. A Huh7 and Huh7.NTCP cells seeded at 20,000 cells/well were infected with HBV pseudotypes (BT10D4 and gtD13) and incubated at 37 °C and 5% CO₂ for 72 h. Infectivity was analysed by a luciferase assay. Data presented as mean relative light units \pm SD. Statistical analysis was carried out utilizing an unpaired t-test set at a 95% confidence interval. **** indicates $p<0.0001$, *** indicates $p<0.001$, ** indicates $p<0.01$, ns indicates $p>0.05$.

4.6 Annexin A2 antibody neutralisation assays

The protein AnxA2 had a 25-fold difference in expression between HepG2 and Huh7, making it an ideal candidate for further investigation. To further investigate AnxA2, a series of neutralisation assays were performed using an anti-annexin A2 blocking antibody to assess its ability to prevent BT10D4 infection in Huh7 and Huh7.NTCP cells (Figure 21).

The cell lines were treated with anti-annexin A2 blocking antibody using a dilution series ranging from 0-10 µg/mL. As controls, HCV pseudotypes and anti-CD81, a well-known anti-receptor antibody for HCV were included together with a negative control (delta E; without incorporation of a surface glycoprotein). The absence of antibody (untreated cell lines) was used as a positive control for infection.

Firstly, no significant difference was observed between infection between untreated Huh7 cell lines compared to those treated with 10 µg/mL anti-annexin A2 blocking antibody ($p=0.7105$) (Figure 21A). Secondly, no significant difference was observed in infection of BT10D4 between untreated (0 µg/mL anti-annexin A2 antibody) and anti-annexin A2 (10 µg/mL) antibody treated Huh7.NTCP cell lines ($p=0.2380$) (Figure 21B). However, there appeared to be a trend of decreasing of pseudotype infectivity as the concentration of anti-annexin A2 increased. The neutralisation assay of H77 (HCV) using anti-annexin A2 shown in Figure 21C, showed no significant difference in infection between untreated and Huh7 cell lines treated with 10 µg/mL of anti-annexin A2 ($p=0.2663$). As expected, H77 infection in Huh7.NTCP cells treated with anti-annexin A2 revealed no significant differences between 0 µg/mL and 10 µg/mL ($p=0.7076$) (Figure 21D). Pre-treatment of Huh7 cells with anti-CD81 infected with BT10D4 (Figure 21E) also showed no significant difference in infection between untreated and 10 µg/mL of anti-CD81 ($p=0.3474$). Again, in Figure 21F, the repeat of this infection in Huh7.NTCP gave rise to no significant difference between 0 µg/mL and 10 µg/mL of anti-CD81 ($p=0.6381$). The infection of H77 in anti-CD81 pre-treated Huh7 and Huh7.NTCP, displayed in Figures 21 G and H, showed a clear significant difference between 0 µg/mL and 10 µg/mL of anti-CD81 ($p=0.0003$). This

reduction in infection in both cell lines, validated the effectiveness of the CD81 antibody.

Despite there being no significant difference, there appeared to be a trend where there was a reduction of BT10D4 infection in Huh7.NTCP cell lines as the concentration of anti-annexin A2 was increased. In addition to confirming the reproducibility of these results, the trend warranted further investigation with more repeats. The experiment was therefore repeated using a higher concentration of antibody (20 µg/mL).

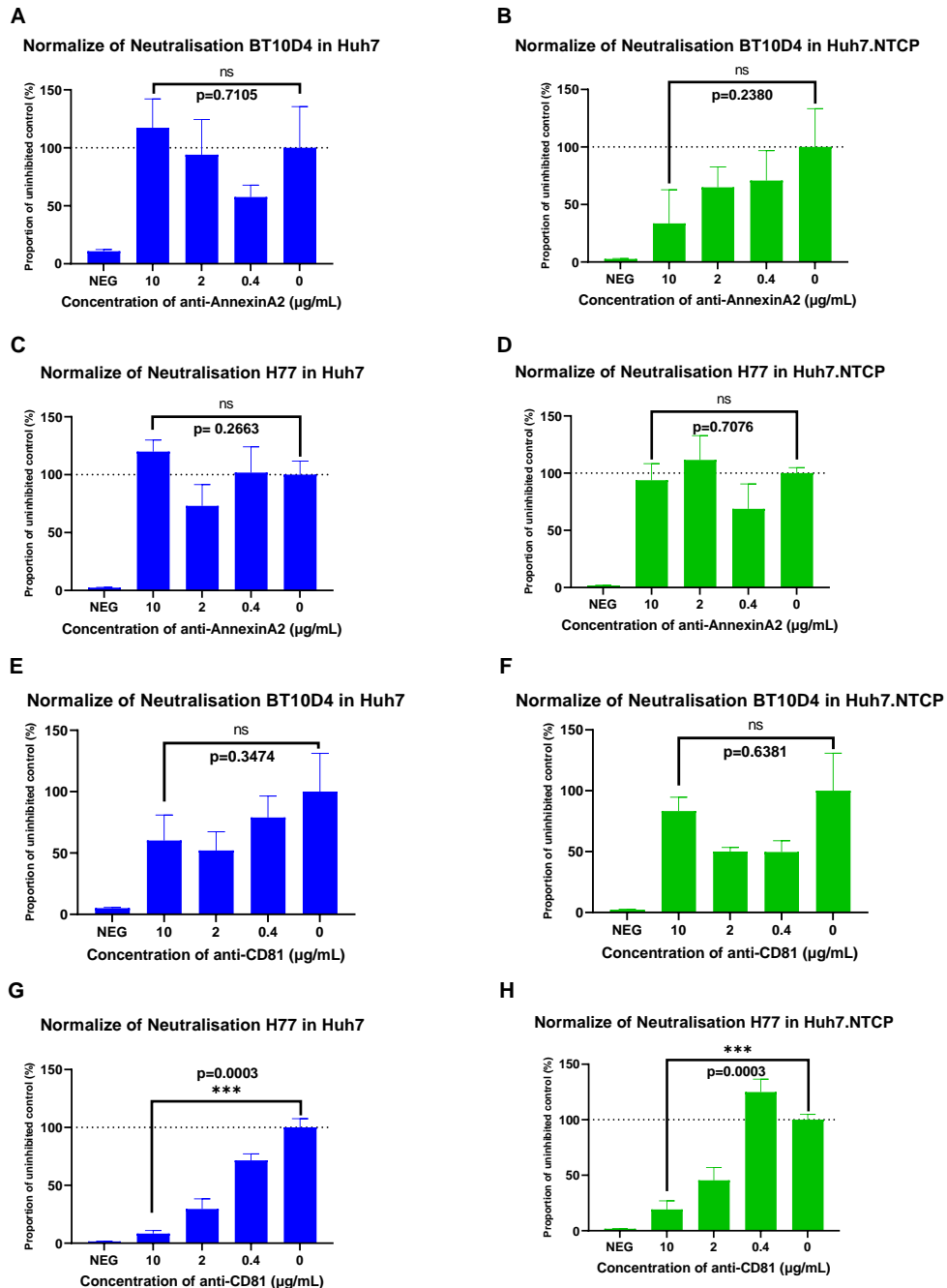


Figure 21: Neutralisation of BT10D4 using anti-annexin A2 and anti-CD81 antibodies in Huh7 and Huh7.NTCP cell lines. Cells seeded at 20,000 cells/well were pre-treated with either anti-annexin A2 or anti-CD81. The cells were then infected with BT10D4 and H77 pseudotyped and incubated at 37 °C and 5% CO₂ for 72 h. Infectivity was analysed by a luciferase assay. Data presented as proportion of uninhibited control (%). The negative control (NEG) was cells not infected with pseudotype. Statistical analysis was carried out utilizing an unpaired *t*-test set at a 95% confidence interval. **** indicates *p*<0.0001, *** indicates *p*<0.001, ** indicates *p*<0.01, ns indicates *p*>0.05.

With this experimental setup, the main finding was that no significant difference in BT10D4 infectivity rates in Huh7.NTCP cells was seen between untreated cells and those treated with a higher 20 µg/mL concentration of anti-annexin A2 antibody ($p=0.0508$) (Figure 22B). However, the statistical value was very close to being significant and showed a similar trend to the previous experiment with BT10D4 infection in Huh7.NTCP treated with 10 µg/mL of anti-annexin A2 (Figure 21B). The neutralisation of H77 using anti-annexin A2 (Figure 22C), demonstrated that there was no significant difference in infection between 0 µg/mL and 20 µg/mL of anti-annexin A2 ($p=0.4403$). As expected, when Huh7 cells were pre-treated with anti-CD81 and infected with BT10D4 (Figure 22E), no significant difference in infection between untreated cells and those treated with 20 µg/mL of anti-CD81 ($p=0.9437$) was observed. The repeat infection experiments (Figure 22F) showed that Huh7.NTCP gave rise to no significant difference between 0 µg/mL and 20 µg/mL of anti-CD81 ($p=0.8537$). The infection of H77 in Huh7 cells pre-treated with anti-CD81, displayed in Figure 26G, showed a clear significant difference between untreated and cells treated with 20 µg/mL of anti-CD81 ($p=0.0002$). This was again seen in Figure 26H, where there was a significant reduction in infection between untreated cells and those treated at 20 µg/mL anti-CD81 in Huh7.NTCP cells ($p=0.0084$).

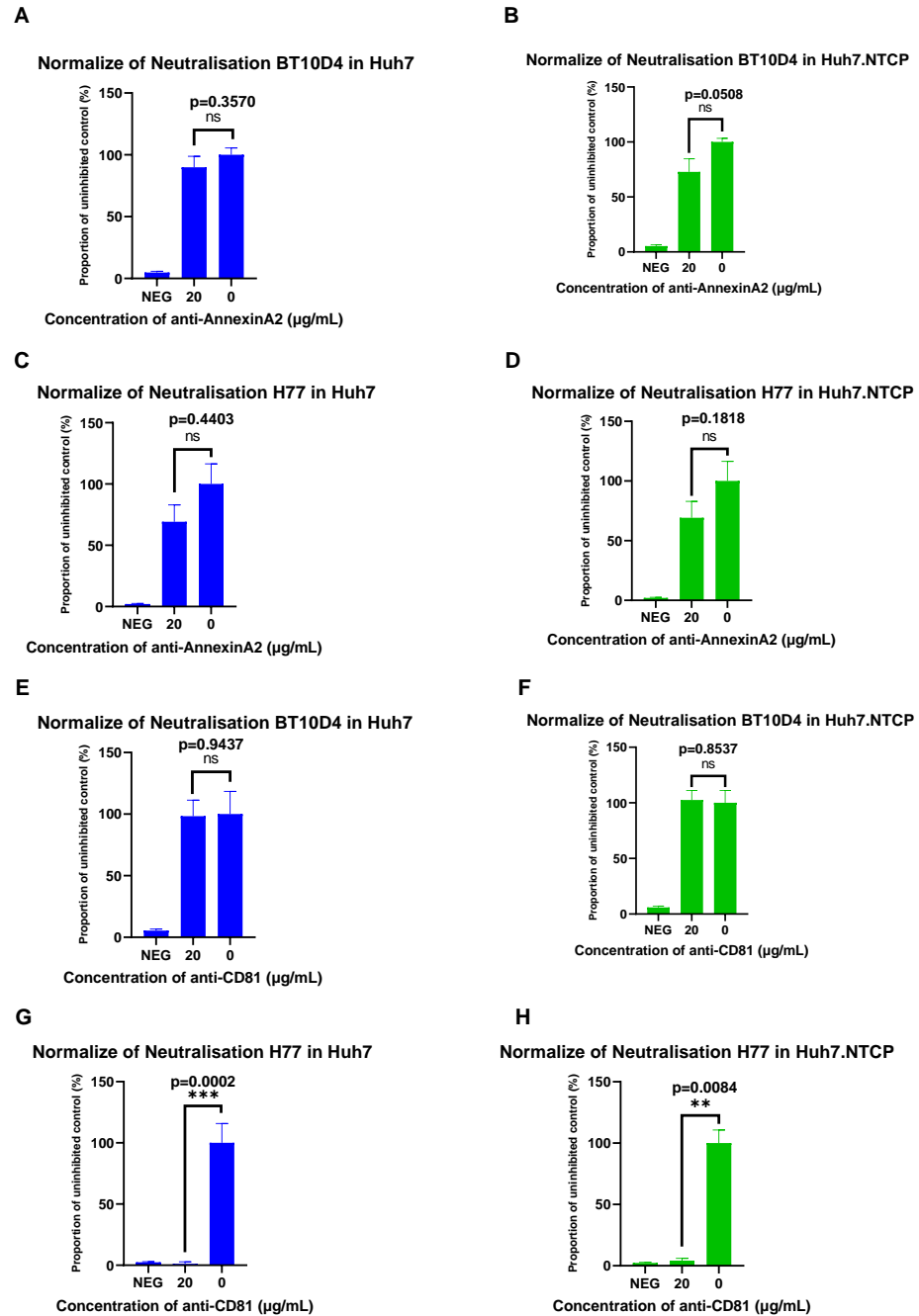


Figure 22: Normalised data of neutralisation of BT10D4 using anti-annexin A2 and anti-CD81 antibodies (Concentrations of 0-20 µg/mL). Neutralisation of BT10D4 in Huh7 and Huh7.NTCP. Cells seeded at 20,000 cells/well were pre-treated with either anti-annexin A2 or anti-CD81. The cells were then infected with BT10D4 and H77 pseudotypes and incubated at 37 °C and 5% CO₂ for 72 h. Infectivity was analysed by a luciferase assay. Data presented as proportion of uninhibited control (%). The negative control (NEG) was cells not infected with pseudotype. Statistical analysis was carried out utilizing an unpaired t-test set at a 95% confidence interval. **** indicates $p < 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, ns indicates $p > 0.05$.

To confirm the trend observed in the previous neutralisation experiment the experiments were then repeated, to identify a relationship between annexin A2 and BT10D4 entry in Huh7.NTCP cells. No significant difference was seen between Huh7 cells treated with anti-annexin A2 and Huh7 cells with no antibody ($p=0.4676$) (Figure 23A). The anti-annexin A2 antibody's inability to inhibit BT10D4's infection of Huh7 cell lines has been observed in all previous neutralisations performed. In addition to this, there was no significant difference between the positive control and Huh7 cells pre-treated with anti-CD81 ($p=0.8076$). The results illustrated in Figure 23B, also show no significant difference between the positive control and Huh7.NTCP cells pre-treated with anti-annexin A2 ($p=0.8570$). Although all experiments of BT10D4 infection in Huh7.NTCP cells have been non-significant, there has either been a trend (Figure 21 B), or the data has been close to being significantly different (Figure 22 B). In contrast, there was no significant difference between the positive control and Huh7.NTCP cells pre-treated with anti-CD81 ($p=0.7304$).

The validity of the experimental set-up was confirmed as the controls worked effectively, both Huh7 and Huh7.NTCP cells pre-treated with anti-CD81 had a significant reduction in infection ($p=0.0003$) and as expected there was no significant difference between the positive controls and both cell lines pre-treated with anti-annexin A2 ($p=0.2184$) ($p=0.6589$) (Figure 23C and D). There did however appear to be slight discrepancies between the neutralisation data of Huh7.NTCP cells pre-treated with anti-annexin A2 and BT10D4 entry. This called for further investigation into variations of this assay to understand if there could potentially be relationship between anti-annexin A2 and Huh7 cells expressing NTCP, thereby causing BT10D4 entry. To achieve this, the experiment was re-created utilising the soluble form of the receptor annexin A2. This was performed by incubating the soluble receptor with the pseudotypes and then infecting Huh7 and Huh7.NTCP cells.

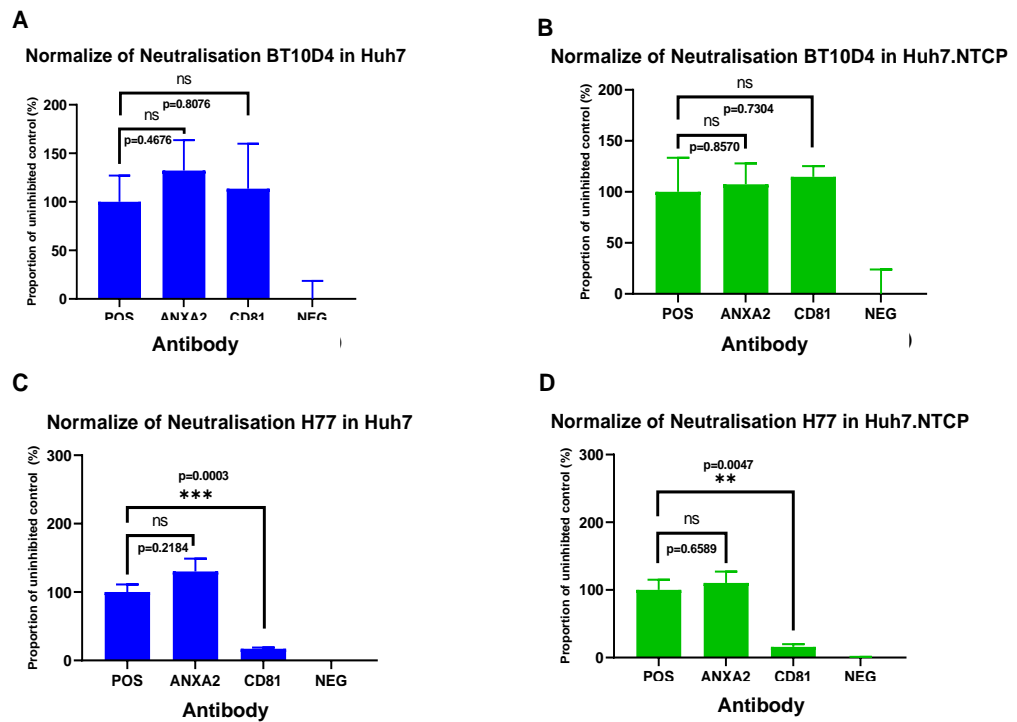


Figure 23: Normalised data of neutralisation of BT10D4 using anti-annexin A2 and anti-CD81 antibodies (Concentrations of 0-20 $\mu\text{g/mL}$) repeated. Neutralisation of BT10D4 in Huh7 and Huh7.NTCP. Cells seeded at 20,000 cells/well were pre-treated with either anti-annexin A2 or anti-CD81. The cells were then infected with BT10D4 and H77 pseudotypes and incubated at 37 °C and 5% CO₂ for 72 h. Infectivity was analysed by a luciferase assay. Data presented as proportion of uninhibited control (%). The negative control (NEG) was cells not infected with pseudotype and the positive control (POS) represented cells infected by pseudotype with no antibody administered. Statistical significance was analysed using a repeated measures one-way ANOVA with the Geisser-Greenhouse correction and the Dunnett's multiple comparisons test, and each antibody was compared to BT10D4 with no antibody (positive control). **** indicates $p < 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, ns indicates $p > 0.05$.

In brief, the soluble of annexin A2 and CD81 (at a concentration 20 $\mu\text{g/mL}$) did not show neutralisation activity for of BT10D4 infection in Huh7 and Huh7.NTCP cells (Figure 24). This experiment included a positive control (no antibody) and a negative control (delta E). There was no significant difference between the infectivity with no annexin A2 and treatment of 20 $\mu\text{g/mL}$ annexin A2 in Huh7 cells ($p = 0.2452$) (Figure 24A). Huh7.NTCP cells infected with BT10D4 that were pre-treated with

annexin A2 also showed no significant changes in infection at 20 µg/mL compared to untreated ($p=0.9085$) (Figure 24B). The neutralisation of H77 using soluble annexin A2 (Figure 24C) also showed no significant difference in infection between 0 µg/mL and 20 µg/mL of soluble annexin A2 ($p=0.3492$). H77 infection in Huh7.NTCP cells treated with soluble annexin A2 revealed changes in the presence of 20 µg/mL of anti-annexin A2 ($p=0.6660$) (Figure 24D). As expected, when Huh7 cells infected with BT10D4 that were pre-treated with 20 µg/mL soluble CD81 (Figure 24E), showed no changes ($p=0.5767$). Whilst infection Huh7.NTCP gave rise to no significant difference with and 20 µg/mL of soluble CD81 ($p=0.4198$) (Figure 24F), the infection of H77 in Huh7, (Figure 24G), showed a clear significant differences with 20 µg/mL of soluble CD81 ($p<0.0001$). There was also a significant reduction in infection of Huh7.NTCP with 20 µg/mL of soluble CD81 ($p<0.0001$) (Figure 24H). The reduction in HCV infection with CD81, validates that the controls worked efficiently and that the assay was functional.

This variation of assay showed clearly that there was no significant difference in infection between the positive control and BT10D4 pre-treated with annexin A2 ($p=0.9085$). This data provides further support that annexin A2 is not the receptor in which the isolate BT10D4 is entering through. To validate the results, the experiment was repeated multiple times.

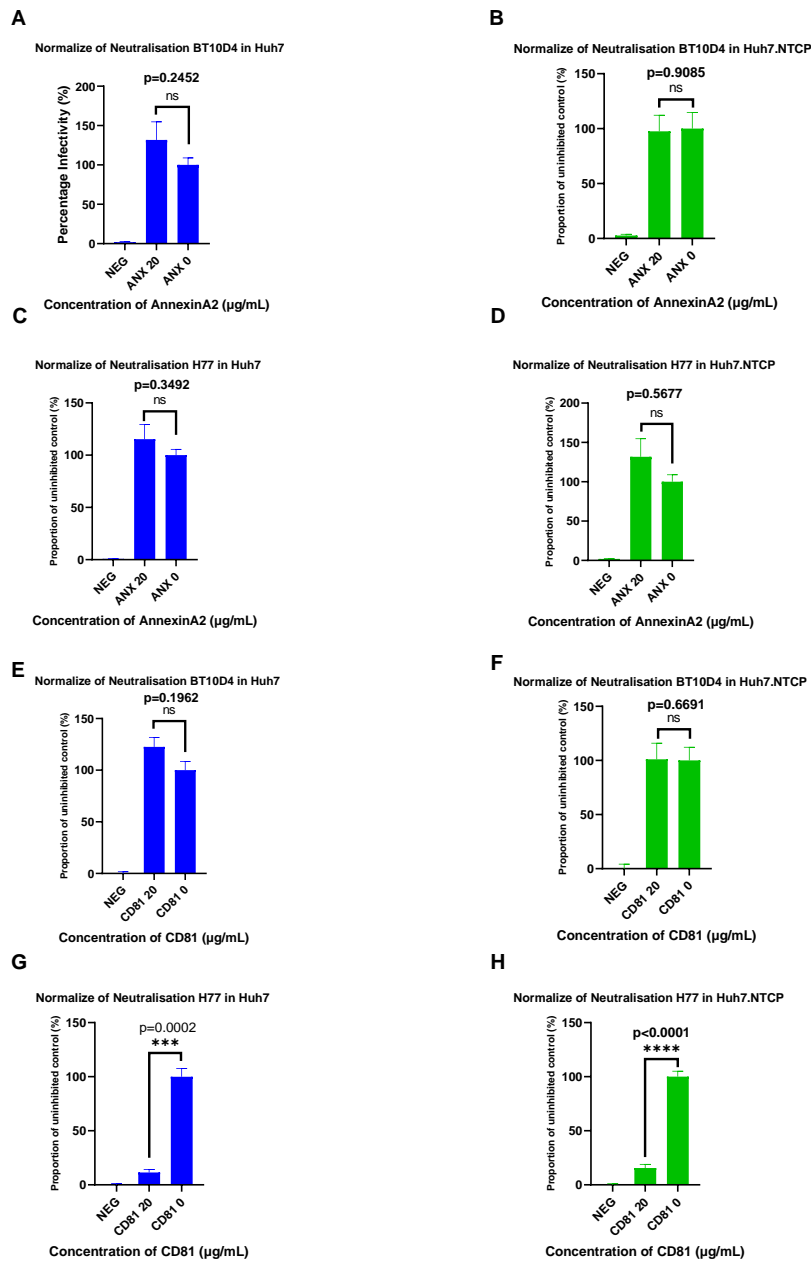


Figure 24. Normalised data of neutralisation of BT10D4 using soluble forms of annexin A2 and CD81 (Concentrations of 0-20 µg/mL). Neutralisation of BT10D4 in Huh7 and Huh7.NTCP. Cells seeded at 20,000 cells/well and then infected with BT10D4 and H77 pseudotypes that were pre-treated with soluble forms of either annexin A2 or CD81 receptor and incubated at 37 °C and 5% CO₂ for 72 h. Infectivity was analysed by a luciferase assay. Data presented as proportion of uninhibited control (%) - normalised data of neutralisation assays of BT10D4 in Huh7 and Huh7.NTCP cells. The negative control (NEG) was cells not infected with pseudotype. Statistical analysis was carried out utilizing an unpaired t-test set at a 95% confidence interval. **** indicates $p<0.0001$, *** indicates $p<0.001$, ** indicates $p<0.01$, ns indicates $p>0.05$.

The results of the repeat experiments for BT10D4 neutralisation are shown in Figure 25. This experiment provided further confirmation that there is no inhibitory effect of soluble annexin A2 on BT10D4 entry. Furthermore, it was important to attempt to elucidate whether there really was a relationship between annexin A2 and Huh7.NTCP.

No significant difference between Huh7 cells infected with BT10D4 that was pre-treated with soluble annexin A2 and the positive control ($p=0.1994$) (Figure 25A) was observed. The inability of annexin A2 in soluble form and as an antibody to inhibit BT10D4 in Huh7 cells was consistent throughout all neutralisation assay experiments. In addition to this, there was no significant difference in infection between the positive control and BT10D4 pre-treated with soluble CD81 in Huh7 cells ($p=0.1500$). Moreover, no significance between the positive control and BT10D4 pre-treated with annexin A2 in Huh7.NTCP cells ($p=0.4847$) was seen. This was also true for the effects of soluble CD81 on BT10D4 in Huh7.NTCP cells ($p=0.1349$) (Figure 25B). However, the experiments were valid as the controls worked effectively: H77 pre-treated with soluble CD81 had a significant reduction of infection in both Huh7 and Huh7.NTCP cells ($p=0.0002$, $p=0.0001$ respectively) and as expected there was no significant difference between the positive controls and H77 pre-treated with soluble annexin A2 in both cell lines ($p=0.1679$) ($p=0.4847$) (Figure 25C and D respectively).

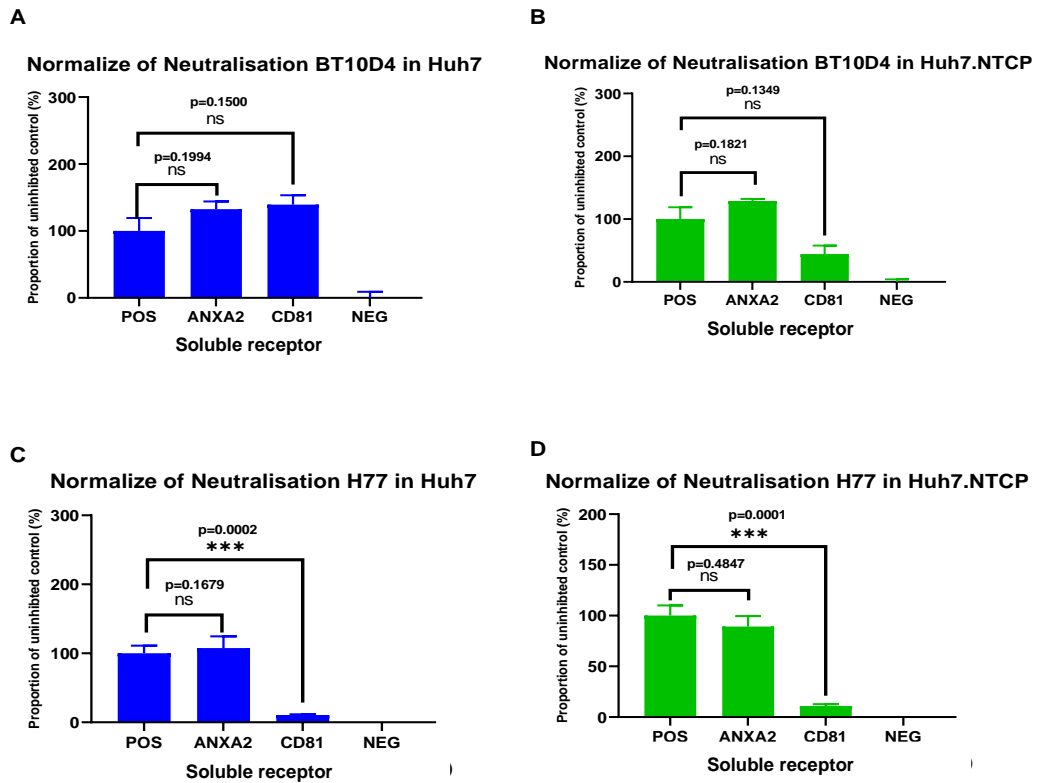


Figure 25. Normalised data of neutralisation of BT10D4 using soluble forms of annexin A2 and CD81 (Concentrations of 0-20 $\mu\text{g/mL}$). Neutralisation of BT10D4 in Huh7 and Huh7.NTCP. Cells seeded at 20,000 cells/well. The cells were then infected with BT10D4 and H77 pseudotypes that were pre-treated with either anti-annexin A2 or anti-CD81 and incubated at 37 °C and 5% CO₂ for 72 h. Infectivity was analysed by a luciferase assay. Data presented as proportion of uninhibited control (%). The negative control (NEG) was cells not infected with pseudotype and the positive control (POS) represented cells infected by pseudotype with no antibody administered. Statistical significance was analysed using a repeated measures one-way ANOVA with the Geisser-Greenhouse correction and the Dunnett's multiple comparisons test, and each antibody was compared to BT10D4 with no antibody (positive control). **** indicates $p < 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, ns indicates $p > 0.05$.

4.7 HBV-Annexin A2 binding assays

The combined results from both types of neutralisation assays (soluble annexin A2 and annexin A2-antibody) provided evidence that BT10D4 did not interact with the receptor annexin A2. In order to achieve a definitive answer on the matter, different variations of binding assays

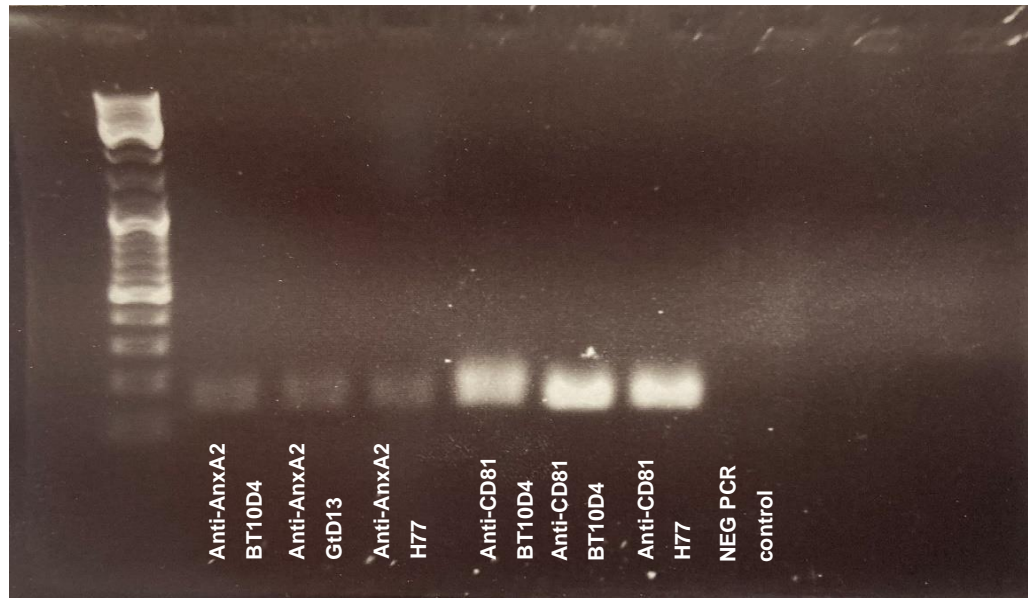
were carried out to consolidate this evidence. The first type of binding assay explored was a co-immunoprecipitation pull-down assay. This technique utilised a magnetic anti-annexin bound beads which were used to identify interactions between soluble forms of annexin A2 and the BT10D4 pseudotype. BT10D4 would bind to the soluble form of annexin A2 and the anti-annexin bound Dynabeads would also bind to the soluble Annexin A2. A magnet would be then used to isolate the complex from suspension and would be heated to break down the glycoprotein envelope and release the capsid containing the luciferase. The sample would be amplified using RT-PCR to test for the presence of luciferase. The presence of Luciferase would suggest that there is interaction between BT10D4 and annexin A2.

To ensure the presence of HBV proteins within the pseudotype preparations, western blotting was performed. This was important as appropriate levels of HBV protein were required for the success of the binding assay. The presence of the p24 antigen (25kD), the HIV-1 capsid protein, can be seen in Figure 26C.1. From this we can see that the lentiviral backbone incorporation into the pseudotype was successful. Three bands representing the small (25kD), medium (35kD) and large (40kD) HBsAg were also observed (Figure 26C.2). Due to the presence all 3 HBsAg components, it can be concluded that glycoprotein production was successful and that particles possess sufficient HBV proteins for the success of the binding assay. In this western blot, the HCV pseudotype was used as a negative control, demonstrating no evidence of incorporation of HBV antigens.

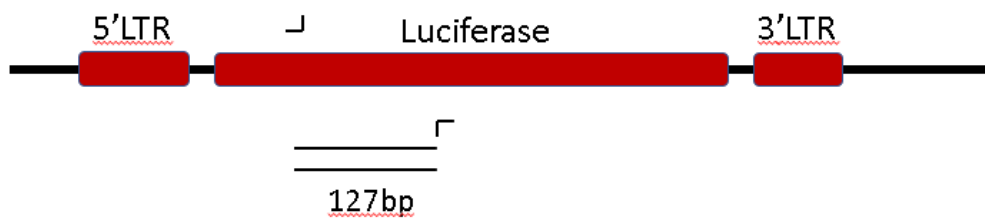
Co-immunoprecipitation pulldown assays of BT10D4 revealed unexpected data revealing HBV interaction with the CD81 control (Figure 26). Interestingly, pseudotypes that were incubated with soluble annexin A2 and mixed with anti-annexin A2 bound beads, there was no pull-down. This can be assumed as there was no presence of the luciferase gene within the sample. However, the experiment revealed that pseudotypes that were incubated with soluble CD81 and mixed with anti-CD81 bound

beads resulted in pull-down of luciferase, irrespective of the glycoprotein expressed on the surface. The interaction with the HCV antibody with the HBV pseudotype questions the validity of the experiment.

A



B



C

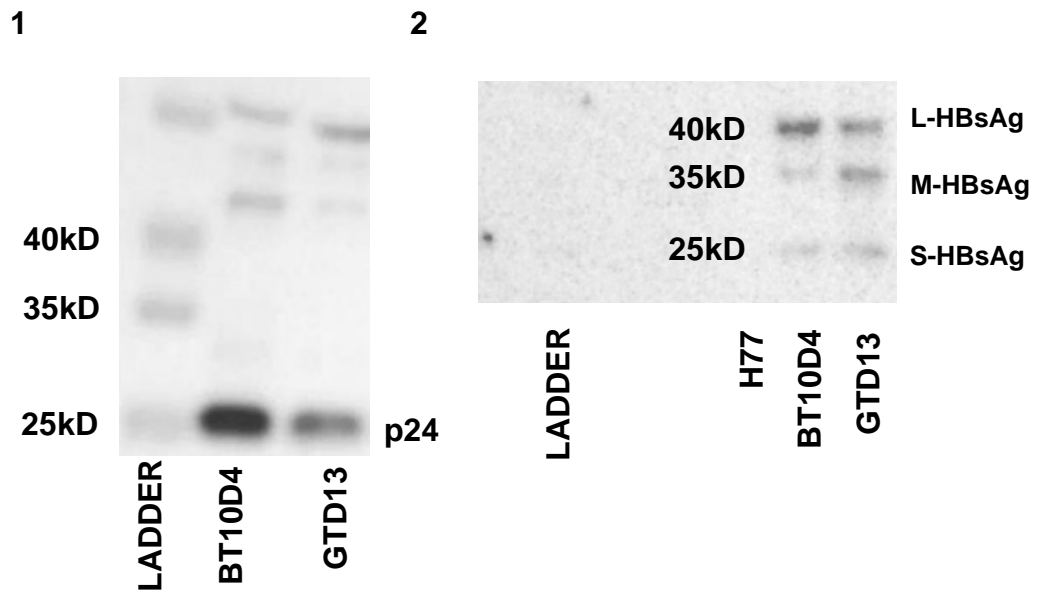


Figure 26 Co-Immunoprecipitation pull-down of BT10D4 assay. **A** PCR analysis for the presence of the Luciferase gene in pseudotype samples in pull-down assay. HBV and HCV pseudotypes were magnetically pulled down by antibody bound dynabeads. The sample was heated to 95oC and amplified using RT-PCR with Luciferase primers. The samples were then run-on agarose gel electrophoresis (AGE) to detect the presence of the Luciferase gene. Thermo scientific Gene Ruler DNA Ladder was used as a molecular marker for comparison. DNA bands were visualised by UV transillumination. **B** PCR product length between these two primers is 127bp. **C** Western blot analysis of HBV and HCV pseudotypes. Pseudotypes from BT10D4, D13 and H77 were run by gel electrophoresis and then transferred to a nitrocellulose membrane. The nitrocellulose membrane was then incubated with a 1:1000 dilution of the mouse monoclonal primary antibody Ma1694 (anti-HBsAg) (2) and ab9071 (anti-HIV-1 p24) (1). The samples were then incubated with rabbit anti-mouse HRP secondary antibody (Sigma) and visualised using a G: BOX F3 gel doc system. A Spectra Multicolour Broad Range Protein Ladder was utilised as a molecular marker for comparison.

To further investigate BT10D4 interactions with annexin A2 further, an ELISA was performed. The ELISA assayed the binding of HBV pseudotypes captured to the plate surface incubating with soluble annexin A2. The primary (anti-annexin A2) and secondary (Horseradish peroxidase) antibodies were then administrated to the mixture, washing

between incubations. Controls were prepared omitting different steps of the assay in order to distinguish any nonspecific binding. The results shown in Figure 27, display the binding of each pseudotype and delta E to soluble annexin A2. No binding of pseudotypes to the target protein was observed, the pseudotype binding appeared to be very similar to the delta E and the results in comparison to the negative control groups were similar. This provided more confirmatory evidence that there is no specific binding of BT10D4 to annexin A2 or any pseudotypes within the experiment. In retrospect, there should have been positive controls using CD81 and anti-CD81 for H77 to demonstrate the signal achieved with a high affinity interaction.

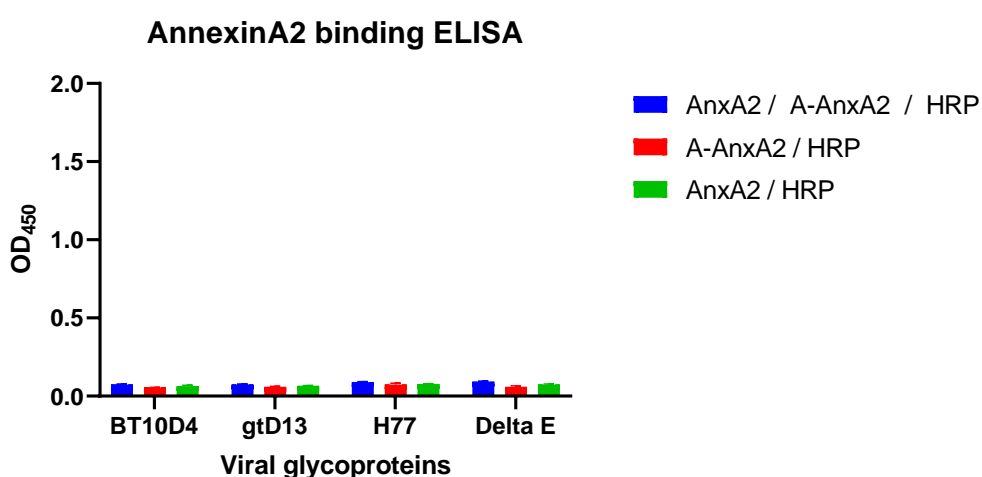


Figure 27. Annexin A2 BT10D4 binding ELISA assay. Annexin A2 BT10D4 binding ELISA assay. The pseudotypes gtD13, H77 and the delta E were used as controls against BT10D4. The group containing soluble annexin A2, anti-annexin A2 and Horseradish peroxidase (HRP) was used to investigate BT10D4 binding. The groups containing anti-annexin A2 and HRP in combination with the soluble annexin A2 and HRP were negative controls. The samples were incubated with 1:1000 of the mouse monoclonal primary antibody annexin A2 and then incubated with 1:2000 of rabbit anti-mouse Horseradish peroxidase (HRP) secondary antibody (Sigma). Results were read at 620nm.

5 Discussion

Main experimental findings

The aims of the study were to (1) successfully produce functional HBV pseudotypes which consisted of the BT10D4 glycoprotein to investigate its unusual phenotype and to identify its NTCP-independent entry mechanisms (2). Demonstrate clearly that BT10D4 is infecting Huh7 cells independently of the main HBV receptor NTCP and compare infection with the majority HBV phenotype. (3) Utilising proteomic data, decipher possible receptor candidates which had the highest levels of expression differences between the Huh7 and HepG2 cell lines. (4) Interrogate the ideal receptor candidate, which in this case was annexin A2, via a series of neutralisation and binding assays.

Initially, it was found that when producing functional pseudotypes it was evidently noticeable that HBV pseudotypes were more difficult to produce than HCV pseudotypes. This observation was made due to the ability to produce function HCV pseudotypes with various ratios of Gag plasmid to glycoprotein. Whereas with HBV pseudotypes with high RLU were only produced at the 'golden ratio' of 1:1(2 ug of Gag plasmid and 2 ug of glycoprotein).

When pseudotypes are expressed, they contain a signal peptide which directs the protein to a particular place in the cell and the retrovirus buds via an internal membrane. The HBV protein needs to be exactly in the appropriate place in the cell for the retrovirus to passively pick it up, as it is not an active process (Lindemann et al., 2001). Therefore, the HBV protein needs to be overexpressed to a point where it starts to leak into all the cellular membranes (plasma, ER and Golgi). As enough of the protein accumulates, wherever the retrovirus is budding from, then the particles become infectious (Condor Capcha et al., 2021). If the protein is over expressed, this can become cytotoxic to the cell, causing cell death (Lei et al., 2019). However, under expression leads to HBV protein potentially not being in the right part of the cell. As a consequence, the

1:1 ratio is the right balance for these factors considered. The processing pathway of HCV proteins may be in a different place to the HBV proteins, which could be a potential reason for HBV pseudotypes being more sensitive. In addition to this, factors such as the health and passage number of the producer and target cells should be considered, as these factors can alter the expression of pseudotypes. Pseudotype production is a lengthy and laborious procedure, each experiment taking more than one week. As such maximal usage of a single harvest is most efficient. This is achieved by storing pseudotypes at 4°C. However, the results revealed that whilst 7 days had no detrimental effect on infectivity, 14 days impaired infection efficiency and should be avoided. This reduction in infectivity could be due to degradation of proteins, membrane, or the clumping of viral particles.

One of the main objectives of this study was to demonstrate that the HBV variant BT10D4 was infecting Huh7 cells independently of the known HBV receptor NTCP. In order to achieve this goal, two cell lines were used in parallel, both Huh7 and Huh7 cells expressing NTCP. Throughout the study, the data reflected that BT10D4 was unquestionably infecting Huh7 cells independently of the expression levels of NTCP. The unusual phenotype of BT10D4 was compared with the dominant HBV tropism, which is NTCP dependent infection. Previous unpublished research from the group has discovered that BT10D4 is unable to infect the cell line HepG2 but can infect Huh7 cells that are NTCP deficient. Therefore, one of the key objectives was to analyse the proteomic expression differences of the genes expressed in Huh7 and HepG2 to identify potential receptor candidates. The ANXA2 gene had the highest difference in proteomic expression (25-fold) between Huh7 and HepG2. The ANXA2 gene produces the pleiotropic protein annexin A2. This is a multifunctional protein that binds lipids and calcium, which is widely expressed in almost all human tissues and is linked to a number of pathologies, immunological functions, and viral infection (Bharadwaj et al., 2013). Furthermore, a study carried out in 2012, demonstrated that AnxA2 is located on the cell surface of epithelial cells, has a role to play in HPV16 infection and co-

immunoprecipitates with HPV16 viral particles. Additionally, the study also demonstrated that when neutralizing HPV16 with anti-annexin A2 antibodies, there was a significant reduction in HPV16 infectivity (Woodham et al., 2012). Due to these factors, blockage assays of AnxA2 were explored using the concentrations found in the HPV study.

Initially, the blockade of AnxA2 was performed using an anti-annexin A2 antibody. This technique of blockade entailed blocking AnxA2 on the cell surface of Huh7 and Huh7.NTCP cells and then infecting with HBV pseudotypes. Interestingly, Huh7.NTCP cells pre-treated with anti-annexin A2 and infected with BT10D4 showed that there was no significant difference between no anti-annexin A2 and 10 μ g ($p=0.2380$). However, there appeared to be a trend of decreasing percentage of pseudotype infectivity, as the concentration of anti-annexin A2 increased. As for the BT10D4 infected Huh7 cells, there was no inhibition. This trend was apparent in the repeat of this experiment, whereby infectivity of BT10D4 was lower in Huh7.NTCP ($p=0.0608$). It was initially thought that there could be an interaction between NTCP and AnxA2 which could be potentially changing the conformation of AnxA2 on the cell surface and inhibiting BT10D4 entry. Alternatively, a third-party molecule involved in the presence of NTCP such as the recruitment of bile acids could be interacting with AnxA2, promoting the binding of anti-annexin A2 to AnxA2. However, the final repeat of this antibody blockade assay demonstrated no inhibition in both Huh7 and Huh7.NTCP cells (pre-treated with anti-annexin A2) which were infected with BT10D4. The blockade assays were then explored using soluble forms of AnxA2, whereby the pseudotype was pre-treated and then the target cells were infected. Throughout all soluble AnxA2 blockade assays, there appeared to be no reduction in infection. Nevertheless, there may be conformational differences in the soluble AnxA2 resulting in different spatial presentation of AnxA2 on the cell surface. This could mean that either the pseudotype binds to the region that is not contained within the soluble form of AnxA2 or the spatial presentation does not allow binding and as a consequence provides a false negative. To attempt to settle the

debate on whether AnxA2 has a role to play in BT10D4 entry, two binding assays were set up. The immunoprecipitation pull-down assay demonstrated some particularly unusual data showing the pull down of the luciferase DNA for anti-CD81 bound pseudotypes which included H77, gtD13 and BT10D4. This is unusual given CD81 is a known receptor for HCV and not HBV. Despite, CD81 being a receptor that is expressed by Huh7 and not HepG2, the subsequent experiments using anti-CD81 as a control showed no inhibition of HBV. A possible explanation for the observed phenomenon is that CD81 can bind HBV envelope proteins, but this interaction does not lead to active HBV entry. Another explanation is that this is due to some level of non-specific binding or human error. As a consequence of these results, this experiment should be repeated and further investigations assessing CD81-HBV interactions conducted. The other binding assay utilised was an ELISA. The ELISA showed no evidence of binding between soluble AnxA2 and BT10D4 with all absorbance readings being the same level (background).

Context of study

Worldwide, CHB infection continues to be a leading factor contributing to liver-related morbidity and mortality. The most recent estimates suggest that only 10% of the 300 million HBV carriers have been clinically diagnosed (Dolgin, 2022). Alarming, less than 5 million people out of 100 million that have CHB have received anti-HBV treatments, despite having severe disease (Razavi-Shearer et al., 2018).

As the literature surrounding HBV entry expands, evidence of other key players in HBV entry are being unveiled. While previous studies in our lab had indicated that there are other entry factors that remain elusive, the data in this study conclusively demonstrates some strains of HBV do not require NTCP for entry.

NTCP-independent HBV variants, despite not being the dominant phenotype, are circulating at low levels in populations. The BT10D4 variant was found serendipitously within the Nottingham cohort in 2 out of the 21 of the patients sampled (9%). Extrapolating this data, it maybe

that these variants not uncommon and could be significantly contributing to the global burden of disease. To achieve eradication of HBV, a full understanding of HBV entry factors, other than NTCP, is urgently needed (Chen et al, 2009).

Historically, there has been uncertainty regarding why some patients fail to respond successfully to anti-HBV therapies. However, given the findings in this study, it is plausible that this alternative receptor pathway, is one mechanism by which HBV could evolve to evade treatment such as Hepatitis B Immunoglobulin (HBIG). Until all HBV receptors are defined, there is a small chance of eliminating the virus completely both in the patient and worldwide. In fact, eliminating major variants of HBV, through therapeutic intervention could drive the emergence of resistant variants and through natural selection, which could then become the dominant species, even if less fit, in any given patient, and potentially could even exacerbate disease.

The WHO aim to eradicate HBV by 2030, however this is an extremely difficult target because of the low rates of (1) treatment (<15%); (2) diagnosis (<20%) and (3) vaccine administration to babies born to infected mothers (50%) (Sohn, 2022). An important strategy to achieve HBV eradication involves interrupting the horizontal transmission route. An increase in the uptake of treatment for CHB patients such as interferon, nucleoside analogue therapy and prophylactic administration of antibodies will help. These can either be used alone or in combination depending on compliance, severity, and stage of the disease (Maisa et al., 2021). However, HBIG is only 75% effective, therefore in 25% of patients the virus breaks through and persists (CDC, 2001). As a result, HBIG is administered in combination with vaccine to increase effectiveness. A possible explanation maybe the presence of NTCP independent or HBIG resistant HBV variants in the quasispecies. Deep sequencing the viral population may uncover the presence of these variants and is an area that requires further work. Understanding their prevalence and the ability of HBIG to neutralise effectively the variants could inform the development of more effective HBIG formulations, which

will consequently prevent horizontal transmission. Furthermore, preventing horizontal transmission would directly decrease the cases of vertical transmission which accounts for a significant proportion of chronic infections. In low- and middle-income countries where chronic infection is most prevalent, strategies are required to encourage the use of the birth-dose vaccine and hepatitis B immunoglobulins (Veronese et al., 2021). Moreover, understanding the molecular signatures of HBV variants that are associated with NTCP-independent infection could pave the way for diagnostic tests to identify patients that may be resistant to HBIG or in which NTCP-inhibitors are unlikely to be effective.

Indeed, an issue that needs to be addressed further is whether NTCP-independent HBV variants are detected by standard HBsAg diagnostic assays, particularly as pre-S1 is different in BT10D4 which may lead to conformational changes to HBsAg. This is important to determine for diagnosis in general and for assessing loss of HBsAg during treatment. Moreover, evasion of HBsAg detection by standard assays (enzyme-linked immunosorbent assay) will have serious implications for detecting infected donor blood and transfusion-associated hepatitis B virus infection (TAHBV). This is in fact a significant issue in third world countries, such as India. Notably, patients who have several transfusions are reported to have a high incidence of TAHBV. It has been shown that certain people who are HBsAg-negative and anti-HBc antigen positive (anti-hepatitis B core antigen positive) still have active hepatitis B viral replication (HBV) (Shastry & Bhat, 2011). The absence of circulating HBV may not always be guaranteed by the presence of negative HBsAg in the blood of patients who appear to be in good health (Schillie et al., 2018). This could be due to the presence of NTCP variants. Therefore, blood containing anti-HBc antigen, whether or not HBsAg is identified, may still be infectious. To combat this problem, an increase in anti-HBc IgM testing should be incorporated into routine screening of donors' blood to lower the risk of transfusion-associated hepatitis B since it has been shown to be a highly accurate indication of occult HBV throughout the window period (Kumar et al., 2007).

One of the best courses of action to eradicate CHB has been suggested to be maximising hepatitis B vaccination rates among all susceptible people, especially among infants. Hepatitis B immunisation is currently highly advised by the WHO for all infants at birth (universal vaccination). Also, children and adolescents who did not receive the hepatitis B vaccine during infancy (catch-up vaccination) because perinatal and early postnatal transmission are the main causes of chronic hepatitis infection (Chang & Chen, 2015). Vertical transmission induces the highest proportion of chronic infection after HBV exposure and so this is an important area of need. Furthermore, vaccination is still advocated for adults who are at a higher risk of contracting HBV. Since the risk of chronic infection is higher in these cohorts, the catch-up immunisation is now prioritised for younger age groups (Pattyn et al., 2021). However, in the context of the data presented in this thesis, further work does need to be done to ascertain whether the current HBV vaccine is as efficacious in producing antibodies that are fully protective against NTCP-independent HBV variants, as escape from this would have dire consequences.

Novel strategies for CHB treatment

Although existing HBV carriers can now be treated successfully and viral loads can decline to undetectable levels, extended use of current nucleos(t)ide analogues against HBV still presents the issue of viral resistance and the expense of long-term therapy remains significant (X. Guo et al., 2018). Additionally, the treatments currently in use to treat patients with CHB lack a long-lasting response. The persistence of HBV covalently closed circular DNA (cccDNA) requires treatment for patients with CHB indefinitely (Yang & Kao, 2014; Yuen et al., 2021). This global health burden requires the emergence of investigations into novel drugs that target distinct steps of the HBV life cycle. Currently, investigations into inhibiting HBV entry into hepatocytes, HBV cccDNA silencing, regulating nucleocapsid assembly, disrupting HBV transcription, and HBsAg inhibition are underway (Watanabe et al., 2022). The next step in

the HBV eradication process is the establishment of a functional cure or permanent HBsAg loss in CHB patients.

Regarding the pathophysiology of CHB, the underlying immune response, and species tropism particularly understanding the entrance mechanism of HBV is crucial. Researchers have learned a great deal more about the HBV life cycle following the discovery that NTCP. Before interacting with NTCP and co-receptor EGFR, HBV has been found to bind to sulfated HSPGs, which are selectively expressed in liver tissues. Furthermore, E-cadherin is essential for HBV infection because it transports NTCP to the plasma membrane. HBV enters the endosomal compartment via clathrin-mediated endocytosis after being bound to NTCP-EGFR. Endosomal fusion appears to take place in late endosomes, although the precise triggers that cause endosomal fusion are yet unknown (Cagno et al., 2019; Herrscher et al., 2020; W. Li & Urban, 2016).

Viral entry serves as the initial stage of viral infection and is a desirable target for antiviral therapies. HBV infection can be suppressed by entry inhibitors before the virus forms its genome or impacts infected cells. During post-exposure prophylaxis, organ transplantation, reactivation following therapeutic immunosuppression, or perinatal transmission from an infected mother to her child, de novo infection can be averted by inhibiting HBV entry (Maravelia et al., 2021). The preS1 domain of the HBV large envelope protein interacts specifically with NTCP. This establishment of NTCP as a cellular receptor for HBV, allowed for the rational development of drugs that block viral entrance. Bile acids and their derivatives, peptides, pharmacological compounds, and neutralising antibodies are only a few examples of the several types of HBV entry inhibitors that have been developed so far that target NTCP-mediated viral infection (Li et al., 2022). The data presented in this thesis confirms NTCP-independent entry by HBV variants and the elucidation of these alternate entry factors need to be considered in the development of therapeutic strategies to block HBV entry. The approach should be similar to the development of substrates that mimic the conformation of

NTCP substrates such as taurocholate, bromosulfophthalein, and tauroursodeoxycholate, which are able to competitively inhibit entry of HBV via NTCP. This is due to the overlapping nature of HBV molecular determinants and uptake of bile salts by NTCP (Yan et al., 2014; Zhao et al., 2021). With this knowledge, derivatives of bile acids are able to be utilised such as 6 α -ethyl-24-nor-5 β -cholane-3 α ,7 α ,23-triol-23 sulfate sodium salt and obeticholic acid (OCA) to inhibit entry of HBV via NTCP blocking (Ito et al., 2021).

In addition to the use of bile acid derivative, there has been various literature advocating the use of monoclonal antibodies to inhibit HBV entry. There are currently a variety of monoclonal antibodies which have the ability to recognise the preS1 HBV region and neutralize infection. Two examples of these monoclonal antibodies is 2H5-A14 and MA18/7 (Glebe et al., 2003; D. Li et al., 2017). Furthermore, NTCP specific antibodies have been developed to recognise the extracellular loop-4 of human NTCP (residues 276 and 277). An advantage of using this method to inhibit HBV entry into hepatocytes *in vitro*, is that there is an insignificant effect on the uptake of bile acids (Takemori et al., 2022).

The linear synthetic N-acetylated lipopeptide Myrcludex B, is made up of 47 amino acids derived from the preS1 region of the large HBV envelope protein (L-protein). The process of generating Myrcludex B is carried out by solid-phase synthesis. One of the therapeutic advantages of Myrcludex B is that NTCP interaction with HBV particles is inhibited in both *in vitro* and *in vivo*, competing for binding with NTCP (Nanahara et al., 2021). Furthermore, the concentration required to inhibit HBV and HDV entry is significantly lower (100-fold) than which is needed to block NTCP transportation of bile acids (Y. Li et al., 2022). Currently, there has only been approval of Myrcludex B in Europe for chronic HDV patients which has been branded Hepcludex®. However, it is important to note that all these strategies targeting NTCP would be ineffective in variants that infect via alternative entry receptors.

Limitations, Future perspectives, and Final conclusions

Overall, the data from this study reveal that HBV variant BT10D4 is able to enter human hepatoma cell line Huh7 independently of NTCP. The data suggests that AnxA2 is not the entry receptor of BT10D4. But, due to some of the differences within the neutralisation data, AnxA2 should be investigated further, especially NTCP-annexin A2 interactions. Alternatively, it is plausible that there are multiple factors involved in BT10D4 entry, not just one. Additionally, higher concentrations of antibody should be used be investigated in future experiments. Although, caution needs to be taken when increasing the concentration of antibody and soluble annexin A2 as there reaches a point whether blockade of the virus is because of nonspecific inhibition due to the high concentration of antibody used. There should also be a repeat of the ELISA performed as a limitation was that there was no established positive control which provided no indication of a binding standard. In this case the positive control could have been HCV pseudotype binding to soluble CD81. Furthermore, repeats of the neutralisation assays with an HBV control (gtD13) would be ideal. In addition to this, another limitation to the experiments is the potential presence of Annexin A2 in the foetal bovine serum (FBS) and secretion of Annexin A2 from the cell lines which may interfere with the assays. In future, Annexin A2 levels should be quantified in the FBS and if present in large quantities cell lines and further experiments should be performed with serum-free media (xeno-free reagents). Furthermore, during these blocking assays, HCV was used as a positive control, however, a better control would be to pseudotype HPV or use full-replicating HPV, that requires AnxA2 for entry, to demonstrate that anti-annexin A2 antibody effectively blocks viral entry of HPV.

One of the next phases in the roadmap would be to create gene library of all the mRNAs expressed by Huh7 cells. The library approach would consist of a lentiviral delivery system which delivers the entire gene repertoire of Huh7 cells to HepG2 cells (Brown et al., 2020). Entry factors that provide Huh7 cells the ability to be infected would then be selected. This process would be similar to the pseudotype assay, however, instead

of having the luciferase in every single copy of the pseudotypes, there would be a generated PCR product which amplifies the ORF from cDNA from the mRNA transcribed by the cells. The RNA from the cells would be extracted and converted into cDNA, using primers which target the poly-A tail. The cDNA would be cloned into expression constructs and each pseudotype would therefore receive one different copy of a gene. In addition to having the gene, they would also have GFP (Green fluorescent protein) tag. The HepG2 cells (BT10D4 infection negative) would then be transduced. In theory, each HepG2 cell would get infected with a different gene which gets integrated and produces a GFP in combination with a target protein of interest (protein X). The pseudotypes would also contain another secondary fluorescent tag, which doesn't overlap GFP such as YFP (Yellow fluorescent protein). HepG2 cells would be then infected with pseudotypes. The second fluorescent gene gets integrated into the cell genome as well. Infected cells then contain GFP, protein X and the secondary marker. The cells uninfected are just GFP positive. The cells are then harvested and run through a flow-cytometer, which collects the cells. The flow-cytometer would be set to specifically collect GFP, YFP dual positive cells. This will allow for the deduction that the collected cells contain the trans gene and are infected as then contain the YFP. The cells would then be harvested again, and a PCR would be performed across the DNA cassette, which express the GFP target gene. This provides the sequence of the GFP (control) and the sequence of the protein X gene that has been induced with it. The sequence data could then be compared against the database of protein or gene sequences and identify the gene in the cell. This process would identify potential entry receptor candidates. An expression construct would be made out of these candidates and transfected into the HepG2 cells with the single gene and then test for infectivity with BT10D4.

Another avenue that was not explored in this study was shRNA knockout of AnxA2 in Huh7 cells. In order to identify whether downregulating the expression of AnxA2 in Huh7 cells affects BT10D4's ability to infect. Experimentally, Huh7 cells would be transduced with a lentiviral vector

which encoded a short hairpin (sh) RNA for the receptor AnxA2 mRNA (shAnxA2). A concern for this experiment is the potential consequence of knocking out AnxA2 on other important processes of the cell. However, a study carried out in 2012 demonstrated that shRNA knockout of AnxA2 in HeLa cells had no effect on endocytosis or intracellular trafficking. Furthermore, the knockout of AnxA2 inhibited internalisation of HPV particles (Woodham et al., 2012). Moreover, another experiment worth considering is recording the protein expression of AnxA2 post-BT10D4 infection. In theory, BT10D4 binding to AnxA2 would stimulate endocytosis of the virus and result in a decrease of AnxA2 protein expression once infected. Despite the experiment being based on the occurrence of endocytosis, it could provide insight into AnxA2 proteomic activity post-infection with BT10D4.

It appears from the data collated that AnxA2 is not an entry receptor for HBV. The next step would be continuing the search through the identified gene candidates and continue to interrogate them. In the future, HBV virus particles could be used to carry out these assays, as a limitation to using pseudotypes to investigate infection is that they are not fully representative of full replicating HBV virions. Furthermore, there has been literature showing some disadvantages of pseudotype systems, whereby the study demonstrated that the HCV E2 protein has a downregulating effect on the LTR-based transcription of HIV-1. Therefore, the expression of E2 in cells will suppress pseudotype production (McKay et al., 2022). A similar pathway of suppression of pseudotype production may occur with HBV proteins. This could inevitably be a contributing factor to difficulty of producing functional pseudotypes at high titres. Potentially in the future experiments other gammaretroviral vectors such as MLV could be used. However, a disadvantage to gammaretroviral vectors is that they can only infect mitotically active cell types (Beard et al., 2007). Performing either pseudotype or full-replicating HBV infection in human hepatocytes rather than hepatoma cell lines would be another route to explore in the future.

In conclusion, this study confirms that certain HBV variants can enter and infect cells via alternative entry receptors than the classical NTCP route. This study has resulted in the formation of more unanswered questions and is the beginning of the journey into NTCP-independent HBV species. Further work is urgently needed to identify these and develop therapeutics for CHB which remains an important and clinically unmet need worldwide.

6 References

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