Development of antiviral therapies for chronic hepatitis B virus infection

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Chapter 1: Introduction

1.1 Background: Epidemiology of HBV infection

Hepatitis B virus (HBV) infection is a major public health problem worldwide with increasingly high prevalence and mortality in the recent past. HBV currently infects an estimated 2 billion people globally, including 400 million individuals with chronic HBV infection (McMahon 2005; Lin and Kao 2008). HBV transmission occurs via parenteral routes that include needle stick injury, blood transfusion, organ transplantation and sexual contact. Horizontal transmission from infected mothers to neonates is also an important route of transmission in areas where HBV is hyper-endemic. Patients such as hemophiliacs who undergo blood transfusion, individuals in occupations such as dentistry and surgery, health care staff in other disciplines, neonates of infected mothers and intravenous drug users are at high risk of HBV infection due to possible frequent exposures. HBV carriers can be a major source of infection for individuals who live in the same household and share razors or toothbrushes (McMahon 2005; Lin and Kao 2008).

The highest prevalence of HBV (>8%) is seen sub-Saharan Africa, Southeast Asia, the South Pacific Islands and the Artic/sub-Artic region, including Western Alaska, the Baffin Archipelago of Canada and all of Greenland (McMahon 2005; Ocama *et al.* 2005). Some regions have an intermediate HBV prevalence. These include much of the Mediterranean region, Southern Europe and North Africa, Eastern Europe, Russia, the Middle East, and the Indian subcontinent and parts of South America (McMahon 2005; Lee *et al.* 2008). HBV transmission in endemic regions mainly occurs through chronically infected carrier mothers to neonates and by unrecognised accidental exposure to infected blood or body fluids, especially in children and adults who are in the same household as infected individuals. The prevalence of chronic HBV infection is inversely correlated with age at the time of infection, hence, >90% perinatal infections progress to chronic HBV infection. Based on a long-term study conducted in Taiwan, >25% of HBV infections in preschool children and <4% of infections in adolescents progressed to chronicity (Mast *et al.* 2005; Osiowy *et al.* 2008).

In regions where HBV is moderately (2-7%) or less (<2%) prevalent, infection of adults is very common. Adults can be infected via unprotected sexual intercourse or by intravenous drug use. It is also possible that HBV transmission is not restricted to those who practice risky behaviours. HBV is viable for a long time in the environment as shown in a study conducted by the Centre for Disease Control (CDC). A highly infective serum placed on a glass slide and left in the environment for a week, was infective to a chimpanzee (McMahon 2005). This suggests that any kind of accidental exposure to articles contaminated by blood and body fluids of HBV carriers could also transmit the infection. In countries where HBV prevalence is low or moderate, immunisation of health care workers is carried out as they are at increased risk. However, immunising specific groups has not reduced the overall prevalence of HBV worldwide (Mast *et al.* 2005; McMahon 2005).

1.1.1 History of HBV

Although viral hepatitis dates back to the 5th century BC, it was only in 1963 that Blumberg and colleagues, who were searching for polymorphic serum proteins, discovered HBsAg in the blood of an Aboriginal Australian (Blumberg *et al.* 1965) and HBV was then distinguished from other serum hepatitis viruses (Blumberg 1977; Brunello *et al.* 1979). A few years later Dane and his colleagues showed the presence of virus-like particles (VLP) in the serum of HBV infected patients using immunoelectron microscopy (Alberti *et al.* 1978). Dane's discovery was confirmed with the identification of an endogenous polymerase activity within HBV core particles (He *et al.* 1985). Subsequently, the proteins and genome of the HBV were identified and HBV became the first recognised human hepatitis virus (Dejean *et al.* 1983; Kobayashi and Koike 1984; He *et al.* 1985).

1.2 Hepadnaviruses

The hepadnaviridae family consists of two genera. Firstly, orthohepadnaviruses infect mammals and include human HBV, naturally occurring HBV strains isolated from non-human primates, woolly monkey hepatitis B virus (WMHBV), woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus (GSHV) and arctic squirrel hepatitis virus (ASHV) (Table 1.1). Secondly, avihepadnaviruses infect avian hosts and include duck hepatitis B virus (DHBV), heron hepatitis B virus (HHBV), snow goose hepatitis virus (SGHBV), Ross

goose hepatitis B virus (RGHBV), stork hepatitis B virus (STHBV) and crane hepatitis B virus (CHBV) (Funk *et al.* 2007). Avihepadnaviruses are transmitted by the vertical route which is also called "*in ovo*" transmission because it occurs via the egg. DHBV has been extensively studied in its natural host as an experimental model for HBV to understand hepadnavirus replication, some aspects of pathogenesis and testing antiviral and immune therapies (Funk *et al.* 2007; Zoulim *et al.* 2008).

All hepadnaviruses primarily infect hepatocytes in the liver. They are small DNA viruses that replicate via reverse transcription of an RNA intermediate. Although they have similar virion morphology and genome organisation, there is only 40-55% nucleotide sequence homology between the genomes of orthohepadnaviruses and avihepadnaviruses (Sprengel *et al.* 1985). There are many similarities and some differences between viral structure and replication between orthohepadnaviruses and avihepadnaviruses. The characteristics of orthohepadnaviruses and avihepadnaviruses are summarised in Table 1.2.

1.2.1 Structure of hepadnavirus particles

HBV virions in general are spiky spherical, double-shelled structures of 42-47 nm in diameter (Figure 1.1A). The outer shell is the viral envelope that contains three types of viral surface protein embedded in host-derived lipid. The three types of proteins are small (S), medium (M) and large (L) HBV surface antigen (HBsAg) (Figure 1.1B). S make up 70% of the surface protein content, while the remaining 30% is constituted of M and L in approximately equal amounts (Figure 1.1C) (Bruss 2004; Skrastina *et al.* 2008). The inner shell is the virus core or nucleocapsid, which is icosahedral in shape and is composed of HBcAg (Bruss 2004). The HBV genome is located within the nucleocapsid and is covalently attached to the HBV polymerase protein. In addition to virions, the serum of highly viraemic carriers also contains non-infectious HBsAg particles, which are present at a titre of up to 90 times greater than that of infectious virions (Sakamoto *et al.* 1983; Kann 2008). Electron microscopy has facilitated the study of these HBsAg particles in HBV infected serum (Dryden *et al.* 2006). There are two types of HBsAg particles and they are spherical bodies of 17-25 nm in diameter and filaments or tubular bodies of variable length with a diameter of 17-25 nm (Figure 1.1C) (Bruss *et al.* 1996). The HBsAg particles, like the HBV envelope, consist of three surface proteins embedded in host-derived lipid, though they

contain predominantly S, with variable amounts of M and only trace amounts of L surface proteins (Heermann *et al.* 1984).

The DHBV virion structure differs from that of HBV in shape, number of envelope proteins and the types of sub-viral surface antigen (DHBsAg) particles present in the infected serum. DHBV virions are double-shelled but pleomorphic structures of 40-45 nm in diameter. The viral envelope is composed of two types of trans-membrane surface proteins embedded in host-derived lipid. These two proteins are small (S) and large (L) DHBsAg (pre-S/S). Only spherical sub-viral DHBsAg particles, and not filaments, are present in DHBV-infected serum. These sub-viral DHBsAg particles are 30-60 nm in diameter, making them difficult to distinguish from similarly sized virions. DHBsAg particles are present in serum at a titre up to 500 times greater than that of virions (Table 1.2) (Jilbert *et al.* 1996; Franke *et al.* 2007).

1.2.2 Genomic organisation of HBV

HBV has a partially double stranded relaxed circular DNA genome (rcDNA) that replicates through a RNA intermediate using reverse transcription. The HBV genome (~3200 base pairs) carries 4 open reading frames (ORFs) arranged in a compact organisation (Jilbert *et al.* 1998). The entire rcDNA genome encodes for proteins and >50% of the nucleotides are used for more than one ORF. In addition, all regulatory signals overlap with coding regions, for example the S ORF fully overlaps with the P ORF (Figure 1.2) (Rehermann and Nascimbeni 2005).

The four ORFs contained within the negative DNA strand are the P, S, pre-C/C and X genes except in DHBV and other avihepadnaviruses, where the X gene is absent, thus making 3 ORFs (Table 1.2) (Sprengel *et al.* 1985).

The P gene occupies >80% of HBV genome encoding for the viral RNA- and DNA poly A signal directed DNA polymerase (P protein) (Nassal 1996; Nassal and Schaller 1996). The central and the C-terminal regions of the P protein contain the DNA polymerase (Pol) and RNase H domains. These Pol and RNase H domains share significant homology to Pol and RNase H domains of related viruses such as human immunodeficiency virus (HIV) (Nassal 1996; Nassal and Schaller 1996). The N terminal domain or the priming domain is separated by a spacer from the catalytically active Pol domain and has a tyrosine residue at

the 63rd position in HBV (Tyr-63) and 96th position in DHBV (Tyr-96) (Figure 1.4). Binding of P protein to the encapsidation signal (ε) at the 5' end of the pgRNA via the Tyr-63 of HBV or Tyr-96 of DHBV will initiate the synthesis of negative strand DNA (Figure 1.4). In addition to DNA synthesis, P protein also contributes to the packaging of pregenomic RNA (pgRNA) genome into the nucleocapsids (Hu and Seeger 1996).

The S ORF contains 3 in frame start codons (pre-S1, pre-S2 and S) sharing a common carboxyl terminus. The translation of the S ORF gives rise to three types of surface proteins, namely small (S), medium (M) and large HBsAg (L) (Heermann *et al.* 1984). S proteins are translated from 2 mRNA transcripts (L protein is from a 2.4 kb mRNA transcript; M and S proteins are from a 2.2 kb mRNA transcript) (Figure 1.2).

The preC/C ORF has two distinct in frame start codons that produce the two proteins, namely hepatitis e antigen (HBeAg) and the core protein that forms the viral nucleocapsid which is also called hepatitis c antigen (HBcAg). Both HBcAg and HBeAg are translated from a 3.5 kb mRNA transcript (Figure 1.2) with two different 5'ends. HBeAg is the secreted form of the core protein that is found in the serum of HBV-infected individuals (Sprengel *et al.* 1985).

The X ORF codes for HBV X protein which is believed to have a role in carcinogenesis. The actual mechanisms involved in this process, however, are not well understood. Nonetheless the X gene is capable of *trans*-activating many viral and cellular growth promoters including genes involved in cellular growth regulation (Sprengel *et al.* 1985; Rehermann and Nascimbeni 2005).

In summary, genomic and subgenomic viral transcripts, all of which utilise the same polyadenylation (polyA) signal (Figure 1.2), are transcribed from cccDNA (Rehermann and Nascimbeni 2005). Viral mRNAs encode the viral core (HBcAg), envelope (HBsAg), Pol, and HBx polypeptides (Rehermann and Nascimbeni 2005). The largest HBV transcript, the pgRNA, is also the template for viral replication and is reverse transcribed by the viral Pol, similar to the replication of retroviruses (Tang and McLachlan 2002).

1.3 Replication

1.3.1 Viral entry, cccDNA synthesis, transcription and translation

Hepadnaviruses are species- and tissue-specific to a great extent and it is believed that this specificity is due to specific cellular receptors, which mediate binding and entry. Hepadnaviruses primarily infect hepatocytes in the liver. Virus entry is believed to occur through receptor mediated endocytosis (RME), but the actual receptors involved have not been identified. A region within the pre-S1 of L-HBs has been identified as being involved in binding to hepatocytes (Neurath *et al.* 1986). More recent data suggest that pre-S1 of L governs the entry process. Only a small number of virions that have pre-S1 with the correct topology will bind and enter the host cell. Furthermore, monoclonal antibodies against amino acid (AA) 31-31 of the pre-S1-domain inhibit the attachment of HBV to the primary human hepatocytes (PHH) supporting the importance of pre-S1 in the entry process (Glebe and Urban 2007). Myristylated peptides represent the amino terminus of pre-S1 block HBV infection (Rabe *et al.* 2006; Jilbert *et al.* 2008). Although the cellular receptor for HBV has not been conclusively identified, a putative receptor (gp180) for DHBV in duck hepatocytes has been identified (Breiner and Schaller 2000). Following endocytosis the nucleocapsid is transported to the nucleus and enters the nucleus, possibly via the nuclear pore complex (Kann *et al.* 1999; Kann 2008).

Upon entering the hepatocyte nucleus, the rcDNA genome is converted to covalently closed circular DNA (cccDNA) through a series of molecular changes such as completion of the positive DNA strand, removal of the covalently bound Pol, removal of the RNA primer, ligation of the gaps in the positive and negative strands, and supercoiling of the DNA (Jilbert *et al.* 2008; Kim *et al.* 2008). These cccDNA molecules are stable and act as a reservoir for hepadnavirus infection. Furthermore, cccDNA is highly resistant to anti-viral therapies and thus it is difficult to clear these molecules from the liver in the absence of hepatocyte division (Zoulim 2005).

1.3.2 Virion assembly and release

Immature nucleocapsids are formed by packaging or encapsidation of pgRNA within nucleocapsids in the cytoplasm of the cell (Figure 1.4) (Bartenschlager and Schaller 1992).

Encapsidation of the pgRNA requires binding of the viral Pol to the ε at the 5' end of the pgRNA (Figure 1.4) (Bartenschlager and Schaller 1992; Knaus and Nassal 1993). The polymerase performs a number of functions in production of the rcDNA genome (Knaus and Nassal 1993). After encapsidation the viral Pol facilitates the conversion of pgRNA to the rcDNA genome. It primes the negative-strand DNA by production of 3 nt (in HBV) or 4 nt (in DHBV) of DNA and translocates to the 3' end of the pgRNA, where the 3-4 nt primer binds to the complementary sequence in a region called direct repeat 1 (DR1) (Figure 1.4) (Rieger and Nassal 1995). The Pol then synthesises the negative DNA strand by RNA-dependent DNA polymerisation and degrades the pgRNA template via its RNase H activity (Figure 1.4). An 18 nt region at the 5' end of the pgRNA, including DR1, escapes degradation and binds to direct repeat 2 (DR2) at the 5' end of the negative DNA strand, acting as a primer for synthesis of the positive DNA strand which occurs through the DNA-dependent DNA polymerase activity of the Pol (Figure 1.4) (Rieger and Nassal 1995). Circularisation occurs during positive-strand synthesis, leading to production of the rcDNA genome (Rieger and Nassal 1996; Jilbert *et al.* 2008). Mature nucleocapsids containing rcDNA genomes are then recycled to the nucleus to form a pool of cccDNA (Figure 1.4), ranging from 30 to 50 copies of cccDNA per cell (Newbold *et al.* 1995), or are exported from the cell via the endoplasmic reticulum (ER), where nucleocapsids are enveloped in surface proteins embedded in host-derived lipid (Figure 1.3) (Block *et al.* 2007; Bruss 2007; Mhamdi *et al.* 2007).

However, there are more studies emerging on the topic of HBV/DHBV virion formation and release. Surface protein synthesis occurs in the ER where the cytoplasmically preformed nucleocapsids are enveloped with these surface proteins and then released via the constitutional secretion pathway (Funk *et al.* 2008). The envelopment results from highly co-ordinated biological interactions between the nucleocapsids and the cytoplasmically exposed pre-S region of the L protein. The S protein plays a major role in the secretion and the budding of virus particles. Budding of surface proteins without the nucleocapsids and virus genome into the ER lumen results in the production of sub-viral particles (SVPs) (Mhamdi *et al.* 2007).

Moreover, the L topology of both DHBV virions and SVP exhibit the same amount of L protein with some subtle differences. Pre-S of intact virions can be enzymatically digested with chymotrypsin whereas in SVPs only a part of pre-S was accessible to enzymatic digestion. DHBV virions have a higher ratio of exposed pre-S to S compared to SVPs. Different surface architectures of virions to that of SVPs support the role of pre-S domain in binding to a cellular receptor of hepatocytes and exclude SVPs as competitors for the receptor binding and entry of virions (Franke *et al.* 2007).

1.4 HBV/DHBV Infection

1.4.1 Infection outcomes

Hepadnavirus infection gives rise to two possible outcomes; acute or chronic infection (Figure 1.5) (McMahon 2005). In humans, 1-5% of adults infected with HBV develop chronic infection, while the remainder clears the infection. Approximately 25-50% of children infected with HBV at ages 1-5 develop chronic infection, as do 90% of infants infected at birth, suggesting an age-dependent infection outcome (Sprengers and Janssen 2005). This age-related relationship of infection outcome has also been demonstrated in DHBV-infected ducks (Jilbert *et al.* 1998), where younger ducks infected with the same dose of virus were more likely to develop chronic DHBV infection than older ducks. It was also shown that if ducks of the same age were inoculated with different doses of virus, the ducks given the larger dose were more likely to develop chronic infection (Jilbert *et al.* 1998; Foster *et al.* 2005). This outcome of DHBV infection depends on the immune response developed against the infection and the outcome of infection is affected by the dose of infecting virus and age at infection (Jilbert *et al.* 1998; Foster *et al.* 2005). As the age at infection increases the immune system develops and is more likely to mount an immune response capable of clearing the infection. Furthermore, neonatal infections with WHV have been comprehensively studied. Neonatal woodchucks infected with WHV resolved the infection when the immune response is able to clear the virus, whereas neonatal WHV infection at most times leads to chronic infection (Menne *et al.* 2002; Wang *et al.* 2004). However, the age and dose related outcome of hepadnavirus infection has not been studied in primates. This may be due to practical and ethical issues associated with the use of primates for such studies.

1.4.1.1 Acute infection

An acute HBV infection occurs when HBsAg is eliminated from the serum and alanine aminotransferase (ALT) levels return to normal in less than 6 months. Although it was initially believed that HBV DNA is eliminated from the serum and liver, traces of DNA may be detectable by sensitive PCR assays (Lok and McMahon 2001). Acute infection lasting for 6 months is quite unusual for a viral infection, as in most cases acute infections occur if the virus is rapidly eliminated, but given the long incubation period of HBV and the resultant slow development of the immune response, this is not surprising. Transient DHBV infections in ducks resolve faster than HBV infections in humans and this may be due to the relatively shorter life span of ducks than that of humans, with surface antigen cleared from the serum by 1-2 months post-inoculation (Jilbert *et al.* 1998; Le Mire *et al.* 2005). Furthermore, residual DHBV infection has been noted in ducks resolving DHBV infection (Le Mire *et al.* 2005; Reaiche 2008) similar to residual HBV infection that follows an acute HBV infection in humans (Lavine *et al.* 1991; Petit *et al.* 2001).

Strong cell-mediated immune (CMI) responses to surface, core and polymerase proteins are described during transient HBV infections, as well as humoral immune responses to the surface, core, e-antigen and polymerase proteins (Kara *et al.* 2004). Both types of immune response can facilitate clearance of the virus. Anti-HBs antibodies produced by the humoral immune response (Figure 1.3A) are able to bind HBV surface proteins, which may serve two purposes. Firstly, they may block receptors necessary for attachment to hepatocytes, and secondly, they may facilitate the removal of virus from the blood by opsonising virus particles for engulfment by phagocytes (Akbar *et al.* 1999). T-cells generated by CMI response may facilitate clearance of the virus in two ways. Firstly, CD8+ T-cells may cause infected hepatocytes to undergo apoptosis and secondly, T-cells may secrete cytokines, which may lead to non-cytolytic clearance of the virus (Guidotti and Chisari 2001). The relative contribution of the two types of immune response in the clearance of HBV infection in humans is not well understood and is discussed in Section 1.4.1.2.

In addition to adaptive immunity, innate immunity may also play a role in the clearance of hepadnavirus infections. An early innate immune response in many viral infections is the production of interferon alpha (IFN-α), interferon beta (IFN-β) and interleukin-2 (IL-2) (Weber *et al.* 2004). These are produced in response to double-stranded RNA molecules

during virus replication. IFN-α has been shown to inhibit DHBV replication *in vitro* (Schultz *et al.* 1999) and HBV replication in the transgenic mouse model (Anderson *et al.* 2005). In addition, various cells of the innate immune system may also have roles in clearing hepadnavirus infection. Natural killer (NK) cells have been shown to play a role in controlling hepadnavirus infections. They have both cytolytic and non-cytolytic antiviral functions, being able to induce apoptosis of cells by down-regulating expression of major histocompatibility complex (MHC) class I, and also producing antiviral cytokines such as IFN-α and tumour necrosis factor alpha (TNF-α) (Scully *et al.* 1990). Macrophages, granulocytes and NK T cells may also play a role in clearance of hepadnavirus infections, although the role of these cells in viral clearance is poorly understood (Dandri and Petersen 2005). Other components of the innate immune response that may be important in clearance of viruses are the toll-like receptors (TLRs). These are a range of molecules able to recognise certain foreign structures which are also called pathogen-specific molecular patterns (PAMPs). They activate cell-signalling pathways leading to maturation of dendritic cells, expression of co-stimulatory molecules and secretion of type I interferons. The TLRs that have been shown to be activated during virus infection are TLR3, TLR9, TLR7, TLR8, TLR2 and TLR4 (Rosenthal 2006) and they may make some contribution to hepadnavirus clearance (Visvanathan *et al.* 2007).

1.4.1.2 Resolution of acute infection

The exact mechanism of resolution of hepadnavirus infections is not well understood. One important aspect of the resolution of hepadnavirus infection is the identity of the immune cells involved. CD8+ T-cells are believed to be the main cell-type responsible for clearance of infected hepatocytes from the liver. Studies on resolution of HBV infection in chimpanzees show that the depletion of CD4+ T-cells does not change in the course of HBV infection, whereas depletion of CD8+ T-cells extends the peak of HBV DNA and HBsAg in the serum and liver, which does not fall until the reappearance of CD8+ T-cells in the circulation (Thimme *et al.* 2003; Bertoletti and Gehring 2006). These results indicate that CD8+ T-cells play an important role in the clearance of infected hepatocytes from the liver. However, it is not known if CD8+ T-cells assist clearance of cccDNA by killing infected hepatocytes or by production of cytokines (Bertoletti and Gehring 2006).

It has been shown that acute hepadnavirus infections can be cleared even after infection of the majority of hepatocytes in ducks (Jilbert *et al*. 1992; Reaiche 2008), woodchucks (Kajino *et al.* 1994; Summers *et al.* 2003; Glebe *et al.* 2009) and chimpanzees (Thimme *et al.* 2003). As discussed in Section 1.4.1.1, there are two mechanisms proposed for how infection can be cleared after a virtual infection of all hepatocytes. The first is non-cytolytic clearance which occurs without destruction of infected hepatocytes. It has been proposed that non-cytolytic clearance of hepadnavirus infections may be mediated by cytokines released by CD8+ T cells. Schildgen *et al.* (2006) demonstrated that TNF-α and IFN-α production are increased during WHV infection and there is some correlation between their changes with levels of WHV load. Initial support for non-cytolytic clearance came from experiments in the transgenic mouse model (Guidotti *et al.* 1996). This study showed that adoptively transferred virus-specific cytotoxic T-cells (CTL) were able to abolish HBV antigen expression and replication without the killing of hepatocytes. This type of CTL response is thought to be mediated by the cytokines TNF-α and IFN-α (Guidotti *et al.* 1996). However, since cccDNA was not produced in the transgenic mouse model used in the study, it may be that cytokines alone are not enough to clear cccDNA (Guidotti and Chisari 2006). Non-cytolytic clearance of hepadnavirus infection has also been demonstrated in chimpanzees where HBV was cleared from the liver before marked apoptosis or elevation in liver enzyme levels were detected (Guidotti *et al.* 1999). This study also showed that T-cell influx into the liver occurred after clearance of the virus, suggesting that the initial decline in markers of HBV infection was due to cells of the innate immune system, possibly NK or NK T-cells (Guidotti *et al.* 1999).

Although, increase in liver transaminases in combination with apoptosis is not a specific qualitative or quantitative marker of hepatocyte death during clearance of HBV infection in primates (Guidotti *et al.* 1999; Thimme *et al.* 2003). In studies of WHV infection, the liver cell proliferation and apoptosis became increased in woodchucks that resolved infection at the time of maximum liver injury indicated by the peak serum sorbitol dehydrogenase (SDH) activity (Glebe *et al.* 2009). In DHBV/duck system increase in transaminases is not pronounced during the death of hepatocytes although there is histological evidence for apoptosis during resolution of acute infection (Reaiche 2008). Non-specific changes in transaminases or lack of changes in transaminase activity in birds is common. Therefore

using transaminase activity is not a conclusive indicator of hepatocellular injury in birds (Lumeij and Westerhof 1987; Cray *et al.* 2008).

The second proposed mechanism for clearance is that of T cell-mediated hepatocyte death, followed by rapid division of the remaining hepatocytes, diluting out the cccDNA pool (Dandri and Petersen 2005). This is supported by an anti-viral therapy study, in which the decline in cccDNA occurred at a level that could be attributed to the death of hepatocytes by the immune response, without additional depletion by non-cytolytic mechanisms (Tang *et al.* 2005). Studies conducted in woodchucks (Summers *et al.* 2003) support virus clearance by death and proliferation of hepatocytes. Furthermore, Summers *et al.* (2003) showed that increases in both apoptosis of hepatocytes, an indicator of cell death and proliferating cell nuclear antigen (PCNA), which in turn is an indicator of DNA synthesis, occurred at times corresponding to clearance of infection, indicating that both death and division of remaining hepatocytes contribute to virus clearance. The pattern of clearance of WHV-infected hepatocytes noted by Summers *et al.* (2003) is similar to the pattern of clearance of DHBV-infected hepatocytes during the resolution of DHBV infection in ducks (Reaiche 2008). This reemphasises the roles played by hepatocyte death and division of remaining hepatocytes in the resolution of acute hepadnavirus infections.

1.4.1.3 Chronic infection

Chronic HBV infection is defined as the failure to clear HBsAg from the serum of a HBV-infected individual for more than 6 months (McMahon 2005). There are three common phases in the natural history of chronic HBV infection (Figure 1.6) (Lok and McMahon 2001).

The first phase is called the immune tolerance phase and this occurs when the infection is contracted during childhood and lasts for 1-2 decades. Individuals in this phase have high levels of serum HBV DNA (10^7-10^{11} copies/mL) with normal ALT levels as the liver necroinflammatory changes are minimal (Valsamakis 2007). HBeAg, a truncated transcriptional product of HBV core gene, is present in the serum during immune tolerance phase. This scenario supports the postulates of HBeAg being a tolerising protein to induce T-cell tolerance.

The second phase is called the immune clearance phase in which there is an increased level of HBcAg expression in the infected hepatocytes presented by the antigen presenting cells (APC) leading to immune mediated death of infected hepatocytes by the CTL response, resulting in increased ALT levels in the serum. However, the immnue response is often not sufficient to eradicate the virus but it contributes to continued liver damage (Rehermann 2000). Based on the nature of the immune response during the immune clearance phase, chronically HBV infected (CHB) patients may develop fibrosis or cirrhosis of the liver (McMahon 2005; Lok and McMahon 2001). After repeated attempts of immune response, virus replication will be suppressed due to the reduced number of infected hepatocytes and then the CHB patients often seroconvert to anti-HBe antibodies (McMahon 2005; Fattovich *et al.* 2008; Yuen and Lai 2008).

Seroconversion to anti-HBe antibodies indicates entry into the third "residual" phase or "inactive" carrier state where patients have lower serum HBV DNA levels (Figure 1.5) and persistently lower ALT levels (Lok and McMahon 2001). This is a long phase where the patients have anti-HBe antibodies and relatively low levels of HBV DNA between 10^3-10^5 copies/mL. Although most patients have normal ALT levels, some may have mild to moderate elevations. During this phase 0.2-2.0% of CHB patients may clear HBsAg from the serum and seroconvert to anti-HBs antibodies (McMahon 2005; Fattovich *et al.* 2008; Yuen and Lai 2008).

Approximately 30% patients will undergo reactivation (some times called the fourth phase) with occasional reversion to HBeAg-positivity. Reactivation after the seroconversion to anti-HBe antibodies is called HBeAg-negative chronic HBV infection. Some think that it is misleading to call reactivation the fourth phase because some patients may have continued liver damage and elevated ALT levels even after seroconverting anti-HBe antibodies (McMahon 2005; Fattovich *et al.* 2008; Yuen and Lai 2008). Moreover, the majority of the patients who acquired the infection in childhood, after seroconverting to anti-HBe antibodies, will go on to the reactivation phase without symptoms of cirrhosis or hepatocellular carcinoma (HCC) (Lok and McMahon 2001; Villeneuve 2005; Dai *et al.* 2009).

In the case of ducks, there has been no evidence that DHBV infection leads to cirrhosis or HCC. Since most congenitally DHBV-infected ducks are infected "*in ovo*" it may be that

they are tolerant to the virus and do not develop self-damaging immune responses that cause liver damage (Jilbert and Kotlarski 2000). This is supported by the fact that humans infected in the neonatal period of life have less liver disease than those who become chronically infected later in life (McMahon 2005). Since oncogenesis requires the accumulation of multiple mutations over time, it is possible that ducks do not develop HCC due to their relatively short life span compared to humans (Schultz *et al.* 2004).

1.5 Treatment of HBV infection

In the majority of adults HBV infection is self-limiting and does not require any treatment similar to many other viral infections. In chronic HBV infection the virus persistently replicates in hepatocytes leading to immune mediated hepatocyte damage. Therapies have been instituted to inhibit viral replication to prevent or at least to delay the progression of hepatitis to cirrhosis and HCC. A number of prophylactic and treatment measures using HBV vaccines, immunomodulators such as IFN-α and pegIFN-α and antiviral agents called nucleotide/nucleoside analogues (NAs) have been developed for the treatment of chronic HBV infection (Hadziyannis *et al.* 2003; Younger *et al.* 2004; Fournier and Zoulim 2008)

1.5.1 Vaccines

Two general types of HBV vaccines are widely used for prophylaxis. The first is a plasma derived vaccine composed of HBsAg particles purified from the serum of chronic HBV carriers. The second is a genetically engineered recombinant vaccine where the HBV S gene is expressed in yeast via a plasmid vector to produce HBsAg VLP. Both of these HBV vaccines are highly immunogenic and result in the production of anti-HBs antibodies and restrict infections caused by all subtypes and genotypes of HBV. Three intramuscular doses of HBV vaccine confer protective immunity in 95-99% of healthy infants, children, and young adults (Mast *et al.* 2005). However, these vaccines fail to produce protective immunity in individuals who are more than 40 years of age and in individuals who are immunocompromised (Fabrizi *et al.* 2004). Immunising neonates born to carrier mothers with immunoglobulins against HBV plus HBV vaccine can prevent the development of chronic infection in >90% of neonates (Mancini-Bourgine *et al.* 2004; Mast *et al.* 2005). Universal neonatal immunisation is practiced in some countries that experience hyper-

endemic HBV infection. Adopting universal neonatal immunisation has been shown to reduce the number of HBV carriers and HBV associated primary liver cancer or HCC in Taiwan (Mancini-Bourgine *et al.* 2004; Mast *et al.* 2005).

1.5.2 Immunomodulators

In CHB patients, HBV-specific humoral and CMI are unable to eliminate HBV from all infected hepatocytes (Fattovich *et al.* 2008; Yuen and Lai 2008; Zoulim and Perrillo 2008). On the other hand, the immune response contributes to the destruction of HBV-infected hepatocytes and this then results in chronic necroinflammatory liver disease (Roh and Kim 2003). The primary aim of immunomodulation therapy in CHB patients is to induce sustained disease remission through viral clearance and prevent serious sequelae associated with chronic HBV infection (Sprengers and Janssen 2005). Moreover, the emergence of drug-resistant HBV mutants and post-treatment relapse as a consequence of long-term NA monotherapy emphasize the fact that the principal goal of anti-HBV therapy must be to stimulate a favourable immune response to control the infection (Cooksley *et al.* 2003; Sprengers and Janssen 2005). IFN-α has been used to treat CHB patients for many years with the objective of facilitating a favourable immune response.

1.5.2.1 IFN-α/Pegylated IFN-α (peg IFN-α) and their mode of action

Interferons are cytokines with immunomodulatory, antiproliferative and antiviral properties. IFN-α in particular has marked immunomodulatory and less pronounced antiviral effects (Zoulim 2004; Sprengers and Janssen 2005). After being extensively researched, this agent was licensed in 1992, and it was the first approved treatment for chronic HBV infection in many countries.

IFN-α inhibits packaging of viral pgRNA into core particles and also enhances expression of HBsAg on hepatocytes and that could help the immune response to clear the virus (Cooksley *et al.* 2003; Sprengers and Janssen 2005).

IFN-α therapy in chronic HBV infection has shown variable degrees of beneficial effects. A sustained post-treatment response to IFN-α improves survival of patients, but the response rate is often low (Hui *et al.* 2005; Mailliard and Gollan 2006). Duration of IFN-α therapy varies widely among different protocols, as the optimal duration of IFN-α therapy

for chronic HBV infection is not well established (Krogsgaard 1998; Sprengers and Janssen 2005). Generally, the duration of therapy depends on patient characteristics and some often need indefinite treatment (Marcellin *et al.* 2005). It has been shown in a large multi-centred study that continuing IFN-α therapy for 32 weeks was beneficial in patients who did not clear HBeAg after 16 weeks (Janssen *et al.* 1999). Several long-term follow up studies have assessed the durability of responses and continued benefit of IFN-α therapy. In North America and Europe 95 to 100% of responders remained HBeAg negative during a follow up period of 5 to 10 years of and ultimately 30 to 86% responders lost HBsAg (Lin *et al.* 1999; Lampertico *et al.* 2003). However, Asian patients showed a relatively low rate of durable response and this may be due to genotypic variability in the virus and hereditary factors (Martin *et al.* 1998; Marcellin *et al.* 2005).

Relapses in IFN-α therapy are very common. HBeAg-negative CHB patients appear to relapse more often after IFN-α therapy than HBeAg-positive patients (Sprengers and Janssen 2005). In HBeAg-negative chronic HBV infection, response rates are highly variable with 6 to 24% of patients maintaining a sustained virological response (SVR) even 12 to 18 months after cessation of therapy (Hadziyannis *et al.* 2003; Lampertico *et al.* 2003).

PegIFN-α, with an attachment of a polyethylene glycol (peg) polymer molecule to increase the half-life, has been introduced for the treatment of chronic HBV infection in the last decade. PegIFN-α has shown considerable improvement in efficacy over conventional IFN-α in chronic HBV infection outcome (Hui *et al.* 2005).

1.5.3 Antiviral agents

Chemical agents that suppress virus replication and inhibit their capability to replicate and reproduce are called antiviral agents. Some anti-viral agents block the attachment of virus or entry of virus into the host cell; others inhibit replication by interfering with viral DNA synthesis. Antiviral drug development has been concurrent with advances in molecular biology (Waxman and Darke 2000; Littler and Oberg 2005).

Antivirals are now available for a wide variety of viral diseases. Ribavirin, available since the mid-1980s, is used to treat respiratory syncytial virus (RSV) that causes severe childhood respiratory infections (Waxman and Darke 2000; Littler and Oberg 2005). It is thought to inhibit messenger RNA. Amantadine and rimantadine are effective against strains

of influenza A and act by interfering with viral uncoating (Littler and Oberg 2005). Alpha herpes viruses including herpes simplex virus 1 (HSV 1) and varicella zoster virus (VZV) can be treated by a highly selective antiviral drug acyclovir, belongs to a group of chemicals called NAs, that interfere with an enzyme critical to viral DNA synthesis (Littler and Oberg 2005). Subsequently, different members of the NA group of antivirals have been shown to be effective against HIV and HBV infections *in vitro* and *in vivo* (Karayiannis 2003).

1.5.3.1 NAs and their mode of action

NAs, currently considered as the primary therapeutic choice for HBV infection, were first identified as antiviral agents two decades ago and are now used in the treatment of viral infections caused by HIV, herpes viruses and HBV (Karayiannis 2003; Zoulim 2004; Mailliard and Gollan 2006). NAs were introduced for the treatment of CHB from the clinical observations of clearance of HBV viraemia during therapy of HIV-infected patients with acquired immunodeficiency syndrome (AIDS) with co-existing chronic HBV infection. Conversely, the similarity between the HBV and HIV replication strategies and polymerase enzymes (Karayiannis 2003) strengthen the use of NAs in treating chronic HBV infection. Apart from potent antiviral activity, other advantages of NAs include oral administration, excellent tolerance, absence of serious side effects, possibility of treatment of chronic HBV infection caused by different HBV genotypes, wild-type and precore mutant HBV strains. The usability of NAs in patients at high risk of side effects after or with contraindications to IFN-α therapy is also an advantage (Karayiannis 2003; Zoulim 2004; Mailliard and Gollan 2006).

On the other hand, NAs especially lamivudine (3TC) and adefovir (AFV) have been used to treat CHB patients for less than a decade and some NAs such as ETV have only been licensed since 2007. It may be fair to say clinical and scientific community is yet to see the side effects of NAs in CHB patients. Some studies indicate that HIV/AIDS patients treated with NAs have experienced side effects due to the effect of NAs on mitochondrial DNA. A range of clinical toxicities from asymptomatic hyperlactaemia to non-specific symptoms are commonly reported among HIV/AIDS patients treated with NAs (Cote *et al.* 2003; Montaner *et al.* 2003; Van Dyke *et al.* 2008). In a recent study, the most common clinical toxicities associated with NAs noted were hepatitis, peripheral neuropathy, lipodystrophy/lipoatrophy and pancreatitis whereas the most common biochemical toxicities

were an elevated anion gap, an increased total amylase level, neutropenia and thrombocytopenia (Van Dyke *et al.* 2008).

The mechanism of action of NAs against HBV replication is via a competitive inhibition of the nucleotide/nucleoside concerned (Karayiannis 2003; Younger *et al.* 2004). NAs are carried across the plasma membrane by the same transporters as natural nucleosides. These analogues can inhibit key enzymes of nucleotide metabolism. In the case of HBV or HIV the enzyme inhibited is the viral polymerase. These drugs function by depleting the natural nucleotide pool, promoting the incorporation of phosphorylated forms of the NA drug into DNA, thus causing inhibition of DNA replication resulting in termination of newly formed DNA often referred to as "chain termination" (Pastor-Anglada *et al.* 1998). Furthermore, the ability of NAs to act as chain terminators will prevent the further release of virus progeny and prevent the formation of new cccDNA molecules from rcDNA (Karayiannis 2003; Younger *et al.* 2004).

1.5.4 Currently approved therapies for chronic HBV infection

Current therapies approved by the Food and Drug Administration (FDA) for chronic HBV infection are IFN-α and pegIFN-α and the NAs, 3TC, AFV and ETV. Permanent loss of HBV DNA including cccDNA and seroconversion from HBeAg to anti-HBe antibodies and/or HBsAg to anti-HBsAg antibodies indicates a positive response to treatment. A positive response to treatment is usually associated with an improvement in necro-inflammation of the liver according to the protocols developed by the World Health Organisation (WHO) for therapy chronic HBV infection (Karayiannis 2003; Younger *et al.* 2004; Marcellin *et al.* 2005; Rapti *et al.* 2007; Fournier and Zoulim 2008). However, more recent data suggest that loss of HBsAg gives a better SVR than the loss of HBV DNA among patients who respond to the therapy with pegIFN-α. Approximately 10% pegIFN-α treated patients who lost HBsAg in the serum at the end of treatment endpoint eventually cleared HBV (Moucari *et al.* 2008).

1.5.4.1 Antiviral resistance to NAs

In general, NA treatments are administered until the patients seroconvert to anti-HBs or anti-HBe antibodies (Karayiannis 2003; Marcellin *et al.* 2005). In many clinical cases seroconversion takes more than a year and in this time HBV becomes resistant to NA,

creating a NA-resistant HBV mutant strain. Emergence of resistant strains is associated with a reduction in the viral fitness due to the inherent rate of mutations that occurs in HBV due to error-prone proof-reading capacity (Karayiannis 2003; Marcellin *et al.* 2005). The selection of additional mutations that favour growth of the virus under a drug treatment modifies the resistance dynamics of the virus (Locarnini and Bathholomeusz 2008).

This has been shown by *in vitro* experiments as replication deficient phenotype of HBV or reduction plasma virus load in treated patients. However, under the selection pressure of a drug, the resistant clone of a HBV can replicate relatively faster due to the growth advantage (Locarnini and Bathholomeusz 2008).

Hence, drug resistance has been widely reported with NA monotherapy, which is the common treatment modality used against chronic hepatitis B virus infection (Marcellin *et al.* 2005; Zoulim 2005). Conventional NA therapies also fail to reduce the levels of cccDNA in infected hepatocytes to a significantly low level at which the immune system can counteract to overcome the infection by targeting the HBV infected hepatocytes either through cytolytic killing or non-cytolytic mechanisms or both (Liaw 2002; Zoulim 2005).

Finding new treatment modalities using novel therapeutic approaches and enhancing existing therapies by combining the appropriate NAs to overcome the emergence of antiviral resistance will lead to better response to treatment for chronic HBV infection. Combination therapies have been shown to be more successful than the monotherapies for a number of infectious diseases such as tuberculosis and AIDS (Liaw 2002; Zoulim 2004; Sasadeusz *et al.* 2007).

1.5.5 Novel antiviral agents in HBV research

Several novel therapeutic agents have been tested against HBV infection with some success. Different herbal extracts have been tested against HBV especially in South East Asia where CHB infection is highly prevalent (Chiang *et al.* 2003; Chang *et al.* 2007; Han *et al.* 2008; Tseng *et al.* 2008). In these studies various plant derivatives were tested for cytotoxicity and general inhibitory activity against HBV infection in the PHH or DHBV infection in the primary duck hepatocytes (PDH). Chiang *et al.* (2003) tested saikosaponins, the extract of *Bupleurum spp.* against HBV infection using HBV transfected hepatoma cell line. Out of 3 constituents tested from *Bupleurum spp.*, only one constituent inhibited HBV infection. The

other two exhibited cytotoxicity to PHH with no antiviral activity against HBV. A crude extract of *Bupleurum spp.*, saikosaponin, inhibited HBV infection by suppressing levels of HBsAg and HBeAg in a HepG2 cell culture system. It is not clear in this study if saikosaponin was cytotoxic or not (Chang *et al.* 2007). Helicoxanthin (HE-145) showed indirect evidence for anti-HBV activity by inhibiting the expression of the HBsAg promoter II and the HBcAg promoter but had no effect on the DHBsAg I or the promoter X gene (Tseng *et al.* 2008). *Oenantha javanica* extract was tested for its anti-HBV activity using the DHBV-duck model. *Oenantha javanica* extract exhibited anti-DHBV activity when the culture fluid of treated PDH and the sera and liver sections of treated ducks were tested for DHBsAg expression (Han *et al.* 2008). These studies appear to provide a basis for investigating novel compounds that have antiviral potential. However, none of these studies included detailed investigations into the ability of these phytochemicals to prevent the infection progressing to chronic infection or to prevent rebound of HBV infection after cessation of treatment, which is a very common phenomenon with current anti-HBV agents in CHB patients (Hui *et al.* 2005; Sasadeusz *et al.* 2007; Seetharam and Lisker-Melman 2009).

1.5.5.1 Amphipathic DNA polymers (APDPs) as anti-HBV agents

DNA-based medicines are increasingly being tested against various virus infections. The traditional approach with this class of molecules has employed anti-sense strategies to target the activity of viral specific genes (Bartlett *et al.* 2007; Bartlett and Davis 2008; Rossin *et al.* 2008; Haasnoot and Berkhout 2009; Schreiber *et al.* 2009). APDPs are a new class of DNA-based medicines that have been shown to inhibit a wide range of enveloped viruses including HIV, HSV, cytomegalovirus (CMV), hepatitis C virus (HCV) and HBV (Vaillant *et al.* 2006; Guzman *et al.* 2007; Bernstein *et al.* 2008). APDPs use the unique characteristics of long, phosphorothioated oligodeoxynucleotides (PS-ONs) as APDPs to target the function of analogous amphipathic chemical domains in viral fusion proteins (Vaillant *et al.* 2006).

APDPs with degenerate or repetitive homo- or hetero-ploymeric sequences have been examined as a novel antiviral approach against HIV-1 infection. Vaillant *et al.* (2006) demonstrated the sequence independent ability of APDPs to inhibit HIV by allowing these

compounds to bind to the core fusion domain of gp41. Thus APDPs bind to the core fusion domain and then prevent conformational changes required for gp41-mediated membrane fusion. The ability of these compounds to inhibit HIV-1 was also shown to be size dependent. Polymers greater than 30 nucleotides in length exhibit the most potent antiviral activity, which is phosphorothioation dependent (Vaillant *et al.* 2006).

Phosphorothioation of APDPs increases their hydrophobicity (Agrawal *et al.* 1991) and thus is the source of their amphipathic nature. This chemical activity in combination with a sufficiently long chain length has been shown to be essential for the potent activity of these compounds in all viruses examined (Vaillant *et al.* 2006; Guzman *et al.* 2007; Bernstein *et al.* 2008).

Clinical trials with several PS-ONs of different lengths and sequences that are pharmacologically similar to APDPs have shown the safety of parenteral exposure of these compounds in humans. The well tolerated pharmacological nature of these compounds is independent of the nucleotide sequence with acute or chronic dosing regimens (Tolcher *et al.* 2004; Badros *et al.* 2005; Morris *et al.* 2005; Chi *et al.* 2008; Liu *et al.* 2008; Moulder *et al.* 2008; Yu *et al.* 2009a; Yu *et al.* 2009b), suggesting that the use of APDPs as antiviral agents in human patients will also be well tolerated.

1.5.6 Combination chemotherapy using NAs

The emergence of resistance to the widely used NA 3TC and low tolerability to IFN-α or pegIFN-α indicates the need for alternative therapeutic approaches such as combination chemotherapy (Fournier and Zoulim 2008). Moreover, there is a great fear among the clinical and scientific communities about multi-drug resistant HBV as there is evidence for HBV strains resistant to AFV and ETV when they are used as monotherapeutic agents (Zoulim and Perrillo 2008). It may be difficult to deal with multi-drug resistant HBV because drug targets for HBV are limited whereas HIV has multiple targets (Yim *et al.* 2006; Zoulim and Perrillo 2008). Combination chemotherapy requires extensive pre-clinical evaluation or toxicity and efficacy in terms of virologic and biochemical responses to different combinations of antiviral agents, which may be used effectively against chronic HBV infection.

Although many have suggested the need for combination chemotherapy to combat the antiviral resistance that results from NA monotherapy, very little has been done to investigate the effectiveness of combination chemotherapy using NAs (Sasadeusz *et al.* 2007; Fournier and Zoulim 2008; Seetharam and Lisker-Melman 2009). In a pre-clinical study using the HepG2 cell culture system, the additive effect of AFV with ETV, emtricitabine (FTC), 3TC and telbivudine (TLB) has been reported. AFV with all other NAs used in the study in dual combination produced an enhanced antiviral activity against HBV (Delaney *et al.* 2004). The antiviral activity of AFV and 3TC in combination gave a better additive effect than other combinations used. Furthermore, no cytotoxic effects were seen in the cells treated with dual combinations (Delaney *et al.* 2004).

A 4 week treatment of DHBV-infected ducklings with combinations of amodoxovir and FTC showed that the dual combination was synergistic in inhibiting viral replication (Seigneres *et al*. 2003). Clevudine (L-FMAU), together with the above mentioned dual combination acted even more potently in inhibiting viral replication (Seigneres *et al.* 2003). The use of combined treatment with two or more NAs seems warranted as administration of a single drug typically requires a higher concentration than that used in combination to achieve a similar level of inhibition of viral replication. Although these combinations were more effective in inhibiting viral replication, they failed to clear the virus and prevent the infection progressing to chronic infection (Seigneres *et al.* 2003). Continuing combination therapy for longer periods may achieve viral clearance at a faster rate with minimal cases of antiviral resistance than conventional NA monotherapies.

In a clinical study of CHB patients, combination therapy with 3TC and famciclovir reduced serum HBV DNA to undetectable levels although the treated patients were unable to clear the virus. In this study the emergence of resistance to 3TC was noted after two years (Sangfelt *et al.* 2002). Antiviral agents for combination therapy need to be selected appropriately to induce synergistic activity with no toxic effects. Many approved antiviral agents or the ones in clinical trials need to be investigated in combination against HBV infection for future clinical applications.

1.6 Regulatory requirements for preclinical testing of drugs

Developing therapeutic and diagnostic products for use in humans is a long and complex process. In an ideal situation, scientists should be able to test thousands of different compounds in humans to determine treatment or vaccination resulted in recovery from infection. However, this can be time consuming and dangerous as most compounds may not be effective and some might cause illness or mortality. Thus researchers often use animals to test the efficacy of drugs and vaccines to make sure that these products are safe (Act 1968; Council 2004).

There are three reasons why animals may be used in scientific experimentation. The first is to ensure the safety of new drugs and possible pharmaceutical compounds. The second is to see whether such drugs/compounds might be effective in humans. The third is for general research into the biology of an animal or the function and the pathogenesis of certain diseases within its body (Act 1968; Council 2004).

In many countries it is a legal requirement that all drugs and vaccines are tested on animals to ensure safety. In the United Kingdom for example, the Medicines Act of 1968 states that all new pharmaceutical products must be tested on at least two different species of live mammal, one of which must be a large non-rodent (Act 1968). This legislation was introduced shortly after the discovery that the drug Thalidomide could cause serious physical deformities in babies born to mothers who had taken it during pregnancy. Thalidomide was not thoroughly tested on animals before it was prescribed to women and this is the root of animal testing safety laws today.

Australia has its codes and practices for the use of animals for scientific research (Council. 2004). Animal safety tests usually come at the end of a long process of safety data collection that may include testing the product '*in vitro*' and using a computer program to simulate what might happen to the drug inside the body (Schultz *et al.* 1973). The regulations for a new product vary from country to country and drug to drug, with most drug authorities requiring *in vitro* and computer-generated data prior to commencement of *in vivo* animal experimentation (Schultz *et al.* 1973).

There are many ethical regulatory requirements necessary for *in vivo* animal experimentation. The real need for the use of animals for a particular drug testing has to be

justified by the researchers. All applications undergo a thorough evaluation by ethical committees before an approval may be granted. This is the widely used practice in many countries in the world to minimise the use of animals when there are alternatives to animal testing and to make sure the experimentation is conducted with minimal distress to the animals (Act 1968; Council 2004).

1.7 DHBV infection as a tool to study human HBV infection

The conversion of rcDNA to cccDNA does not occur in cell lines that are available to grow HBV. In the recent past, cccDNA molecules were diluted in some cell lines but these cccDNA molecules have a very short or unpredictable half-life due to cell turnover (Zhou *et al.* 2006; Guo *et al.* 2007). Transgenic mouse models also failed to produce cccDNA and that limited their use in many laboratories (Yang *et al.* 2002). There is some evidence now that *in vivo* gene transfer of a plasmid encoding HBV DNA can establish chronic viral replication in a non-transgenic mouse model, which involves, at least in part, new synthesis of cccDNA episome, hence recapitulating only a part of human HBV infection (Takehara *et al.* 2006).

In experimental animal models the only animal species that can be infected by HBV are primates. Unfortunately, many practical problems make this model unavailable to many laboratories for studies of hepadnaviruses and HBV.

Other members of the hepadnaviridae family and their natural hosts are used for experimental studies. DHBV in its natural host, Pekin ducks (*Anas domesticus platyrhynchos*), can be used as an animal model to study various aspects of treatment outcomes and vaccine trials, at the pre-clinical level in both *in vitro* and *in vivo* experiments. Much of what is known especially of viral replication and infection outcomes has been developed using the DHBV-duck system due to the similarity of age and dose-related infection outcome of DHBV infection of ducks to HBV infection of humans (Jilbert *et al.* 1998; Foster *et al.* 2005; Jilbert *et al.* 2008). This model is less expensive than chimpanzees or woodchucks and reagents are readily available. Moreover, this model is less hazardous to the laboratory staff as DHBV is unable to infect humans.

Several vaccine and antiviral studies have been performed in this model. For example, the therapeutic efficacy of acyclovir (a synthetic nucleoside analogue) and foscarnet

(phosphonic acid derivative) was studied in the DHBV model using a total of 112 ducks inoculated with DHBV at 11 days of age. Three days later, these ducks were injected IP twice daily for 10 days with acyclovir (25 mg/kg) or foscarnet (250 mg/kg) or PBS. Serum samples were analysed before, during and up to 4 weeks post-treatment for DHBV DNA and liver tissue at autopsy was examined for DHBV DNA and for histological changes to determine the anti viral effect of acyclovir and foscarnet (Freiman *et al.* 1990).

In an *in vitro* study, a purine nucleoside analogue penciclovir (PEN) was tested for its anitiviral activity against DHBV. DHBV-infected PDH harvested from congenitally infected ducklings were treated with PEN to determine its inhibitory effect of DHBV infection (Shaw *et al.* 1994). The effects of pyrimidine and purine analogue combinations were investigated in the DHBV model to study the effect of these combinations against an ongoing DHBV infection using acutely infected ducks for a short term (Seigneres *et al.* 2003).

ETV, a potent selective inhibitor of HBV, treatment efficacy of ETV on age- and dose-related outcomes of DHBV infection was investigated in ducks (Jilbert *et al.* 1998; Foster *et al.* 2005). Moreover, a number of immune therapy studies in combination with ETV or without ETV therapy have been performed. In all of these studies therapy with different types of DNA vaccines was started at the time of DHBV infection to show the ability of the therapy to prevent the development of chronic DHBV infection. Using a DHBV inoculum size (5×10^8 DHBV virus genome equivalents) that is known to cause persistent DHBV infection in 14-day-old ducks (Jilbert *et al.* 1998; Foster *et al.* 2005), these studies were aimed to test the efficiency of the prophylactic immunisation with or without ETV therapy (Foster *et al.* 2003; Miller *et al.* 2006a; Miller *et al.* 2006b; Miller *et al.* 2008).

There have been other studies that discuss the availability of different reagents available to study pathogenesis and immunological aspects of hepadnavirus infection using DHBV-duck model (Cova 2005; Vickery *et al.* 2006). The idea of noting these studies is to not discuss their results but to emphasise the point that DHBV infection in ducks has been widely used for antiviral and immune therapy studies as an animal model system to perform HBV pre-clinical studies. Hence this system provides a good platform for the current research.

Moreover, the ability to harvest PDH from the livers of young ducks through collagenase perfusion and culture in the laboratory has made an immense contribution to hepadnavirus

studies *in vitro* (Zoulim *et al.* 2008). DHBV-infected PDH have been used to study the infection kinetics, outcomes, anti-NA resistance during NA monotherapies and a few studies have used this system to evaluate the efficacy and safety of NA combinations (Seigneres *et al.* 2003; Schultz *et al.* 2004). A combination of amodoxavir, FTC and L-FMAU was tested for its efficacy in PDH. The combination was able to produce a favourable response in terms of antiviral efficacy when assayed to test the presence of DHBsAg-positive PDH but unable to prevent the conversion of rcDNA to cccDNA (Seigneres *et al.* 2003). Another triple NA combination of PEN, 3TC and AFV was tested in DHBV-infected PDH to examine the synergistic effect and toxicity. These three drugs were synergistic and resulted in no toxicity to the PDH (Colledge *et al.* 2000).

In summary, several studies have been performed to study the antiviral activity of different NAs or new phytochemicals (Section 1.6.3) in both in *vitro* and *in vivo* systems of DHBV infection.

1.8 Research outline and aims

The current research aimed to examine the potential of a novel therapy for chronic HBV infection (using APDPs) and of a combination therapy approach using nucleotide and nucleoside analogues in combination. All studies were performed with the DHBV model for HBV infection.

This Ph.D. thesis aimed to:

- Test the novel antiviral APDPs for cytotoxicity in PDH, antiviral efficacy against DHBV infection *in vitro* including the role of chemistry (hydrophobicity, polymer size and uptake of APDPs) on anti-DHBV activity (Chapter 3).
- Then studies tested the novel antiviral APDPs (REP 2006 and REP 2031) for their antiviral efficacy against DHBV infection *in vivo* (Chapter 4).
- Subsequent studies tested the antiviral efficacy and dose optimisation of novel therapeutic APDP REP 2055 against DHBV infection *in vivo* (Chapter 5).
- The ability of REP 2055 to prevent rebound of DHBV infection and its potential to act as a therapeutic agent against DHBV infection was tested using prophylactic and therapeutic experimental protocols (Chapter 6).
- Subsequently, the ability of REP 2055 to prevent rebound of DHBV infection and its potential to act as a therapeutic agent against persistent DHBV infection was comprehensively studied using therapeutic experimental protocols (Chapter 7).
- As a method of testing the combination chemotherapy approach using NAs, pharmacokinetics (PK) of nucleotide analogue tenofovir (TFV) and tenofovir disoproxil fumarate (TDF) (oral formulation of TFV) in DHBV negative healthy ducks was investigated. This was followed by testing the antiviral efficacy of TFV and FTC, either of the drugs alone or in combination in ducks with persistent DHBV infection (Chapter 8).

Table 1.1: Members of hepadnavirus family

Orthohepadnaviruses	*Avihepadnaviruses*
Hepatitis B hepatitis virus (HBV)[a]	Duck hepatitis B virus (DHBV)[a, b, c]
Woodchuck hepatitis virus (WHV)[a, d]	Heron hepatitis B virus (HHBV)[e, c]
Ground squirrel hepatitis virus (GSHV)[f, g]	Snow goose hepatitis virus (SGHBV)[h, c]
Arctic squirrel hepatitis virus (ASHV)[i]	Ross goose hepatitis B virus (RGHBV)[h, c]
Woolly monkey hepatitis B virus (WMHBV)[j]	Crane hepatitis B virus (CHBV)[k, c]
	Stork hepatitis B virus (STHBV)[l, c]

[a] (Mason *et al.* 1980);
[b] (Triyatni *et al.* 2001);
[c] (Funk *et al.* 2007);
[d] (Galibert *et al.* 1982);
[e] (Sprengel *et al.* 1988;
[f] (Marion *et al.* 1980);
[g] (Seeger *et al.* 1984);
[h] (Chang *et al.* 1999);
[i] (Testut *et al.* 1996);
[j] (Lanford *et al.* 1998);
[k] (Prassolov *et al.* 2003);
[l] (Pult *et al.* 2001).

Table 1.2: Comparison of ortho and avihepadnaviruses

Characteristics	*Orthohepadnaviruses* Prototype (HBV)	*Avihepadnaviruses* Prototype (DHBV)
Tropism	Primarily hepatotropic[a, b]	Primarily hepatotropic[c]
Genome	Partially double stranded DNA[d, e]	Partially double stranded
Size	3200 ntd[d, e]	DNA[d, f]
		3021-3027 nt[d, f]
Sequence homology	40-50% to avihepadnaviruses[d]	40-50% to orthohepadnaviruses[d]
ORFs	4 ORFs P,S, pre-C/C and X genes[e]	3 ORFs as X gene is absent or
		has unconventional start codon[g]
Entry to target cell	Receptor mediated endocytosis (RME)[h, i, j]	RME[h, i, j]
Replication	Reverse transcription (RT) of an RNA intermediate[k]	RT of an RNA intermediate[k]
Virion structure	Spherical double-shelled structures of 42-47 nm[l, m, n]	Pleomorphic double-shelled structures of 40-45 nm[h]
Surface Ag particles	HBsAg particles are present in spherical and tubular forms[l, m, n]	Only the spherical DHBsAg particles are present[h]
	90 times higher in number than that of infective virions[l, m, n]	500 times higher in number than that of infective virions[h]
Envelope proteins	Large, medium and small envelope proteins are present (3types)[l, m, n]	Only large and small envelope proteins are present (2 types)[h]

[a] (Howard *et al.* 2002); [b] (Wang *et al.* 2006);

[c] (Tagawa *et al.* 1996); [d] (Sprengel *et al.* 1985);

[e] (Kobayashi *et al.* 1984); [f] (Triyatni *et al.* 2001);

[g] (Funk *et al.* 2007); [h] (Franke *et al.* 2007);

[i] (Glebe *et al.* 2007); [j] (Mhamdi *et al.* 2007);

[k] (Tang *et al.* 2002); [l] (Sakamoto *et al.* 1983);

[m] (Heermann *et al.* 1984); [n] (Bruss *et al.* 1996).

Figure 1.1: Structure of hepatitis B virus (HBV) including the surface appearance, different structural components and electron micrographic details.

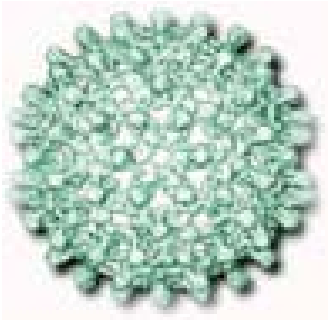
Panel A: Spiky surface appearance resulting from 3 types of surface proteins (Bruss 2004; Skrastina *et al.* 2008).

Panel B: Schematic diagram of HBV showing different structural components.

a: Large (L) surface protein;
b: Medium (M) surface protein;
c: Small (S) surface protein;
a, b and c: HBV surface antigen (HBsAg);
d: HBV core antigen (HBcAg);
e: Partially double stranded rc DNA genome;
f: HBV polymerase.

Panel C: Electron micrograph showing Dane particles and circular and spherical and filamentous HBsAg particles from HBV infected serum (Dryden *et al.* 2006).

A



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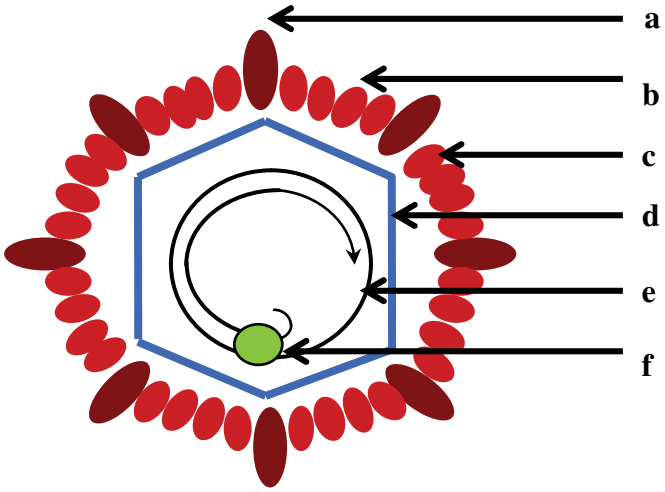
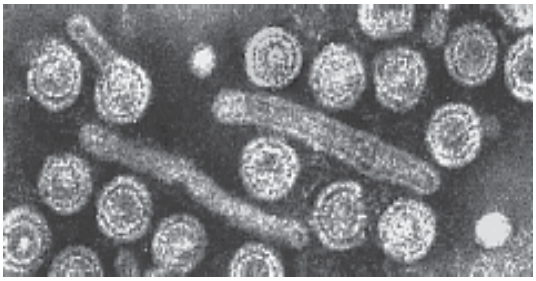


C



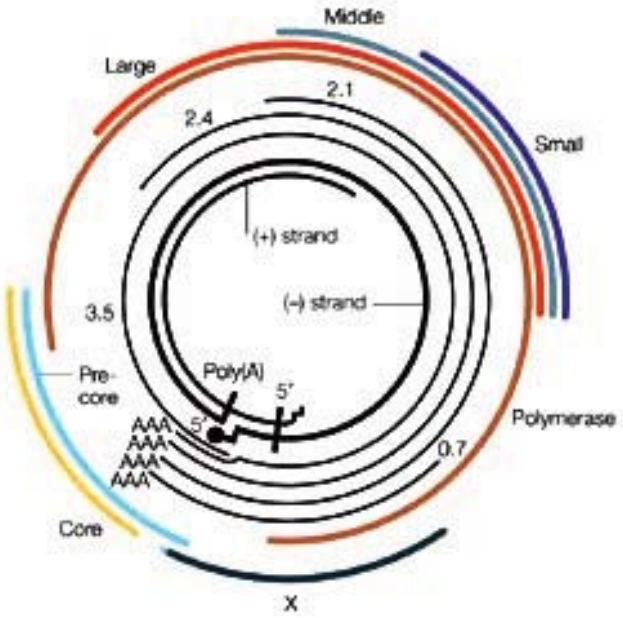


Figure 1.2: Genomic structure of HBV. The inner circles show the full-length negative (-) strand (with the terminal protein attached to its 5' end) and the incomplete positive (+) strand of the HBV genome.

The thin black lines represent the 3.5, 2.4, 2.1 and 0.7 kb mRNA transcripts, which are all terminated near the poly A (polyadenylation) signal.

The ORF of the HBV large (L), middle (M) and small (S) HBV surface proteins, polymerase protein, X protein, core and pre-core proteins are shown in the outermost coloured lines (Rehermann and Nascimbeni 2005).

Figure 1.3: Schematic diagram showing the different stages of hepadnavirus life cycle during replication (Adapted from Jilbert *et al.* 2008).

Figure 1.4: Reverse transcription of DHBV pgRNA by polymerase to produce rcDNA in the cytoplasm of infected hepatocytes (Adapted from Thorpe 2007).

Panel A: Pol synthesises a 4nt DNA primer using sequence from the ε buldge as a template and resulting in covalent attachment of this primer to the enzyme. The Pol-primer complex translocates to DR1*, where binding occurs due to homology between the primer and DR1* sequence.

Panel B: Pol extends the DNA primer by reverse transcription (RT), creating the (-) DNA strand of the genome, using the (+) sense pgRNA as a template.

Panel C: The above process occurs concurrently with degradation of the pgRNA template, by the RNase H activity of Pol, however this lags behind by 18nt. The covalent attachment of the elongating (-) DNA strand to Pol causes the reverse transcribed genome to adopt a circular structure.

Panel D: Pol is unable to degrade the final ~18nt of the pgRNA, which includes part of DR1.

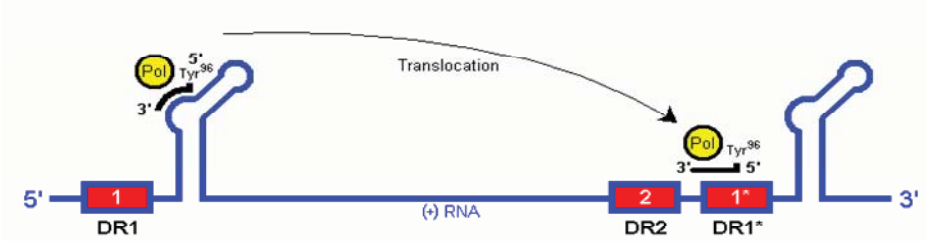
Panel E: Owing to the sequence homology between all DR regions, the remaining positive RNA segment translocates to DR2 of the nascent negative DNA strand, which has been brought into close proximity by the covalent attachment of the strand to Pol.

Panel F: Pol is then able to 'jump' to the RNA strand, and using this as a primer, synthesise the positive sense DNA of the genome.

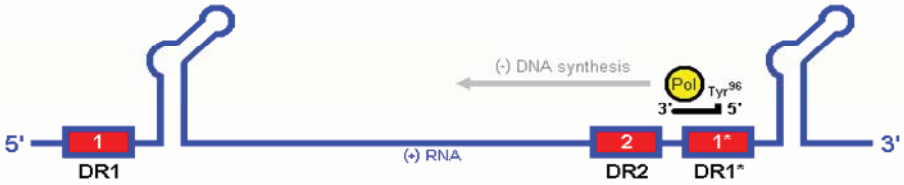
Panel G: Reverse transcription of the pgRNA to form rcDNA is completed by Pol, save for the RNA primer, which forms a gap in the positive strand of the genome. Also present in rcDNA is a nick in the negative strand, owing to Pol remaining covalently attached to the newly-formed genome.

Panel H: Upon entering the hepatocyte nucleus, the rcDNA genome is converted to covalently closed circular DNA (cccDNA) as described in Section 1.3.1.

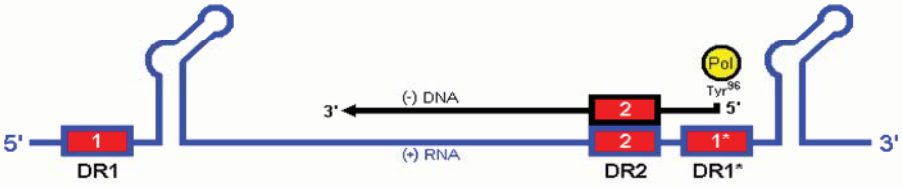
A


Pol
Tyr96
5'
3'
Translocation
5'
1
DR1
(+) RNA
2
DR2
1*
DR1*
3'

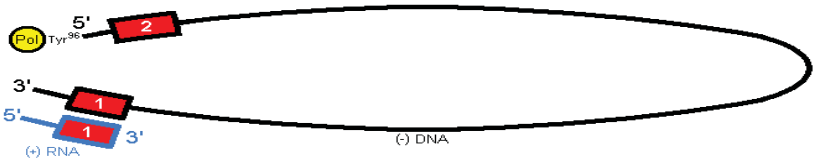
B


(-) DNA synthesis
Pol
Tyr96
3'
5'
5'
1
DR1
(+) RNA
2
DR2
1*
DR1*
3'

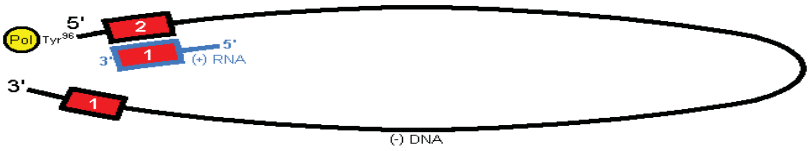
C


Pol
Tyr96
5'
3'
(-) DNA
2
2
5'
1
DR1
(+) RNA
2
DR2
1*
DR1*
3'

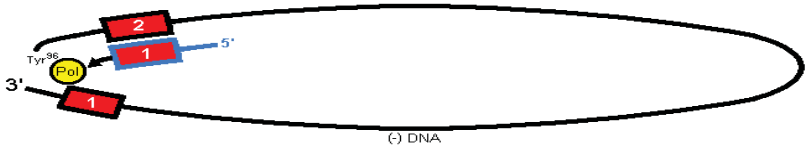
D


Pol
Tyr96
5'
2
3'
1
5'
1
3'
(+) RNA
(-) DNA

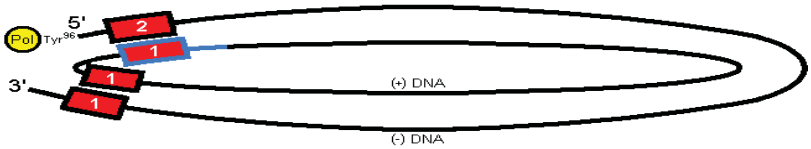
E


Pol
Tyr96
5'
2
3'
1
5'
(+) RNA
3'
1
(-) DNA

F


2
1
5'
Tyr96
Pol
3'
1
(-) DNA

G


Pol
Tyr96
5'
2
1
1
3'
1
(+) DNA
(-) DNA

H


2
1
1
1

Figure 1.5: Serological profile of virological and immunological markers following acute (Panel A) and chronic HBV infection (Panel B) (Adapted from Bowden 2008).

Panel A: Acute HBV infection - HBsAg appears as the first marker of infection followed by anti-HBc antibodies. Resolution of HBV infection occurs with the appearance of anti-HBs antibodies between 2 to 3 months. Anti-HBe antibodies appear after ~14 weeks. Anti-HBc, anti-HBs and anti-HBe antibodies last for many years.

Panel B: Chronic HBV infection - HBsAg appear as the first marker of infection and persists for many years. Anti-HBc antibodies appear 6 weeks after the exposure and persist at a high titre. In some cases anti-HBe antibodies appear after 1-2 years indicating and immune control of infection but not the resolution of HBV infection.

NOTE:
These figures are included in the print copy of the thesis held in the University of Adelaide Library.

Figure 1.6: Natural history illustrating the different phases of chronic hepatitis B infection (Adapted from Yuen and Lai 2008).

HBeAg: Hepatitis B virus e antigen;

HBsAg: Hepatitis B virus surface antigen;

HCC: Hepatocellular carcinoma;

ALT: Alanine aminotransferase.

Chapter 2: Materials and Methods

2.1 Experimental Animals

DHBV-negative 1-day-old Pekin ducks (*Anas domesticus platyrhynchos*) were obtained from a commercial poultry supplier (Pepe's ducks, New South Wales, Australia) and housed and maintained in the animal care facilities at the Institute of Medical and Veterinary Science (IMVS), Frome Road, Adelaide, South Australia. Ducks were cared for in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific purposes (Council 2004). Ducks were carefully examined every day for any abnormalities in feed and water intake, weight loss and gait or changes in behaviours. Findings were recorded using a clinical monitoring sheet for future reference (Appendix I).

Approval to conduct the studies using ducks was obtained from the Animal Ethics Committees of the IMVS and the University of Adelaide.

2.2 Preparation of primary duck hepatocytes (PDH)

PDH were derived from 1-2-week-old ducklings and were prepared by cannulating the heart (via the right atrium) to allow reverse perfusion of the liver with draining of the perfusate via the portal vein. Perfusion was continued using a perfusate containing supplemented minimal essential medium (SMEM) (GIBCO^{TM}, USA), collagenase (0.5 mg/mL, Sigma Chemicals, Cat. NO: C9722), aprotinin (0.02 mg/mL, Sigma Chemicals, Cat. NO: C6103) and trypsin inhibitor (0.02 mg/mL, Sigma chemicals, Cat. NO: C9003) until the liver became marbled, soft and spongy. The liver was then aseptically removed and homogenised gently using the sterile beaker and the forceps in approximately 50 mL of Leibovitz's L15 growth medium (GIBCO^{TM}, USA) containing 10% foetal calf serum (FCS) (GIBCO^{TM}, USA), penicillin (6 µg/mL, Sigma Chemicals, Cat. NO: P7794) and gentamycin (16 µg/100 mL, Sigma Chemicals, Cat. NO: G1272), nystatin (10 U/mL, Sigma Chemicals, Cat. NO: N4014), hydrocortisone hemi-succinate (2.8 µg/mL, Sigma Chemicals, Cat. NO: H4881), glutamine (2 mM, Invitrogen, Australia) and insulin (25 µg/mL Sigma Chemicals, Cat. NO: I4014). Then the PDH homogenate was filtered through 4 layers of sterile gauze and PDH were seeded at a density of 2 x 10^5 cells/well of a 24-well tissue culture plate (FALCON®,

USA). PDH were cultured in Leibovitz's L15 growth medium containing the same constituents as above and incubated at 37°C without CO_2. From the 3rd day, Leibovitz's L15 maintenance medium with the same constituents as above was used to avoid excessive growth of PDH but with 5% FCS.

2.2.1 Cytotoxicity assay to test for cytotoxic changes in PDH

The cytotoxicity assay was performed using a 96-well plate. The plate was prepared for reading after 7 days of treatment with REP 2006, REP 2031 and REP 2086. The details of the preparation of REP 2006, REP 2031 and REP 2086 are provided in Section 2.4.

A 96-well plate (FALCON®, USA) was seeded with 2 x 10^4 PDH per well in L-15 growth medium as described in Section 2.2. For each 96-well plate, 0.5 ml of 10 µM of each REP 2006, REP 2031 and REP 2086 were needed, *i.e.* 0.5 mL total for 5 wells diluted from 10-0.001 µM in L-15. The highest concentration of REP compounds (10 µM) was applied in two rows of wells in quadruplicate and all other concentrations (1- 0.001 µM) were applied to 1 row of wells in quadruplicate (Tables 2.1 and 2.2). Fresh REP 2006, REP 2031 and REP 2086 dissolved in L-15 were added to the plate every other day. The PDH were examined each day for any visible changes using an inverted phase contrast microscope (Olympus CX31).

A fresh mixture of XTT-PMS solution was prepared. To a clean 5 mL tube, 50 µl of 1 mg/mL of tetrazolium salt, XTT (Polysciences, Warrington, PA) was added and dissolved in RPMI without phenol red (GIBCO™, USA). To this XTT solution, 0.15 µl of 1.55 mg/mL of phenazine methosulfate (PMS, Sigma Chemicals, Cat. NO: P9625-1G) previously dissolved in phosphate buffered saline (PBS) was added to make the XTT-PMS solution.

On the 7th day of treatment, a 200 µl of XTT-PMS solution was added to each REP 2006-, REP 2031- and REP 2086-treated and control well and the plate was then incubated for 4 h at 37°C.

After thorough mixing in the plate reader (Spectra Max M2, Molecular Devices, Millennium Science, USA), the absorbance at 450 nm was measured. XTT salt was reduced to an orange colour product, XTT-formazan. This reaction occurs if there are healthy cells in

the wells, as mitochondrial dehydrogenase present in viable cells metabolically reduces the XTT to a water-soluble formazan product (Zhao *et al.* 2002).

2.2.2 Detection of DHBV surface antigen (DHBsAg) by immunofluorescence assay (IFA)

Culture medium in the 24-well tissue culture plate was removed by suction and the cells were washed with PBS at RT. Then cold PBS was added and removed by suction and the same was repeated with cold ethanol acetic acid (EAA: 95:5). EAA solution was added a second time, the plate was incubated for 5 min and EAA was removed by suction. The plate was dried before adding 500 µl PBS. The PBS was removed after 5 min and then the same was repeated with distilled water.

The plate was dried and stored for 1-2 weeks until the assay was performed. Otherwise the dried plate was incubated at 37°C with 1/50 dilution of normal duck serum (NDS) in PBS in a pre-warmed container for 30 min. NDS was removed and the plate was then washed with PBS. The plate was incubated with anti-DHBV preS monoclonal antibodies, 1H.1 (Pugh *et al.* 1995) for 1 h at 37°C. The plate was washed with PBS twice for 5 min and then incubated with secondary goat anti-mouse polyclonal antibodies labelled with Alexa flour 488 (Molecular probes, Australia) for 1 h at 37°C. Both primary and secondary antibodies were diluted 1/200 in 1/50 NDS in PBS. The final PBS wash was performed prior to adding a few drops of 90% glycerol in 10 mM Tris in PBS. DHBsAg-positive PDH were visualised at a wave-length of 488 nm using an Olympus inverted confocal microscope at the IMVS confocal microscopy unit.

2.3 Experimental procedures in ducks

2.3.1 DHBV stock and inoculation of ducks

The DHBV stock was derived from a pool (Pool 8) of serum from 34-day-old congenitally DHBV-infected ducks infected with the Australian strain of DHBV (AusDHBV) (Triyatni *et al*. 2001). The serum was filtered through a 0.2 µm filter, aliquoted and stored at -80^{o}C. The Pool 8 was previously determined to contain 5 x 10^{9} DHBV genomes/mL and 50 µg/mL of DHBsAg (Jilbert *et al.* 1996; Triyatni *et al.* 1998; Meier *et al.* 2003).

14-day-old ducks were infected intravenously (IV) via the jugular vein with 100 µl of Pool 8 serum containing 5 x 10^8 DHBV genomes. Infection of 14-day-old ducks with this dose of DHBV results in rapid spread of DHBV infection in the liver of infected ducks and invariably causes chronic DHBV infection (Jilbert *et al.* 1998; Foster *et al.* 2005; Miller *et al.* 2006a; Miller *et al.* 2006b; Miller *et al.* 2008).

2.3.2 Intraperitoneal (IP) administration of therapeutic agents to ducks

IP injection was performed after disinfecting the mid ventral abdominal area with 70% ethanol. Therapeutic agents such as REP 2006/REP 2031/REP 2055/normal saline (NS)/tenofovir (TFV)/emtricitabine (FTC) were delivered to the peritoneal cavity using a 23G needle during the first four weeks of treatment followed by a 21G needle during the remainder of treatment. 23G needle was chosen as that is strong enough to penetrate the skin efficiently and blunt enough to not to pierce intra-abdominal organs in ducks that are <4 weeks of age. As the ducks grow the thickness of the skin and abdominal fat increase, hence, to avoid the bending of needles while still allowing efficient penetration of the skin, 21G needle were used in ducks that are >4 weeks of age.

Peritoneal penetration was carefully done by lifting up the skin below the tip of the sternum. This method of IP delivery of therapeutic agents was used to avoid accidental piercing of the liver or any other internal organs in the vicinity. Immediately after piercing, the syringe was drawn back on to check for blood to provide an indication of a possible piercing of blood vessels or the liver. This was done to further minimise the chance of accidental piercing of internal organs (Fleet *et al.* 1990).

2.3.3 Venipuncture

Blood samples were collected IV through a venipuncture of the ducks' jugular veins. Blood samples were collected during experiments or at autopsy in ethylenediamine tetra acetic acid (EDTA) impregnated 9 mL blood collection tubes (VACUETTER, Grenier Bio-One GmbH, Austria) for a complete blood evaluation (CBE). Blood samples were also collected in blood collection tubes using a clot activator (SARSTEDT, Australia) to separate serum for liver enzyme analysis, detection of DHBsAg by enzyme-linked immunosorbent assay (ELISA) and DHBV DNA extraction for quantitative polymerase chain reaction (qPCR).

2.3.4 Liver biopsy

Ducks were surgically biopsied using a standard liver biopsy protocol. Ducks were fasted by withdrawing feed the night before the surgery to empty the gut to induce and maintain anaesthesia without complications. Primary anaesthesia was performed for ~10 min using a rubber anaesthetic balloon and a cup using 5% isofluorane (Veterinary Companies Pty Ltd, Australia):O_2, (5%:400 mL/min). The duck was then either intubated with an endotracheal tube (size 2.0 oral; Mallinckrodt Medical, Ireland) and the anaesthetic delivery was transferred to the tube or directly anaesthetised via the balloon. A scavenger tube was set up to collect any excess anaesthetic.

During surgery the breathing of the duck was monitored and the concentration of isofluorane was lowered to 5.0 to 3.5% depending on the situation. Feathers were trimmed from the lower right abdomen and the site was disinfected with Betadine® (povidone iodine). The surgical site was sprayed with bupivacaine hydrocholoride (0.5%, 0.25-0.5 mL) to provide analgesia and local anaesthesia to synergise the effect of operative and post-operative analgesia. A 1-2 cm incision was made in the mid ventral abdominal wall (linea alba) to locate the liver. A lower section of the right lobe of the liver was clamped using long forceps and 200-300 mg of liver tissue was removed. Blood loss from the cut liver surface was minimal. The wound was sutured with Vicryl (4-0, FS-2, ETHICON Inc. USA), anaesthetic was removed and the duck would stand and was fully alert within 5-10 min. Ducks were also injected intramuscularly (IM) with 5 mg/kg of ketoprofen (Troy Laboratories Pty Ltd, Australia) into the leg muscle at the end of surgery as a post-operative analgesia.

2.3.5 Autopsy

Ducks were first given an overdose of barbiturate anaesthetic (Lethobarb®, Virbac; 100 mg/kg) to the jugular vein to perform a humane euthanasia. Blood was collected through cardiac puncture for CBE, liver enzyme tests, ELISA and DHBV DNA extraction for qPCR. Furthermore, 25-50 mLs of blood was removed from the heart to minimise the congestion of blood in the internal organs with the view of getting a clear histological picture of liver and other organs for haematoxylin and eosin (H & E) and immuno-staining. The abdomen was then washed with 70% ethanol. The abdominal and chest walls (both external and internal

abdominal and chest muscle layers) were dissected to expose the liver, pancreas, spleen and kidneys. These tissues were collected and fixed as described below in Section 2.3.6.

2.3.6 Tissue samples for future analysis

Biopsy samples of liver and autopsy samples of liver, pancreas, spleen and kidneys were placed in green tissue cassettes containing the biopsy pads (BP 10803; Blue Sky Scientific, South Australia) and fixed in 10% neutral buffered formalin in PBS prior to wax embedding and sectioning. Tissue samples were also collected in biopsy pads housed in orange cassettes and fixed in EAA (3:1 v/v) for 30 min and then transferred to 70% cold ethanol prior to wax embedding and sectioning.

Both formalin and EAA fixed specimens were taken to the Tissue Pathology Division at the IMVS for processing for wax embedding and sectioning. Then at least one section from formalin fixed tissues of each duck was stained using H & E for histological analysis. Sections from EAA fixed tissues were subjected to immuno staining for DHBsAg (Section 2.8.2).

Pieces of liver tissue collected from each duck were also placed in screw capped tubes and snap frozen in liquid nitrogen prior to storage at -80^{o}C.

2.4 Preparation of antiviral APDPs

Sodium salts of the APDPs REP 2006, REP 2031 and REP 2055 were synthesised and supplied in a powdered form by REPLICor Inc, Canada. These compounds were dissolved in NS at a concentration of 10 mg/mL by heating at 65^{o}C for 15 min until the compound was fully dissolved. The solution was then cooled to RT and filtered through a 0.2 µm filter, aliquoted and stored at -20^{o}C in a non-cycling freezer to maintain the integrity of the compound. APDPs, REP 2006, REP 2031 or REP 2055 were administered IP to ducks using a 23G needle for ducks of <4 weeks of age and a 21G needle for ducks of >4 weeks of age on a 3- or 5-mL syringe depending on the dosage and the volume of the drug required for the application as described in Section 2.3.2.

2.5 Preparation of NAs

ETV was synthesised and supplied in a powdered form by Bristol-Myers Squibb, USA. An ETV solution of 1.0 mg/mL was prepared by adding 10 mg of ETV powder to 10 mL of distilled water followed by heating at 65^{o}C for 45 min until the compound was fully dissolved. ETV stock solutions were stored at 4^{o}C for up to 5 days. Prior to administration the ETV solution was allowed to reach RT. ETV was administered orally to ducks daily using a 2.0 cuffless oral/nasal tube attached to a 3- or 5-mL syringe depending on the dosage and volume of the drug required for the application.

TDF the prodrug of TFV was synthesised and supplied in a powdered form by Gilead Sciences Pty Ltd, USA. A TDF solution of 1.0 mg/mL was prepared by adding 10 mg of TDF powder to 10 mL of distilled water (DW) followed by adding a few drops of 0.25 mM NaOH solution until the compound was fully dissolved. The TDF solution was then administered orally to ducks using a 2.0 cuffless oral/nasal tube attached to a 3- or 5-mL syringe depending on the dosage and volume of the drug required for the application.

TFV, the active drug of TDF was synthesised and supplied in a powdered form by Gilead Sciences Pty Ltd, USA. A TFV solution of 1.0 mg/mL was prepared by adding 10 mg of TFV powder to 10 mL of PBS followed by adding a few drops of 1M NaOH until the compound was fully dissolved. Then the solution was filtered through a 2 µm filter, aliquoted and stored at -20^{o}C. The TFV solution was then administered IP to ducks using a 21G or 23G needle and 3- or 5-mL syringe depending on the dosage and volume of the drug required for the application.

FTC was also synthesised and supplied in a powdered form by Gilead Sciences, USA. A 50 mg/mL FTC solution was prepared by adding 5 g of FTC powder to 100 mL of PBS followed by vigorous shaking until the compound was fully dissolved. Then the solution was filtered through a 2 µm filter, aliquoted and stored at -20^{o}C. The ETC solution was then administered IP to ducks using a 23G or 21G needle and 5- or 10- mL syringe depending on the dosage and volume of the drug required for the application.

Pyrogen-free NS (Abbott Pharmaceuticals, USA) was supplied by the IMVS and administered IP to ducks in the control groups of all *in vivo* experiments. The volume of NS injected was the same volume as each drug used for each application.

2.6 CBE analysis

Blood samples collected for CBE (Section 2.3.5) were sent to the Division of Haematology of the Diagnostic Clinical Pathology unit at the IMVS for CBE with special reference to total red blood cell (RBC), white blood cell (WBC) and platelet counts. CBE was performed using Sysmex automated CBE system (Sysmex, model XE2100, Suppliers: Sysmex Cooperation 2000). During the latter part of the research (Chapter 7) the blood samples were analysed for CBE by the IDEXX International Veterinary Laboratory Service, South Australia.

2.7 Liver enzyme analysis

Sera were separated for liver enzyme analysis by centrifugation at 800g for 10 min at 20^{o} C. Sera (1 mL of serum from each duck) were sent to the Diagnostic Clinical Pathology Unit at IMVS for liver enzyme tests with special reference to liver transaminases, γ glutamyl transferase (GGT), alanine amino transferase (ALT) and aspartate transferase (AST). During the latter part of the research the serum samples were analysed by the IDEXX International Veterinary Laboratory Service, South Australia for liver transaminases, GGT, ALT and AST.

2.8 Serological Assays

ELISAs developed by (Miller *et al.* 2004) to assess the levels of serum DHBsAg, anti-DHBs and anti-DHBc antibodies were used in this Ph.D. research project with minor modifications whenever necessary.

2.8.1 Qualitative detection of DHBsAg by ELISA

Sera harvested from blood samples were tested to determine levels of DHBsAg by ELISA as previously described (Foster *et al.* 2003; Foster *et al.* 2005; Miller *et al.* 2006a; Miller *et al.* 2006b; Miller *et al.* 2008). Costar 3590 flat-bottomed 96-well microtitre plates (Corning Incorporated, USA) were coated in duplicate with 100 µl of test serum samples diluted 1/100 in PBS. Pool 8, high titre DHBsAg-positive control, (Section 2.3.1) and NDS (negative control) were coated in the similar manner. Wells in the last column were coated

with PBS only to determine the background absorbance. Microtitre plates were wrapped in plastic film (equivalent to a plate sealer) and incubated at 37^{o}C overnight (O/N).

Plates were washed in PBS with 0.05% Tween 20 (PBS-T) (Sigma Chemicals, USA) and then coated with 200 µl of 5% skim milk in 0.05% PBS-T (skim milk PBS-T) to block any non-specific binding sites. After washing in PBS-T, plates were then coated with 1/5000 primary anti-DHBV preS monoclonal antibodies, 1H.1 (Pugh *et al*. 1995) diluted in skim milk PBS-T. After washing in PBS-T, plates were coated with 1/4000 secondary horse radish peroxidase (HRP) conjugated sheep anti mouse polyclonal antibodies (GE Healthcare UK Limited, UK; Cat. NO: NA9310V, Batch NO: 342829) diluted in skim milk PBS-T. Microtitre plates were wrapped in plastic film and incubated at 37^{o}C for 1 h for primary and secondary antibody steps. All washes were done using the PBS-T or PBS.

Following the secondary antibody step, microtitre plates were washed 3 times in PBS without tween 20. The o-phenylenediamine dihydrochloride (OPD) HRP substrate was made using a Sigma-Fast^{TM} kit (Sigma-Aldrich, Germany) according to the manufacturer's instructions. OPD (100 µl) solution was added to each well. Plates were then incubated for 15 min in the dark. Fifty µl of 2.5 M H_2SO_4 was added to each well to stop the reaction. Optical density (OD) values were read using an automated plate reader (Spectra Max M2, Molecular Devices, Millennium Science, USA) at 490 nm. OD values were then transferred to Microsoft Excel 2007 for further analysis. Qualitative detection of DHBsAg by ELISA was used in experiments (see Chapters 4, 5 and 6).

2.8.2 Quantitative detection of DHBsAg by ELISA

Quantitative detection of DHBsAg using ELISA was undertaken in experiments (see Chapters 7 and 8).

Costar 3590 flat-bottomed 96-well microtitre plates (Corning Incorporated, USA) were coated in duplicate with 100 µl of test serum samples diluted 1/100 in PBS. Pool 8, high titre DHBsAg positive control containing 50 µg/mL DHBsAg was coated in duplicate with doubling dilutions from 1/500 to 1/64000 to establish a standard curve. Wells in the first column were coated with PBS only to determine the background absorbance. Microtitre plates were wrapped in plastic film and incubated at 37^{o}C O/N.

Plates were washed in PBS-T and then coated with 200 µl of skim milk PBS-T to block any non-specific binding sites. After washing in PBS-T, plates were then coated with 1/5000 primary anti-DHBV preS monoclonal antibodies, 1H.1 (Pugh *et al.* 1995) diluted in skim milk PBS-T. After washing in PBS-T, plates were coated with 1/4000 secondary HRP conjugated sheep anti-mouse polyclonal antibodies (GE Healthcare Limited, UK; Cat. NO: NA9310V, Batch NO: 342829) diluted in skim milk PBS-T. Microtitre plates were wrapped in plastic film and incubated at 37^{o}C for one h for primary and secondary antibody steps.

Plates were washed 3 times in PBS-T for all the previous steps and 3 times in PBS without tween 20 for the final step of prior to adding the HRP substrate using an automated plate washer (ELx405 Select, BioTek®,USA). The next step in OPD treatment was performed as described in Section 2.8.1.

OD values were read using an automated plate reader (Spectra Max M2, Molecular Devices, Millennium Science, USA) at 490 nm. OD values were then transferred to a Microsoft Excel 2007 and Graph Pad Prism 5.03. A standard curve was constructed and the DHBsAg concentration of test samples was then determined.

2.8.3 Detection of anti-DHBs antibodies by ELISA

Detection of anti-DHBs antibodies was performed using an antibody capture ELISA as described by Miller *et al.* (2004). Serum samples of ducks in experiments discussed in Chapter 7 were tested for anti-DHBs antibodies.

Costar 3590 flat-bottomed 96-well microtitre plates (Corning Incorporated, USA) were coated with 100 µl of 1/5000 anti-DHBV preS monoclonal antibodies, 1H.1 (Pugh *et al.* 1995) in 0.1 M $NaHCO_3$ (pH = 9.6). Plates were then incubated at 37^{o}C for 1 h followed by 4^{o}C O/N. The next morning, plates were washed in PBS-T and then coated with 200 µl of skim milk PBS-T to block any non-specific binding sites. Plates were washed in PBS-T and then coated with 100 µl of sucrose purified DHBsAg (1 ng/µl) (Miller *et al.* 2004) in 0.05% PBS-T and incubated at 37^{o}C for 1 h.

After the PBS-T wash, the plates were coated with 5-fold dilutions of individual test serum samples starting at a dilution of 1/500. Serum sample 262 (a positive control duck serum containing high titre anti-DHBs and anti-DHBc antibodies) and pre-bleed serum

sample 182 (a negative control for anti-DHBs and anti-DHBc antibodies) were coated in the similar manner. After the PBS-T wash to remove the unbound antibodies, plates were coated with 100 µl rabbit anti-duck IgY polyclonal antibodies (Bertram 1997) at a dilution of 1/4000 in skim milk PBS-T and incubated at 37^{o}C for 1 h. Plates were washed in PBS-T and then coated with 100 µl of HRP-conjugated goat anti-rabbit polyclonal antibodies (KPL Inc. Gaithersburg, MD, USA) at a dilution of 1/4000 in skim milk PBS-T and incubated at 37^{o}C for 1 h. All washes were performed using an automated plate washer (ELx405 Select, BioTek®, USA). The next step in OPD treatment was performed as described in Section 2.8.1.

OD values were read using an automated plate reader (Spectra Max M2, Molecular Devices, Millennium Science, USA) at 490 nm. OD values were then transported to Microsoft Excel 2007 and Graph Pad Prism 5.03. The anti-DHBs antibody titre in each test serum sample was defined as the highest serum dilution that resulted in an OD 490 nm of 0.4.

2.8.4 Detection of anti-DHBc antibodies by ELISA

Detection of anti-DHBc antibodies was performed using an antibody capture ELISA as described by Miller *et al.* (2004). Serum samples of ducks in experiments discussed in Chapter 7 were tested for anti-DHBc antibodies.

Costar 3590 flat-bottomed 96-well microtitre plates (Corning Incorporated, USA) were coated with 100 µl of purified recombinant DHBcAg (Jilbert *et al.* 1992) at a concentration of 1 µg/mL in PBS. Plates were then incubated at 37^{o}C for 1 h followed by 4^{o}C O/N. The next morning, plates were washed in PBS-T and then coated with 200 µl of skim milk PBS-T to block any non-specific binding sites.

After washing in PBS-T, plates were coated with 5-fold dilutions of individual serum samples starting at a dilution of 1/1000. Serum sample 262 (a positive control high titre anti-DHBs and anti-DHBc antibodies) and pre-bleed serum sample 182 (a negative control for anti-DHBs and anti-DHBc antibodies) were coated in a similar manner. The plates were then incubated with 100 µl rabbit anti-duck IgY (Bertram 1997) at a dilution of 1/4000 in 5% skim milk PBS-T and incubated at 37^{o}C for 1 h. Plates were washed in PBS-T and then coated with 100 µl of HRP-conjugated goat ant-rabbit antibodies (KPL Inc. Gaithersburg,

MD, USA) at a dilution of 1/4000 in skim milk PBS-T and incubated at 37^{o}C for 1 h. All washes were performed using an automated plate washer (ELx405 Select, BioTek®, USA). The next step of OPD treatment was performed as described in Section 2.8.1.

OD values were read using an automated plate reader (Spectra Max M2, Molecular Devices, Millennium Science, USA) at 490 nm. OD values were then transported to a Microsoft Excel 2007 and Graph Pad Prism 5.03. The anti-DHBcAg antibody titre in each test serum sample was defined as the highest serum dilution, which resulted in an OD 490 nm of 0.4.

2.9 Histology and immuno-staining of tissue sections

Histological analysis of liver, pancreas, spleen and kidneys was performed to evaluate the histological appearance of these tissues during the antiviral studies using APDPs REP 2006, REP 2031 and REP 2055 and NAs, ETV, TDF, TFV and FTC. Immuno-staining was done to enumerate the DHBsAg-positive hepatocytes in the liver of treated and untreated DHBV infected control ducks.

2.9.1 Histological studies

Formalin-fixed wax-embedded tissue sections for H & E staining were processed and stained at the Tissue Pathology Laboratories at the IMVS. H & E stained microscopic slides were examined for histological changes in the liver at Hepatitis Research Laboratory at the University of Adelaide.

2.9.2 Immuno-staining to detect DHBsAg

Wax-embedded EAA-fixed tissues were sectioned at 6 μm and adhered to glass microscopic slides. Immuno-staining was used to identify the DHBsAg-positive hepatocytes in biopsy and autopsy liver tissues as previously described (Miller *et al.* 2004; Foster *et al.* 2005; Miller *et al.* 2006a; Miller *et al.* 2006b; Miller *et al.* 2008; Miller *et al.* 2008). In brief, sections of wax-embedded tissue were de-waxed in xylene (MERCK, Australia) and rehydrated through 100% ethanol to PBS and then treated with 0.5% H_2O_2 (BDH Chemicals, Australia) in PBS to inactivate endogenous tissue peroxidases. The slides were then washed in PBS and the tissues on the slides were blocked with 1/30 normal sheep

serum (NSS) in PBS for 30 min at RT in a humid box to avoid non-specific binding thus make a clear differentiation between the DHBsAg-positive hepatocytes and the background field. After removing the NSS gently from the tissues without washing, the slides were then treated using a dilution of 1/500 primary anti-DHBV preS monoclonal antibodies, 1H.1 (Pugh *et al.* 1995) and secondary HRP-conjugated goat anti-mouse polyclonal antibodies (GE Healthcare Limited, UK; Cat. NO: NA9310V, Batch NO: 342829).

Bound HRP was visualised by treating slides with 0.05% diaminobenzidine tetrahydrochloride (DAB; Sigma Aldrich, Germany) and 0.04% H_2O_2 (BDH Chemicals, Australia) in PBS in the dark and then counter stained with haematoxylin. Slides were subjected to dehydration in 100% ethanol (MERCK, Australia) and then xylene (MERCK, Australia) treatment prior to mounting using DePex mounting medium (BDH Laboratory Supplies, UK; Cat. NO: 61254D).

Microscopic examination was carried out using an Olympus microscope with a digital imaging system at Hepatitis Research Laboratory at the University of Adelaide. DHBsAg-positive hepatocytes were examined as markers of DHBV infection in the liver. The hepatocyte cytoplasm that stained brown indicated the positive DHBsAg. Hepatocytes with brown cytoplasm were counted to calculate the percentage of infected hepatocytes. An eyepiece graticule (250 x 250 μm) aided the counting of grid fields to enumerate the total haematoxylin stained hepatocyte nuclei (containing ~100,000 hepatocytes) in each liver section. The minimum sensitivity of detection of DHBsAg-positive hepatocytes in liver biopsy and autopsy tissue was 0.001%. Counting of hepatocytes was performed under 20 X 1000 magnification and images were photographed using 40 X 1000 magnification.

2.10 Methods for extracting & detecting viral & cellular DNA

2.10.1 DNA extraction from serum for qPCR

Viral DNA (DHBV DNA) was isolated from 200 μl of serum by using an Invitrogen Charge Switch viral nucleic acid extraction kit (Cat. NO: CS11040) according to the manufacturer's instructions. DHBV DNA was eluted from the filter column with 50 μl of elution buffer and samples were stored at –20^{o}C until further analysis.

2.10.2 DNA extraction from liver for Southern blot hybridisation and qPCR

Frozen liver tissues collected as described in Section 2.3.6 were dissected aseptically using a sterile scalpel blade in a plastic petri dish and total DNA extractions were performed using 25 mg of frozen liver tissue to extract total cellular DNA using a QIAGEN DNeasy kit (Cat. NO: 69504) following the manufacturer's instructions. In brief, samples were lysed by proteinase K digestion followed by DNA binding to the DNeasy mini column, ethanol washing steps, and elution of DNA with 200 µl of elution buffer. DNA present in the final eluent was ethanol precipitated and re-dissolved in a volume of 30 µl of elution buffer for Southern blot hybridisation.

For the extraction of cellular DNA from liver tissues in experiments discussed in Chapter 7, a modification was introduced to the above protocol. In this, after proteinase K digestion of liver tissue, the lysate was incubated with 4 µl of RNase A (100 mg/mL, QIAGEN, Cat. NO: 19101) for 5 min at RT after a thorough mixing by vortexing. The RNase A treatment was performed to remove RNA because liver is a transcriptionally active tissue rich in RNA (QIAGEN, DNeasy Handbook July 2006: 28–30).

2.10.3 Detection of DHBV DNA by Southern blot hybridisation

The cellular and viral DNA in each sample was measured using a ND-1000 Spectrophotometer (Nanodrop, USA). 2 µg of DNA was mixed with 4 µl loading buffer (Appendix II) and 26 µl of water in a 1.5% agarose gel in TAE buffer (Appendix II) and electrophoresed overnight using a current of 0.01mA and 40V.

DHBV plasmid pBL 4.8 (Triyatni *et al.* 2001) digested using *Pvu I* and *EcoRI* (Figure 2.1) and run on a 1% agarose gel to yield bands of 3.027, 2.339 and 1.044 kb. Diluted *Pvu I* and *EcoRI* digested plasmid samples containing 10 and 100 pg were used as the standard. Southern blot hybridisation was performed using a standard laboratory protocol (Appendix III). Briefly, gel contents were transferred to a nitrocellulose membrane using our standard Southern blot hybridisation transfer procedure and hybridised using a ^{32}P-labelled genome length DHBV DNA probe (Triyatni *et al.* 2001); Genbank NO: AJ006350) labelled using a random primer kit (Roche Molecular Diagnostics, Cat. NO: 1585606). The Southern blot hybridisation mixture contained $>10^6$ cpm per mL of hybridisation mixture. The

nitrocellulose membrane was exposed to an X-ray film in a cassette at -80^{o}C for 12, 24 or 72 h and then developed using an automatic X-ray processing machine.

2.10.4 Detection of DHBV DNA in serum by qPCR

Serum DHBV DNA genomes were detected by qPCR using the ABI Prism 7000 or AB StepOnePlus^{TM} Real Time PCR machine (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems, Cat. NO: 4309155) and the primers shown in Table 2.1.

Standard curves for quantification were established by using the plasmid standard pBL DHBV 4.8 dimer in which the DHBV whole genome has been cloned into pBL as a head-to-tail dimer (pBL DHBV 4.8 X 2) (Figure 2.2) (Triyatni *et al.* 2001; Le Mire *et al.* 2005). A 5 µl aliquot of pBL 4.8 X 2 DHBV plasmid DNA was added to reaction tubes containing a total reaction volume of 20 µl (Table 2.3). pBL DHBV 4.8 X 2 plasmid stock was stored in aliquots at a concentration of 90 ng/µl at -20^{0}C. A 1:10 dilution of the 90 ng/µl stock gives 10^{10} copies and then a continuous 10-fold serial dilution until 10^{1} copies was made. For the standard curve, only 10^{8} to 10^{1} copies were used. A volume of 5 µl of each serial dilution was added to the PCR wells.

A 5 µl aliquot of template DNA was added to each 0.2 mL reaction tube containing a total reaction volume of 20 µl (Table 2.3). qPCR was performed with the following ABI Prism 7000 machine conditions using ABI prism 7000 SDS software, Version 1 or StepOnePlus^{TM} machine (Applied Biosystems). In both machines the same qPCR conditions were used.

The qPCR conditions and the standard curve were set up with an initial denaturation step at 50^{o}C for 2 min and then activation of polymerase at 95^{o}C for 10 min. The amplification conditions were 40 cycles or 15 sec at 95^{o}C and 1 min at 60^{o}C.

2.10.5 Detection of DHBV DNA in liver tissue by qPCR

Extraction of total cellular DNA was performed using a QIAGEN DNeasy kit (Cat. NO: 69504) as explained in Section 2.10.2. Total liver DNA was quantified with the assistance of a ND-1000 Spectrophotometer (Nanodrop, USA).

DHBV viral genomes were quantitated by qPCR using the ABI Prism 7000 machine and SYBR Green PCR Master Mix (Applied Biosystems, Cat. NO: 4309155) and the primers shown in Table 2.1. A 5 µl (150 ng) of aliquot of liver DNA was added to a total reaction of volume of 20 µl (Table 2.3).

A minor modification of *EcoRI* digestion was performed to the DHBV standards and individual liver DNA extracts used in the experiments in Chapter 7. Prior to using the pBL DHBV 4.8 X 2 (Figure 2.2) in the qPCR, the plasmid was digested with *EcoRI*. 5 µg of plasmid DNA was digested with 1 µl of *EcoRI* (20 units) and 10 µl *EcoRI* NE buffer at 37^{o}C for O/N. The digested stock was then stored in aliquots at a concentration of 90 ng/µl at -20^{o}C. A 1:10 dilution of the 90 ng/µl stock gives 10^{10} copies and then a continuous 10-fold serial dilution until 10^{1} copies was made. As in Section 2.10.4, for the standard curve, only 10^{8} to 10^{1} copies were used. A volume of 5 µl of each serial dilution was added to the PCR wells. After this 150 ng (1 µl) of normal duck liver DNA (NDL DNA) was added to each tube containing the DHBV DNA standards (10^{8}- 0^{1}) (Table 2.3).

Duck liver DNA extracted using the DNeasy kit including the *RNase* digestion step was also treated in the similar way as the standard above. Prior to use, ~2-5 µg of DNA was digested at a concentration higher than 150 ng/µl with *EcoRI* similar to the standard. After digesting, the DNA was nano-droped again and diluted to 30 ng/µl. 5 µl of DNA (30 ng/µl) was used in the qPCR reaction with a final concentration of 150 ng per well.

The qPCR was performed using an AB StepOnePlus Real Time PCR machine and SYBR green as the detector (Applied Biosystems, Cat. NO: 4309155) and the primers shown in Table 2.1. The qPCR conditions and the standard curve were set up with an initial denaturation step at 50^{o}C for 2 min and then activation of polymerase at 95^{o}C for 10 min. The amplification conditions were 40 cycles at 15 sec at 95^{o}C and 1 min at 60^{o}C.

2.10.6 Detection of cccDNA in liver tissue by qPCR

The same protocol discussed in Section 2.10.5 was used to quantitate the cccDNA except in that cccDNA specific primers, CC2 and MG1 (Table 2.2; Figure 2.3) were added instead of 390 and 666c. The constituents used to amplify the cccDNA are given in Table 2.3.

Table 2.1: PCR primers to amplify total DHBV DNA

Primers	Site	Product size (bp)	Sequence 5' to 3'
390	[a]390-410	276	[b]CAGATCTCCCTCGCCTAGGA
666c	[c]666-646		[d]ATTGCCTCATGCTGCATCAC

Table 2.2: PCR primers to amplify cccDNA of DHBV

Primers	Site	Product size (bp)	Sequence 5' to 3'
CC2	[a]2462-2481	56	[b]CCTGATTGGACGGCTCTTAC
MG1	[c]2618-2599		[d]AAAGGTACAGTCAAGGCTGA

[a] The nucleotide position of primer 390 (Table 2.1) or CC2 (Table 2.2) relative to the AusDHBV genome (Triyatni *et al*. 2001; AusDHBV Genbank NO: AJ006350);

[b] The sequence of primer 390 (Table 2.1) or the sequence of primer CC2 (Table 2.2);

[c] The nucleotide position of primer 666c (Table 2.1) or MG1 (Table 2.2) relative to the AusDHBV genome (Triyatni *et al*. 2001; AusDHBV Genbank NO: AJ006350);

[d] The sequence of primer 666c (Table 2.1) or the sequence of primer MG1 (Table 2.2).

Table 2.3: PCR mixture to detect total DHBV DNA or cccDNA in liver extracts

Components of 20 µl reaction	**Volume**
SYBR Green PCR Master Mix	10 µl
[a]Primer 390 or [b]CC2 (at 10 µM)	0.6 µl
[a]Primer 666c or [b]MG1 (at 10 µM)	0.6 µl
DW	3.8 µl
[c]pBL DHBV 4.8 X 2 OR [d]Serum extract OR [e]Liver DNA extract	5 µl

[a] Primers 390 and 666c are shown in Table 2.1 and Figure 2.3;

[b] Primers CC2 and MG1 are shown in Table 2.2 and Figure 2.3;

[c] pBL DHBV 4.8 X 2 (Figure 2.2) were prepared as described in Sections 2.10.4 and 2.10.5;

[d] Serum extracts were prepared as described in Section 2.10.5;

[e] Liver DNA extracts were prepared as described in Sections 2.10.5 and 2.10.6.

Appendix I

RESEARCHER ID: ..... PROJECT NO: .....

ANIMAL ID: .....

CLINICAL RECORD SHEET DUCKS

DATE	FOOD/WATER INTAKE	FAILURE TO GROOM	ABNORMAL MOVEMENT	DEPRESSION	TOTAL SCORE	OTHER COMMENTS

YES = 1; NO = 0; TOTAL SCORE OF 3 = THE ANIMAL NEEDS TO BE MONITORED 3 TIMES/DAY.

TOTAL SCORE OF MORE THAN 3 SEEK VETERINARY ADVICE.

IMVS 814/A

Appendix II: Buffers

DNA loading buffer (10X)

60% sucrose, 1% sarkosyl (Sigma Chemicals, Cat. NO: 61747), 1X TAE buffer, 0.1% bromophenol blue (Sigma Chemicals, Cat. NO: B0126), 0.1% xylene cyanol (Sigma Chemicals, Cat. NO: X4126) and DW to make a 10 mL solution. DNA loading buffer (10X) is stored as 500 µl at 4^{o}C.

50X TAE buffer (pH 7.5-7.8)

Tris, glacial acetic acid, EDTA and to make one litre of 50X TAE buffer.

0.05M NaOAc (pH 4.2)

Make 0.2M NaOAc by dissolving 16.4g/L NaOAc (MW 82.03) in DW, then adjusting pH to 4.2 with concentrated glacial acetic acid (~50 mL/L). Store at RT and do not autoclave.

Blot I (pH >8.0)

1.5M NaCL (MW 58.44; 88g/L), 0.5M NaOH (MW 40.0; 20g/L).

Blot II (pH 8.0)

1.0M NH_4OAc (MW 77.08; 77g/L), 0.02M NaOH (0.8g/L).

Appendix III: Laboratory protocol for Southern blot hybridisation

1. Prepare DNA samples from cellular and viral DNA extracted from liver samples as described in Section 2.10.2.
2. Prepare 1.5% horizontal agarose gel (~5-7 mm thick) in 1X TAE buffer.
3. Aliquot DNA samples (usually 2-6 µl) into eppendorf tubes, increase the volume by adding 16 µl TE8 to the eppendorf tubes with DNA samples, add ~2 µl of 10X TAE loading buffer to each sample, mix and load onto gel.
4. Run in samples at 20 mA until both bromophenol blue and xylene cyanol markers have entered the gel (~20 min). Flood gel with 1X TAE buffer and run at ~50-60 mA overnight or until the BPB dye has reached the end of the gel.
5. Remove gel from tank, stain with ~0.3 µg/mL ethidium bromide (50 µl 10 mg/mL Ethidium Bromide in 1.5L 1X TAE) for 30 min at RT. Photograph. Note that ideally all gel treatment steps should be performed with gentle shaking.
6. Treat gel in O.05M NaOAc pH 4.2 for 1 h at RT.
7. Treat gel in 0.05M NaOAc pH 4.2 for 1 h at 55^{o}C.
8. Treat gel in Blot I (recipe is given Appendix II) for <1 h at RT.
9. Treat gel in Blot II (recipe is given Appendix II) for >1 h at RT.
10. Transfer gel to nitrocellulose in Blot II: Place 3 sheets of Whatmann 3 MM (~25 x 25 cm) saturated with Blot II, on a clean, flat surface. Exclude all air bubbles from the saturated 3 MM using a clean pipette as you would use a rolling pin. Position gel face down on the centre of the 3 MM, overlay sequentially with: (i) Schleicher and Schuell BA85 nitrocellulose presoaked for 2-3 min in Blot II (if possible place the NC exactly and do not move once in contact with the gel); (ii) 3 sheets of 3 MM soaked in Blot II (again at this point exclude all air bubbles using a pipette); (iii) 2 dry blotting pads (BRL Cat. NO: 1058HY); (iv) 7-8 cm of absorbent towels; (v) a glass gel plate and (vi) ~100 g weight. Add an extra ~50 mL of Blot II to the original 3 MM sheets and cover the entire structure with Gladwrap to minimise evaporation. Transfer O/N at RT. The gel should have reduced in thickness to 1-2 mm.
11. Dismantle structure, carefully remove the NC and wash in 2X SSC twice for 5 min at RT. Bake between 2 sheets of 3 MM placed between 2 glass plates for 2 h at 80^{o}C in a vacuum oven.

12. Place the NC carefully into the glass hybridisation bottle also called roller bottle.
13. Pre-hybridise at 42^{o}C for 4 h in ~ 5-10 mL prehybridisation solution (recipe is given below) in rotating hybridisation oven.
14. Decant pre-hybridisation fluid and replace with ~5-10 mL of hybridisation solution (recipe is given below) containing 3-6 x 10^6 cpm/mL of ^{32}P-labelled specific DNA. Hybridise at 42^{o}C O/N.
15. Remove hybridisation mix and save into 50 mL blue capped tube, store probe at -20^{o}C to reuse (heat at 68^{o}C for >60 min before reuse).
16. Wash NC (in hybridisation bottle in rotating oven) with 2X SSC twice for 5 min at RT.
17. Wash NC in 2X SSC, 0.1% SDS twice for 30 min at 55^{o}C in hybridisation oven.
18. Perform high stringency wash in 0.1X SSC, 0.1% SDS twice for 30 min at 55^{o}C. Blot dry, wrap in Gladwrap and expose to Kodak X-Omat film in a cassette at -80^{0}C. Develop 10-100 pg plasmid DNA samples included in the gel should be visible after 30 min of exposure.

Prehybridisation Mix	**Stock**	**43 mL**
50% deionised formamide	100% at -20^{o}C	20 mL
500 µg/mL denatured carrier DNA	10 mg/mL at -20^{o}C	2 mL
0.1% PVP Ficoll	2% filtered at 4^{o}C	2 mL
1% glycine	10% filtered at 4^{o}C	4 mL
50 mM Na_2HPO_4, NaH_2PO_4, pH 6.5	1M at RT	2 mL
1 mg/mL BSA	40 mg/mL at -20^{o}C	1 mL
5X SSC	20X SSC	10 mL

Heat the prehybridisation mix to 37^{o}C prior to disperse the carrier DNA.

Hybridisation Mix	**Stock**	**40 mL**
50% deionised formamide	100% at -20°C	20 mL
100 µg/mL carrier DNA	10 mg/mL at -20°C	0.4 mL
0.02% PVP Ficoll	2% at 4°C	0.4 mL
25 mM Na_2HPO_4, NaH_2PO_4, pH 6.5	1M at RT	1 mL
0.2 mg/mL BSA	40 mg/mL at -20°C	0.2 mL
5X SSC	20X SSC	10 mL
10% dextran sulphate	50% in DW at 4°C	8 mL

Heat the hybridisation mix to 37°C prior to adding the probe to disperse the base-treated DNA. To use in hybridisation add 5-10 x 10^6 cpm DNA probe per mL. Before adding to hybridisation mix, denature probes at 100°C for 5 min and then chill on ice.

This protocol was developed and optimised based on methods described by (Thomas *et al.* 1976; Bodkin and Knudson 1985). Since then this protocol has been used at the Hepatitis Research Laboratory, University of Adelaide for more than 15 years.

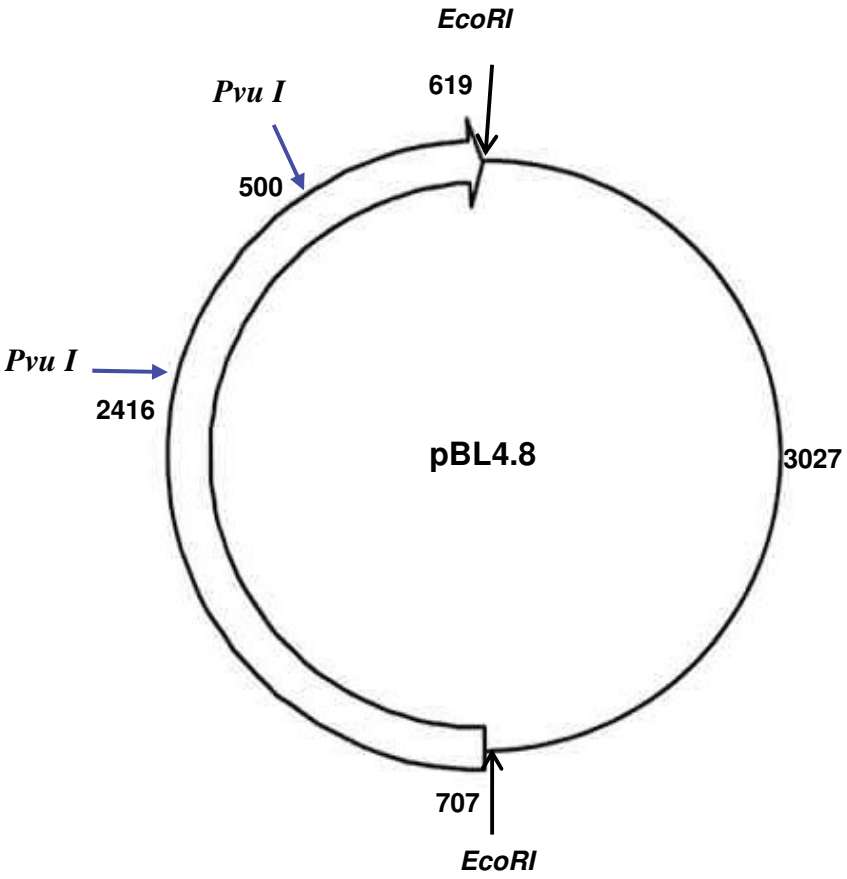


Figure 2.1: DHBV plasmid pBL 4.8 (Triyatni *et al.* 2001) showing the restriction sites for *Pvu I* (blue arrows) and *EcoRI* (black arrows).

Digestion of pBL 4.8 with *Pvu I* and *EcoRI* yielded bands of 3.027, 2.339 and 1.044 kb.

Pvu I and *EcoRI* digested plasmid samples containing 10 and 100 pg were used as the standard for Southern blot hybridisation as described in Section 2.10.3 and shown in Figures 4.3, 5.5, 5.6, 6.3 and 6.5 .

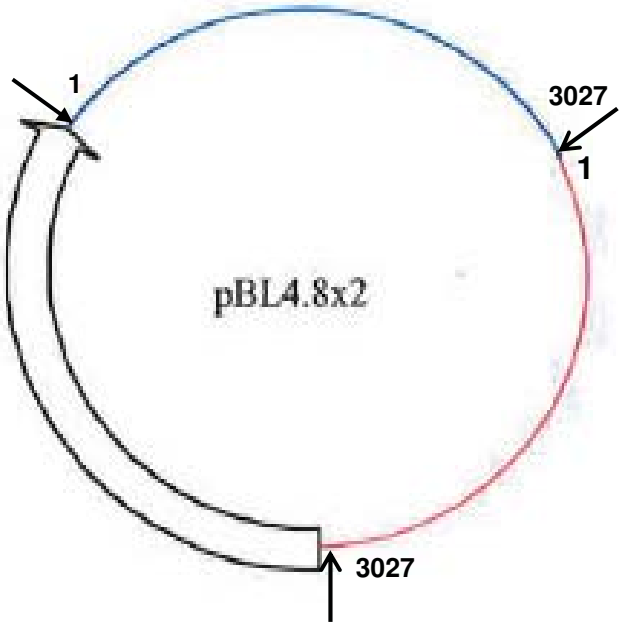


Figure 2.2: DHBV plasmid pBL 4.8X2 (Triyatni *et al.* 2001; AusDHBV Genbank NO:AJ006350) showing the *EcoRI* (black arrows) restriction sites.

Diluted *EcoRI* digested plasmid samples (a volume of 5 µl from each serial dilution of 10^8 to 10^1 copies) were used as the standard for qPCR as described in Section 2.10.4.

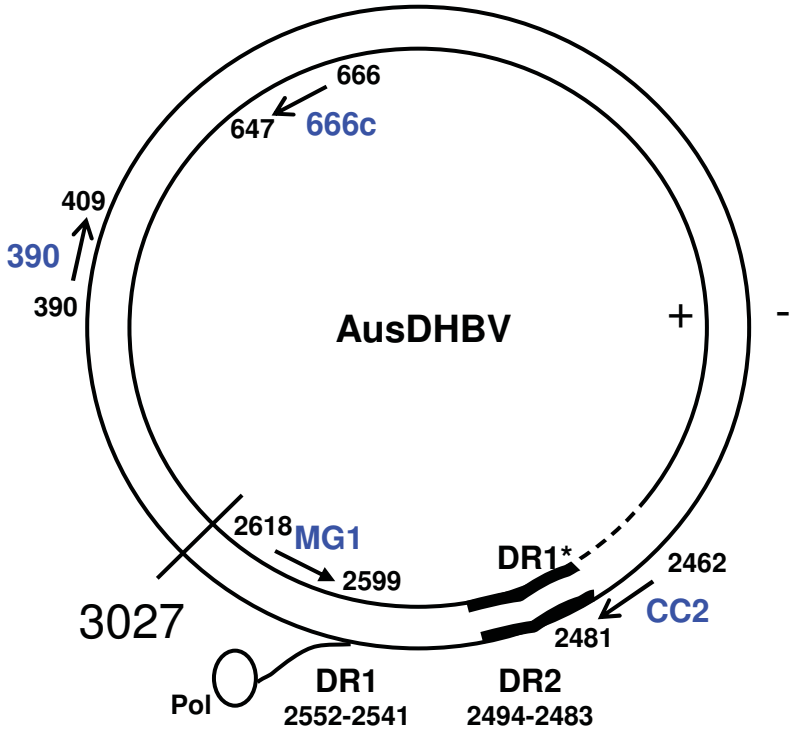
Figure 2.3: Schematic diagram of the DHBV genome depicting the positions of the primers used to amplify total DHBV DNA and cccDNA by qPCR.

Panel A: The AusDHBV rcDNA genome showing the positions of the primers 390 and 666c that were used to amplify total DHBV DNA and primers MG1 and CC2 that were used to selectively amplify cccDNA.

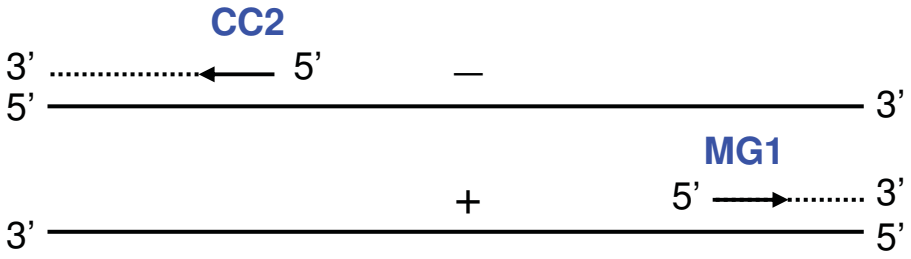
Panel B: Denaturation of rcDNA during the PCR reaction gives rise to two SSDNA molecules. cccDNA primers MG1 and CC2 flank the cohesive overlap regions of the rcDNA and are positioned divergent from each other and do not amplify rcDNA.

Panel C: Annealing of the first product of PCR may occur due to the homology of the DR region. Extension of the annealed first round product forms an amplicon identical to that produced from a cccDNA template. This may compromise the specificity of the assay.

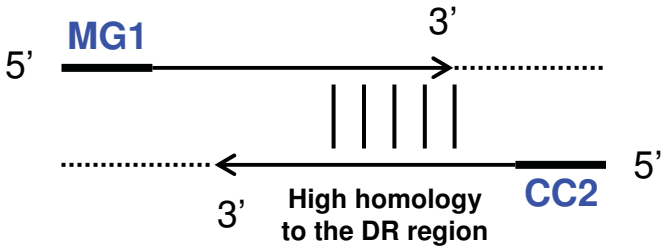
A


666
647
666c
409
390
390
AusDHBV
+
-
2618
MG1
2599
DR1*
2462
CC2
2481
3027
Pol
DR1
2552-2541
DR2
2494-2483

B


CC2
3'
5'
5'
3'
MG1
+
5'
3'
3'
5'

C


MG1
5'
3'
5'
3'
High homology
to the DR region
CC2

Chapter 3: Antiviral efficacy of APDPs against DHBV infection *in vitro*

3.1 Introduction

HBV infection is one of the most common viral infections in the world accounting for 2 billion individuals infected at some point in their life time. Of the 2 billion people who have HBV, over 400 million suffer from chronic HBV infection which manifests as: firstly, persistent HBV DNA and HBsAg in serum; and secondly, continuous production of HBV antigens and HBV DNA in the liver (Fattovich *et al.* 2008). The consequences of chronic HBV infection include cirrhosis, HCC, liver failure and these end stage disease outcomes cause over a million deaths each year (McMahon 2005; Fattovich *et al.* 2008; Lee *et al.* 2008).

As explained in Chapter 1, poor response rates to existing treatment options, adverse reactions to the commonly used immunomodulators, IFN-α and pegIFN-α and resistance to NAs justify the need for research into new therapeutic agents for HBV (Younger *et al.* 2004; Marcellin *et al.* 2005). For this reason the current project examines the antiviral effect of the novel therapeutic APDPs, REP 2006, REP 2031 and REP 2086 in DHBV-PDH system.

The APDPs REP 2006 and REP 2031 can be produced as sodium salts (SS) under the conditions of good manufacturing practice (GMP) grade or as ammonium salts under non-GMP conditions. REP 2006 and 2031 are PS-ONs, which have been designed to interact with the function of a broad spectrum of viral fusion proteins. They interfere with conserved alpha-helical domains through amphipathicity and size dependent, but sequence independent mechanisms (Kocisko *et al.* 2006; Vaillant *et al.* 2006; Guzman *et al.* 2007; Bernstein *et al.* 2008). Phosphorothioation of APDPs increases their hydrophobicity and makes them amphipathicity. Moreover, phosphorothioation increases their stability and therefore APDPs become more resistant to degradation by nucleases (Figure 3.1). The amphipathic nature of APDPs plays a major role in their antiviral activity. For example, longer APDPs ≥30 nucleotide lengths have increased amphipathicity and are more potent in blocking the hydrophobic interactions involved in the gp41 six-helix bundle formation and inhibiting the

HIV-1-mediated cell-cell fusion than shorter APDPs with <30 nucleotides (Vaillant *et al.* 2006). This novel antiviral mechanism of action of long APDPs has future applications for therapy against HIV-1 infection and other enveloped viruses including herpes viruses (Vaillant *et al.* 2006; Guzman *et al.* 2007; Bernstein *et al.* 2008).

The effective broad spectrum antiviral activity of REP 2006 and REP 2031 against many members of the herpesviridae family including, HSV-1 and HSV-2, CMV, VZV, Epstein-Barr virus (EBV) and human herpes viruses 6A and 6B (HHV 6A and 6B), has been demonstrated in *in vitro* infection models at concentrations of 0.02 to 15 μM (Bernstein *et al.* 2008). Furthermore, the murine microbicide model of genital HSV-2 was also used to evaluate the activity of REP 2006 and REP 2031 *in vivo*. REP 2006 (275 mg/mL) protected 75% of animals from infection and disease when administered 5 or 30 min prior to vaginal challenge. When an acid stable REP 2031 was used, 75% of mice were protected when treated with 240 mg/mL for 5 to 30 min prior to infection, while a lower dose (100 mg/mL) protected 100% of the mice (Bernstein *et al.* 2008). The reason for the 75% protection with 240 mg/mL given at 5 min prior to infection is not understood. The authors, however, suggest that this may be due to an unoptimised formulation as the 240 mg/mL dose was more effective than the 100 mg/mL dose with a longer interval from treatment to infection.

In contrast to HIV and herpes viruses, the entry mechanism used by HBV is not well understood but HBV is thought to enter through RME (Treichel *et al.* 1997; Jilbert *et al.* 2008). In RME, a late fusion event occurs between the virus envelope and the target cell membrane. We hypothesise that this late fusion event can be blocked by APDPs that are amphipathic in nature.

Preliminary data by REPLICor Inc. suggested that APDPs can prevent DHBV infection *in vitro* when PDH and DHBV were pre-treated prior to infection of the PDH (Vaillant *et al.* unpublished). The broad spectrum antiviral activity of APDPs against various families of enveloped viruses (Vaillant *et al.* 2006; Guzman *et al.* 2007; Bernstein *et al.* 2008) suggest that APDPs may have significant antiviral activity against DHBV infection.

The current study was undertaken to investigate the antiviral efficacy of REP 2006 and REP 2031 (Figure 3.1) to understand their cytotoxicity, the roles of their structural chemistries in eliciting the antiviral activity and their ability to colocalise to cell membranes and in the cytoplasm of treated cells. This is a descriptive study in which mean percentage of

DHBsAg-positive PDH was used as the marker to assess the level of DHBV infection. Furthermore, the anti-DHBV activity of APDPs or non-APDPs was measured by comparing the mean percentage of DHBsAg-positive PDH with untreated control (UC) PDH.

Optimum working concentrations of REP 2006 and REP 2031 were determined for their usability in the larger *in vitro* experiment (Section 3.4). The specificity of anti-DHBV activity of REP 2006 and REP 2031 treatment were determined by comparing them to the non-APDP control, REP 2086. REP 2086 is non-amphipathic due to a structural modification in which 2' O-linked methyl group on the ribose has replaced the phosphorothioation, an essential structural aspect necessary for the candidate APDPs' antiviral activity. On the other hand, 2' O-linked methyl group on the ribose modification is used to stabilize the oligonucleotide in the absence of the phosphorothioation, hence, the non-APDP, REP 2086 has comparable stability to the candidate APDPs (Vaillant *et al.* 2006). This allows the testing to occur of the role of amphipathicity on the antiviral activity of APDPs independent of stability. The structures of the APDPs, REP 2006, REP 2031 and REP 2055 and non-APDP, REP 2086 are shown in Figures 3.1 and 3.2 and Table 3.1.

REP 2107, a phosphorothioated molecule with a 2' O-linked methyl group on the ribose; REP 2117, an abasic phosphorothioated molecule; and REP 2118, a propane phosphorothioate; were also tested to elucidate the role of the amphipathic nature of APDPs. REP 2117 and REP 2118 are important non-amphipathic controls (Table 3.1) with lowered hydrophilic activity in contrast to REP 2086, which has lowered hydrophobic activity (Figure 3.2).

The effect of polymer size (10-80mers) on the antiviral activity of APDPs (Figure 3.4) and the uptake of CY3-labelled APDPs by PDH (Figure 3.7) were also investigated.

3.2 Experimental details

Details of ethical approval to conduct the experiments on animals, duck supply, preparation and culture of PDH, source of DHBV and detailed method for the cytotoxicity assay are provided in Sections 2.1-2.3.

3.2.1 Infection of PDH and multiplicity of infection (MOI)

Based on previous work, 1 mL of Leibovitz's-15 growth medium containing 2 x 10^5 PDH/mL was used to seed each well of a 24-well tissue culture plate to achieve a confluent monolayer of PDH. In initial experiments, a range of MOI of 25, 50, 100, 250 and 500 DHBV DNA genomes per PDH were tested. It was found that a MOI of 100 or 250 DHBV DNA genomes per PDH were able to produce DHBV infection of >50% of the PDH in the confluent monolayer. Thus in all subsequent experiments PDH were infected at a MOI of 100 or 250 DHBV DNA genomes per PDH using DHBV-positive pooled serum.

The pooled serum contains 5 x 10^9 copies of DHBV DNA/mL (Jilbert *et al.* 1996; Meier *et al.* 2003). Four and 10 µl of pooled DHBV-positive serum were added to each well to achieve a MOI of 100 and 250 DHBV DNA genomes per PDH, respectively.

3.2.2 Candidate drugs and their concentrations for *in vitro* studies

The APDPs REP 2006, REP 2031 and REP 2086 used in the preliminary (Section 3.3) and the larger *in vitro* experiments (Section 3.4) were non-GMP ammonium salts due to limited supply of non-GMP ammonium salts from the manufacturer. Each APDP was dissolved in Leibovitz's-15 growth medium containing 10% FCS and other constituents (as explained in Section 2.2) at concentrations of 0.001-10 µM for the preliminary and at concentrations of 0.01-10 µM for the larger *in vitro* experiments.

The GMP SS of APDPs, REP 2006, REP 2031 and REP 2055, dissolved in Leibovitz's-15 growth medium containing 10% FCS, were also tested for their *in vitro* antiviral activity against DHBV infection in a small scale experiment. Testing the antiviral activity of SS of APDPs became necessary as these were used in subsequent *in vivo* studies for two major reasons; 1. non-GMP ammonium salts are toxic for *in vivo* application; 2. non-GMP ammonium salts cannot be synthesized in large quantities for *in vivo* application due to high cost.

3.2.3 Cytotoxicity assay

The cytotoxicity assay (XTT-PMS assay) was performed to test the viability of PDH in a 96-well plate on the 7th day of APDP treatment. The detailed procedure for the XTT-PMS assay is described in Section 2.2.1.

Briefly, on the day 7th day of the APDP treatment, 50 µl of 1 mg/mL of XTT and PMS were added to wells containing the PDH. PDH were harvested on the 7th day to assess the cytotoxicity based on previous studies in which cells were tested at this time point (Shaw *et al.* 1994; Zhao *et al.* 2002). The plate was incubated for 4 h and after a thorough mixing in the plate reader, the absorbance at 450 nm was measured. XTT is reduced to an orange colour product. XTT-formazan in the presence of healthy cells as mitochondrial dehydrogenase present in viable cells metabolically reduces the XTT to a water-soluble XTT-formazan product (Zhao *et al.* 2002).

3.2.4 Detection of cytoplasmic DHBsAg

DHBsAg was detected using anti-DHBV pre-S monoclonal antibodies, 1H.1 (Pugh *et al*. 1995) and secondary goat anti-mouse polyclonal antibodies labelled with Alexa fluor 488 (Molecular probes, Australia). Both primary and secondary antibodies were diluted 1/200 in 1/50 NDS in PBS and the fluorescence was detected at a wave length of 488 nm as described in Section 2.2.2.

As also explained later in the experiments in Chapters 4 and 5, it was not possible to use PCR or qPCR assays to quantify the DHBV DNA as a means to test the antiviral activity of APDPs. Cations such as Mg^{2+} used in the PCR amplification can be partially or fully inhibited by APDPs (Vaillant and Liang unpublished) at high concentration in APDP-treated PDH cultures. Thus any possible interference in the PCR amplification process can give rise to false negative results for DHBV DNA which can be interpreted as an antiviral effect of APDPs.

3.2.4.1 Calculation of the mean percentage of DHBsAg-positive PDH

The mean total for PDH in 1 camera field was calculated by averaging the sum of PDH in 5 camera fields. The mean percentage of DHBsAg-positive PDH was derived by averaging the sum of DHBsAg-positive PDH in 5 camera fields. Means of the total PDH and the DHBsAg-positive PDH in 1 camera field were used to calculate the percentage of DHBsAg-positive PDH.

For example, in the preliminary experiment (Table 3.5):

Untreated wells of 2 at 100 MOI had 33 DHBsAg-positive PDH in 5 camera fields

Mean DHBsAg-positive PDH in 1 camera field = 33/5 = 6.6

Mean total hepatocytes in 1 camera field = 400/5 = 80

Mean percentage of DHBsAg-positive PDH = 6.6/80 x 100

= 8.25%

3.2.5 Experimental design for the preliminary *in vitro* experiment

PDH were seeded at a density of 2 x 10^5 PDH/well in 24-well tissue culture plates as described in Section 2.2. Prior to infection with DHBV, both the PDH and the DHBV inoculum were pre-treated with APDPs. Pre-treatment of DHBV with REP 2006, REP 2031 and REP 2086 for 1 h at 37^{o}C and pre-treatment of PDH with REP 2006, REP 2031 and REP 2086 for 45 min to 1 h at RT were performed under sterile conditions.

The PDH were then infected with DHBV in the presence of REP 2006 or REP 2031 or REP 2086 at concentrations of 0.001-10 μM. Fresh REP 2006 or REP 2031 or REP 2086 were added during each media change every second day. The PDH were harvested on day 7 p.i. using EAA fixation followed by IFA as described in Section 2.2.2.

3.2.6 Experimental design for the larger *in vitro* experiment

PDH were seeded at a density of 2 x 10^5 PDH/well in 24-well tissue culture plates using a similar method to that used in the preliminary experiment. Fresh medium containing appropriate concentrations of REP 2006 and REP 2031 was added to the PDH test Groups in duplicates for each concentration when necessary. An untreated control (UC) Group of DHBV infected PDH was designed to compare the antiviral efficacy of APDPs. PDH were infected at MOI of 250 DHBV DNA genomes per PDH.

3.2.6.1 Assessing the antiviral activity of APDPs using pre- and post-treatments

Test Group A: This test Group was designed to test whether pre-treatment of DHBV with APDPs (REP 2006 and REP 2031) had the ability to inhibit the DHBV infection of PDH.

The DHBV inoculum was treated with REP 2006 and REP 2031 for 1 h at 37^{o}C prior to infection. No REP 2006 or REP 2031 was added after the first treatment.

Test Group B: Was designed to test whether pre-treatment of PDH with APDPs (REP 2006 and REP 2031) for 1 h at 37^{o}C had the ability to inhibit the DHBV infection. PDH were pre-treated with REP 2006 and REP 2031 for 1 h and then infected with DHBV.

Rationale for test Groups A and B: In Groups A and B, the drug treatment with REP 2006 or REP 2031 was performed only once and then the system was maintained drug free until the harvest but was nourished by providing fresh medium to fully evaluate the entry inhibitory effect of APDPs.

Test Group C: Was designed to test whether APDPs have the ability to elicit antiviral activity against an established DHBV infection. PDH were infected with DHBV 12 h prior to starting the treatment with REP 2006 and REP 2031.

Rationale for test Group C: In Group C, fresh REP 2006 and REP 2031 were added every second day during media change. This test group was designed to determine if continuous administration of APDPs can inhibit the release of progeny virus particles and prevent the spread of DHBV infection.

3.2.6.2 Testing the ability of APDPs to block cell-to-cell spread of DHBV

Test Group D: Determined whether APDPs were able to block cell-to-cell spread of DHBV infection when compared with suramin treatment.

Suramin was used to treat PDH 12 h after infection to allow the first round of DHBV infection to take place and then block the cell-to-cell spread resulting from subsequent rounds of infection (Funk *et al.* 2004; Okabe *et al.* 2006). Suramin was dissolved in DMSO and filtered through a 2 µm filter and then aliquots were stored at -20^{o}C. A working solution of 100 µg/mL of suramin was prepared in the culture medium and added to the system 12 h after DHBV infection. Fresh suramin (100 µg/mL) was added every second day during media change.

Rationale for test Group D: In Group D, suramin was used to treat DHBV-infected PDH 12 h after infection. Suramin inhibits the first round of DHBV infection due to the lectin like binding (Petcu *et al.* 1988). Hence, the results of suramin treatment can be directly

compared to Groups A and B which were designed to test the true antiviral effect of APDPs with a single drug administration either to the DHBV inoculum (Group A) or to the PDH (Group B).

3.2.6.3 Comparing the antiviral efficacy of APDPs to that of ETV

Test Group E: To compare the antiviral efficacy of APDPs to ETV. The PDH were treated with ETV 1 h prior to DHBV infection and fresh ETV was added every second day during media change.

ETV, a guanosine analogue developed by BMS inhibits HBV (Julander *et al.* 2003; Mazzucco *et al.* 2008; Suzuki *et al.* 2008) and DHBV infections (Marion *et al.* 2002; Foster *et al.* 2003; Foster *et al.* 2005). ETV was dissolved in deionised water and heated at 55^{o}C for 10 min and then the solution was allowed to cool and was filtered through a 2 μm filter. A 1 mM stock was used to prepare the appropriate concentrations of ETV in culture medium.

3.2.7 Testing the effect of APDPs on the intensity of fluorescence signals

This experiment was designed to test whether the addition of REP 2006, REP 2031 and REP 2086 had affected the intensity of fluorescence signals. REP 2006, REP 2031 and REP 2086 were added at a concentration of 10 μM 24 h prior to harvest.

3.2.8 Testing the antiviral efficacy of SS of APDPs *in vitro*

To test the antiviral efficacy of SS of the APDPs REP 2006, REP 2031 and REP 2055 against DHBV infection *in vitro*.

Both DHBV and PDH were pre-treated with the SS of REP 2006, REP 2031 and REP 2055 for 1 h at 37^{o}C prior to DHBV infection. Fresh SS of REP 2006, REP 2031 and REP 2055 were added every second day during media change at concentrations of 0.001-10 μM.

3.2.9 Hydrophobicity/amphipathicity *vs* anti-DHBV activity

Ammonium salts of REP 2006, REP 2086, REP 2107, REP 2117 and REP 2118 with different hydrophobicities were tested for signs of antiviral activity against DHBV infection (Table 3.1; Figure 3.5).

PDH were seeded at a density of 2 x 10^5 PDH/well in 24-well tissue culture plates. Fresh medium containing REP 2006, REP 2086, REP 2107, REP 2117 and REP 2118 at a concentration of 10 μM were added to the PDH at the time of DHBV infection. Fresh medium containing 10 μM of these REP compounds was changed every second day. PDH were fixed on day 7 p.i. and tested for the presence of DHBsAg-positive PDH using IFA as described in Section 2.2.2.

3.2.10 Polymer size *vs* anti-DHBV activity

Ammonium salts of REP polymers were synthesized from 10 to 80 nucleotides in length (10, 20, 30, 40, 60 and 80mers) and tested to evaluate the role of polymer size on anti-DHBV activity (Fugure 3.6).

PDH were seeded at a density of 2 x 10^5 PDH/well in 24-well tissue culture plates. Fresh medium containing the appropriate concentrations (0.001-1μM) of different size REP polymers was added to PDH at the time of DHBV infection. Fresh medium with appropriate REP polymers were changed every second day. PDH were fixed and then tested for the presence of DHBsAg-positive PDH using IFA as described in Section 2.2.2.

3.2.11 Colocalisation of CY3-labelled REP 2006 and REP 2086 in PDH

CY3-labelled ammonium salts of REP 2006 and REP 2086 were used to study the colocalisation of these compounds in uninfected and DHBV-infected PDH. PDH were treated 12 h p.i. with 250 nM CY3-labelled REP 2006 or REP 2086. Fresh medium containing 250 nM of CY3-labelled REP 2006 or REP 2086 was changed every second day.

PDH were fixed on days 1, 4 and 7 p.i. using 3 different fixation methods: 1% formalin in PBS; EAA (Section 2.2.2); and 2% para formaldehyde (PFA) in PBS to increase the chance of detecting the colocalisation of CY3-labelled REP 2006 and REP 2086.

For formalin fixation, after removing the tissue culture medium PDH were washed in cold PBS prior to adding 1% formalin in PBS. PDH in formalin were incubated for 30 min at RT. PDH were washed in cold PBS twice prior to perform IFA for DHBsAg detection (Section 2.2.2).

EAA fixation of PDH was performed using procedures discussed in Section 2.2.2 followed by DHBsAg detection by IFA (Section 2.2.2).

For PFA fixation, after removing the tissue culture medium PDH were washed in cold PBS prior to adding 2% PFA in PBS. PDH in PFA were incubated for 15 min at RT. PDH were washed in PBS at RT twice prior to performing the IFA for DHBsAg detection (Section 2.2.2).

After IF staining PDH were examined to test for the presence of cytoplasmic DHBsAg and colocalisation of CY3-labelled REP 2006 and REP 2086, using a confocal microscope at the Confocal Unit of the IMVS.

3.3 Results: Preliminary *in vitro* experiment

The aims of the preliminary *in vitro* experiment were to test the following: REP 2006, REP 2031 and REP 2086 for cytotoxicity in PDH; to determine the optimum working concentration of REP 2006 and REP 2031 for the larger *in vitro* experiment (Section 3.4); and to test the specificity of the anti-DHBV activity of REP 2006 and REP 2031 using the non-APDP, REP 2086 as a control.

3.3.1 Cytotoxicity assay

The cytotoxicity assay showed that REP 2031 and REP 2086 were not highly cytotoxic to PDH *in vitro*. However, the REP 2006-treated PDH had mean OD values that were 3 times higher than that of untreated-PDH indicating some degree of cytotoxicity in REP 2006-treated PDH (Table 3.4). No morphological changes were observed in cellular architecture on daily microscopic examination of each of the REP 2006-, REP 2031- and REP 2086-treated PDH (data not shown).

Orange colour was observed in all wells despite the difference in the colour intensity among different wells. XTT salt was reduced to an orange colour product, XTT-formazan. This reaction occurs if there are healthy cells in the wells. Although the original seeding density of the 96-well tissue culture plate was 2×10^4 PDH per well, the possible reason for the difference in colour intensity among different wells may be due to a variation in the number of PDH attached to the well surface. Any unattached PDH would be removed when medium was changed each day, and this may explain the difference in OD values under similar experimental conditions (Tables 3.2, 3.3 and 3.4).

3.3.2 Optimum working concentration of REP 2006 and REP 2031

Treatment of PDH with REP 2006 and REP 2031 reduced the mean percentage of DHBsAg-positive PDH in a dose-dependent manner at concentrations between 0.001-10 μM. This dose dependent anti-DHBV activity of REP 2006 and REP 2031 was observed at both 100 and 250 MOI (Table 3.5).

The mean percentage of DHBsAg-positive hepatocytes decreased when the concentration of REP 2006 and REP 2031 increased except in the case of REP 2031 treatment at a MOI of 100 DHBV DNA genomes per PDH. Treatment with REP 0.01 μM 2031 inhibited DHBV infection resulting in DHBsAg in 5% PDH while REP 0.001 μM 2031 restricted infection to 1.75% of PDH (Table 3.5). The inhibition of DHBV infection as determined by mean percentage of DHBsAg-positive hepatocytes between these 2 concentrations was not dose dependent and the reason for this discrepancy was not clear.

In contrast, the mean percentage of DHBsAg-positive PDH in cultures infected with both 100 and 250 MOI was not affected by an increase in concentration of REP 2086 (Table 3.5). REP 2086 is a non-APDP and does not elicit antiviral activity against DHBV and was used as a control to test the importance of structural chemistry of APDPs in eliciting the antiviral activity as explained in Sections 3.1, 3.5.1 and 3.6 (Figures 3.1 and 3.2; Table 3.1).

3.4 Results: Larger *in vitro* experiment

3.4.1 Testing the antiviral efficacy of REP 2006 & REP 2031 (Ammonium salts)

REP 2006 demonstrated higher anti-DHBV activity than REP 2031 in all three PDH treatment Groups A, B and C (Table 3.6; Figures 3.3a to 3.3f). REP 2031 showed very low anti-DHBV activity against established DHBV infection as shown in the results (Table 3.6; Figure 3.3f).

3.4.1.1 Antiviral activity of REP 2006 against DHBV infection

When DHBV inoculum was pre-treated with REP 2006 prior to infecting the PDH (Group A-3 to A-6), DHBV infection was inhibited to ≤0.62% DHBsAg-positive PDH between the

concentration of 0.01-10 μM. In contrast, the wells with untreated PDH had a mean percentage of 12.18 DHBsAg-positive PDH (Table 3.6; Figure 3.3a).

When PDH were pre-treated with REP 2006 1 h prior to DHBV infection (Group B-3 to B-6), DHBV infection was inhibited to undetectable levels of DHBsAg-positive PDH at all concentrations of REP 2006. In comparison, the untreated wells had a mean of 10% DHBsAg-positive PDH (Table 3.6; Figure 3.3b).

In treatment Group C-3 to C-6, REP 2006 treatment was applied 12 h after DHBV infection. In this treatment, DHBV infection was inhibited to ≤3.43% DHBsAg-positive PDH between the concentration of 0.01-10 μM. Conversely, the untreated wells had a mean percentage of 11.56 DHBsAg-positive PDH (Table 3.6; Figure 3.3c).

3.4.1.2 Antiviral activity of REP 2031 against DHBV infection

Pre-treatment of DHBV with REP 2031 (Groups A-III to A-VI), inhibited the DHBV infection to 6.56% DHBsAg-positive PDH between the concentration of 0.01-10 μM whereas the untreated wells had a mean percentage of 8.43 DHBsAg-positive PDH (Table 3.6; Figure 3.3d).

Pre-treatment of PDH with REP 2031 (Group B-III to B-VI), inhibited the DHBV infection to ≤5.31% DHBsAg-positive PDH between the concentration of 0.01-10 μM, whereas the untreated PDH had a mean percentage of 13.43% DHBsAg-positive PDH (Table 3.6; Figure 3.3e).

Post-treatment of DHBV-infected PDH with REP 2031 12 h after virus inoculation (Groups C-III to C-VI), failed to inhibit the DHBV infection when compared with the mean percentage of untreated PDH. The mean percentage of DHBsAg-positive PDH remained 18.75 to 52.18% between the concentrations of 0.01-10 μM. The untreated PDH cultures, however, had a mean percentage of 25.31 DHBsAg-positive PDH. This result indicated that REP 2031 demonstrated very low or no antiviral effect against established DHBV infection *in vitro* (Table 3.6; Figure 3.3f).

3.4.1.3 Comparing the DHBV inhibitory activity of suramin and APDPs (Group D)

Suramin was also tested against DHBV infection by treating the PDH with suramin from 12 h post-DHBV infection. It is well known that suramin blocks the virus entry and cell-to-cell

spread of DHBV but not the intracellular virus replication (Offensperger *et al.* 1993; Funk *et al.* 2004; Okabe *et al.* 2006). Hence, the effect of suramin treatment can be compared to the potential entry inhibitory effect of REP 2006 and REP 2031 *in vitro*.

As the first round of DHBV infection was not blocked during suramin treatment, suramin-treated PDH had a mean percentage of 17.18% DHBsAg-positive PDH, whereas untreated PDH had a mean of 24.68% DHBsAg-positive PDH indicating that the suramin has prevented the cell-to-cell spread of DHBV (Table 3.6). Pre-treatment of DHBV or PDH with either REP 2006 or REP 2031 (PDH Groups A and B) reduced the mean DHBsAg-positive PDH to less than the mean percentage of suramin treated PDH (17.18%). This indicated that REP 2006 and REP 2031 had a higher efficacy in preventing the first round of DHBV infection than suramin (Table 3.6).

Furthermore, REP 2006 was effective against established DHBV infection by decreasing the DHBsAg-positive PDH to ≤3.5% in all the concentrations tested (Table 3.6; Figure 3.3c). However, when DHBV-infected PDH were treated 12 h p.i., REP 2031 treatment was unable to elicit an effective anti-viral activity against already existing DHBV infection (Table 3.6; Figure 3.3f).

3.4.1.4 Comparing the DHBV inhibitory activity of ETV and APDPs (Group E)

As expected ETV showed high anti-DHBV activity when the treatment began 24 h prior to DHBV infection (Table 3.4; Group H).

Measurable anti-DHBV activity of ETV pre-treatment 24 h prior to DHBV infection was similar to that of pre-treatment with REP 2006 1 h p.i. (Table 3.6).

3.4.1.5 The effect of APDP treatment on fluorescence signals

APDPs were added to the PDH containing wells 24 h prior to PDH harvest to test whether APDPs have the ability to interfere with immunological detection of DHBsAg. REP 2006, REP 2031 have been shown to interact with glycosylated viral glycoproteins (Vaillant *et al.* 2006). In this respect, addition of REP 2006 and REP 2031 prior to PDH harvest ensured that these compounds were present intact inside cells at high concentration after fixation. If

there was a strong interference with antibody interaction it would be impossible to visualise the immunoreactivity resulting in fluorescence signals.

The Alexa fluor 488 fluorescence signal for DHBsAg had lower intensity in some REP 2006, REP 2031 and REP 2086-treated wells than the fluorescence signals observed in untreated PDH. This observation may be due to mild interference of REP compounds with the fluorescence signal.

Although adding REP 2006, REP 2031 and REP 2086, 24 h before harvest reduced the intensity of the Alexa fluor 488 fluorescence signal for detection of DHBsAg, it did not markedly interfere the detection of DHBsAg-positive PDH (Figure 3.4). Thus the presence of the compounds did have a mild inhibitory effect on immunoreactivity in these experiments, however, they did not prevent the detection and quantification of infected cells.

3.4.1.6 Antiviral activity of SS of REP 2006, REP 2031 and REP 2055 against DHBV infection

As the SS of REP 2006, REP 2031 and REP 2055 were used to treat ducks in experiments (discussed in subsequent chapters), the *in vitro* antiviral activity of SS of REP 2006, REP 2031 and REP 2055 were tested.

When SS of REP 2006 pre-treated DHBV was used to infect PDH, DHBV infection was inhibited to ND to 6.25% DHBsAg-positive PDH. In contrast, the wells with untreated PDH had a mean of 11.88% DHBsAg-positive PDH (Table 3.7).

When SS of REP 2031 pre-treated DHBV was used to infect PDH, the DHBsAg-positive PDH was reduced to ND to 3.75%, whereas the wells with untreated PDH had a mean of 8.13% DHBsAg-positive PDH (Table 3.7).

When SS of REP 2055 pre-treated DHBV was used to infect PDH, the DHBsAg-positive PDH was reduced to ND to 2.2%, whereas the wells with untreated PDH had a mean of 13.15% DHBsAg-positive PDH (Table 3.7).

In summary, SS of REP 2006 and REP 2031 showed similar anti-DHBV activity *in vitro* to that of the ammonium salts of REP 2006 and REP 2031 used in the preliminary and larger *in vitro* experiments.

3.4.1.7 Comparing the activity of ammonium and SS of REP 2006

It should be noted that there was an apparent discrepancy in the activity of ammonium salts of REP 2006 and SS of REP 2006 between the experiments summarized in Table 3.6 and Table 3.7. The experiment (Table 3.7) in which the SS of REP 2006-treated DHBV did not fully inhibit the DHBV infection to an undetectable level. The reason for this discrepancy is not clear but it may be due to different salts used in these experiments producing an experiment to experiment variation.

3.5 Results: Investigating the chemistry of antiviral activity

3.5.1 Hydrophobicity *vs* anti-DHBV activity

The anti-DHBV activity increases in the presence of optimal hydrophobic or amphipathic structural chemistry in the DNA polymer and this was tested by detecting the presence of DHBsAg-positive PDH in cultures treated with polymers with different degrees of hydrophobicity at the same time of DHBV infection.

The percentage of DHBsAg-positive PDH was higher in untreated-, REP 2086-, REP 2117- and REP 2118-treated PDH cultures than REP 2006- and REP 2107-treated PDH (Figure 3.5).

3.5.2 Polymer size *vs* anti-DHBV activity

The effect of polymer size on anti-DHBV activity was tested using polymers that are similar to REP 2006 in chemistry (Figure 1.1; Table 3.1) but of different lengths that ranged from 10-80mers. Each different length polymer was tested at 1 μM concentration and was used to treat PDH at the same time of DHBV infection.

The percentage of DHBsAg-positive PDH was higher in the 10-30mer treated PDH cultures than the 40-80mer treated PDH cultures (Figure 3.6). The anti-DHBV activity increased with polymer size and reached its maximum when the polymer size was 40 (as in REP 2006, REP 2031 and REP 2055). The same antiviral effect was observed with polymers of 50, 60 and 80 bases (Figure 3.6).

3.5.3 Colocalisation of CY3-labelled REP 2006 and REP 2086 in PDH

CY3-labelled REP 2006 and REP 2086 were added to uninfected PDH 24 h after seeding with the fresh culture medium. CY3-labelled REP 2006 and REP 2086 were added 12 h p.i. to DHBV-infected PDH to test the ability of REP 2006 and REP 2086 to colocalise on the cell surface and in the cytoplasm of the PDH.

CY3-labelled 2006 was taken up more effectively by both uninfected and DHBV-infected PDH than CY3-labelled 2086 when DHBV inoculum was pre-treated 1 h prior to infection or DHBV infected PDH were treated 12 h post-DHBV infection. CY3-labelled 2006 showed a more diffuse distribution in the cytoplasm than CY3-labelled 2086 (Figures 3.7a and 3.7c). Nuclear colocalisation was absent in CY3-labelled REP 2006 treated PDH (Figures 3.7a and 3.7b) while nuclear colocalisation was present in REP 2086-treated PDH (Figures 3.7c and 3.7d). Nuclear distribution of REP compounds has not been demonstrated in previous studies (Vaillant *et al.* unpublished) and it appears to be an artefact as some PDH had no nuclear colocalisation of REP 2086 (Figure 3.7d Panel B lower bottom white arrows).

CY3-labelled 2006 inhibited DHBV infection (Figures 3.7a and 3.7b) better than non-APDP, REP 2086 (Figures 3.7c and 3.7d) when added 1 h prior to or 12 h p.i. Based on this qualitative data it appears to be in agreement with the findings of the semi-quantitative data from the preliminary experiment (Table 3.5).

As shown in Figures 3.7a and 3.7b Panel A, 1% formalin fixation assisted the detection of DHBsAg-positive PDH in CY3-labelled 2006 treated PDH while PDH fixed by other 2 fixatives did not have DHBsAg-positive PDH. This may be due to the anti-DHBV activity of PDH in CY3-labelled 2006 or a fixation effect. In this respect, use of 3 different fixatives increased the chance of detecting the colocalisation of CY3-labelled 2006 in the DHBV-infected PDH (Figures 3.7a and 3.7b, Panel A). In contrast, PDH fixed by all 3 fixatives assisted the detection of DHBsAg-positive PDH in CY3-labelled 2086 treated cells (Figures 3.7c and 3.7d) except in 2% PFA fixed PDH in one occasion (Figure 3.7d Panel C). Detection of DHBsAg-positive PDH in CY3-labelled 2086 treated cells was expected as non-APDP, REP 2086 does not inhibit DHBV infection to a level similar to that of REP 2006 (Tables 3.5 and 3.6).

3.6 Discussion

Data presented here shows that APDPs REP 2006, REP 2031 and REP 2086 were not cytotoxic to cultured PDH between the concentrations of 0.001-10 μM. Regular daily microscopic examination of the REP 2006-, REP 2031- and REP 2086-treated PDH cultures showed that the APDPs did not obviously change the normal physiology of the PDH, which would have resulted in morphological changes.

APDPs REP 2006 and REP 2031 demonstrated excellent anti-DHBV activity against DHBV infection in cultured PDH according to the preliminary *in vitro* experiment (Table 3.5). In that the DHBV and PDH were pre-treated with REP 2006, REP 2031 and REP 2086 prior to infection with addition of fresh APDPs during each media change. This experiment also showed the specificity of REP 2006 and REP 2031 in eliciting the anti-DHBV activity compared with the anti-DHBV activity of the non-APDP, REP 2086. The latter does not have the phosphorothioation modification; instead it has a structural modification with 2' O-linked methyl group on the ribose. This modification does not enhance the amphipathicity which is an essential property of APDPs to elicit their antiviral activity (Kocisko *et al.* 2006; Vaillant *et al.* 2006; Guzman *et al.* 2007; Bernstein *et al.* 2008; Matsumura *et al.* 2009). Hence REP 2086 was unable to inhibit DHBV infection whereas its phosphorothioated counterparts, REP 2006 and REP 2031, inhibited DHBV infection between the concentrations of 0.001 and 10 μM (Table 3.5).

Data from the larger *in vitro* experiment with 3 different PDH treatment Groups (A, B and C) showed that REP 2006 had higher anti-DHBV activity than REP 2031 (Table 3.6; Figures 3.3a to 3.3f). This finding indicates that REP 2006 was able to elicit a better antiviral effect in all the treatment conditions than REP 2031. The latter elicited very low/no activity against an established DHBV infection (Table 3.6; Figure 3.3f). This may be due to structural alteration in REP 2031 resulting in a conformational change at this treatment condition. Conversely, REP 2031 being a poly C compound may form a quadruplex structure under low pH conditions (Manzini *et al.* 1994; Huertas and Azorin 1996; Kanehara *et al.* 1997; Pataskar *et al.* 2001; Kanaori *et al.* 2004) that make the compound therapeutically inactive. Hence, it was unable to elicit anti-DHBV activity when used to treat PDH 12 h post-DHBV infection.

Moreover, when polymers with structural modifications to elicit different degrees of amphipathicity were tested against DHBV infection *in vitro*, only REP 2006 and REP 2107 were able to inhibit DHBV infection. REP 2086, REP 2117 and REP 2118 had minimal amphipathic properties and were not able to inhibit DHBV infection to a similar extent to that of REP 2006 and REP 2107 when treated at the same time of DHBV infection (Figure 3.5). REP 2086 is an example of a non-amphipathic polymer that is predominantly hydrophilic while REP 2117 and REP 2118 are predominantly hydrophobic. The latter two are non-amphipathic due to lowered hydrophilic property. Hence REP 2086, REP 2117 and REP 2118 showed very minimal anti-DHBV activity when compared with REP 2006. REP 2107 combines phosphorothioation with 2' ribose O-methylation and serves as a control for the inhibitory effects of the 2' ribose O-methylation on antiviral activity in the presence of phosphorothioation. This experiment clearly showed that amphipathicity is essential to the mechanism of anti-viral activity of APDPs.

The APDPs used in this study were 40mers, REP 2006 has a completely degenerate nucleotide sequence (random AGTC), REP 2031 is a poly C compound and REP 2055 has AC bases alternatively in its sequence. It can be hypothesised that the DHBV interaction with hepatocyte membrane during the early events of DHBV infection such as fusion and entry or DHBV release from infected hepatocytes or both involves a large amphipathic domain of ≥40 nucleotide in length. APDPs have also been shown to inhibit a broad range of enveloped viruses including different members of herpesviridae family and HIV. These findings collectively show the structural conservation of amphipathic alpha helical domains in type 1 viral glycoproteins. Conversely, all 3 polymers demonstrated anti-DHBV activity, suggesting that the antiviral activity was sequence independent. This suggests that APDPs act through a non-anti-sense mechanism that involves structurally conserved amphipathic domains in the virus and host target cell membrane interaction of many enveloped viruses.

In addition, treatment of DHBV-infected PDH with different size polymers starting from 10mer to 80mer showed that anti-DHBV activity increased from 10mer to 40mer and at this point the DHBV infection was completely inhibited with no detectable DHBsAg-positive PDH. From 40 to 80mers, the anti-DHBV activity stayed at its maximum (Figure 3.6), reinforcing the fact that a large amphipathic domain of ≥40 nucleotide length has a major role in the DHBV entry or release or both.

Furthermore, experiments with CY3-labelled 2006 and 2086 showed that REP 2006 was readily taken up by both uninfected and DHBV-infected PDH more effectively than REP 2086 (Figures 3.7a to 3.7d). This finding shows that amphipathicity of REP 2006 is important for it to accumulate at the site of action whereas its non-amphipathic counterpart REP 2086 showed less diffuse colocalisation in the exterior and inside the uninfected and DHBV-infected PDH (Figures 3.7a to 3.7d). These experiments suggest that the amphipathicity and polymer size are important characteristics that determine the antiviral activity of APDPs through a sequence independent mechanism.

In conclusion, the larger *in vitro* experiment has emphasized that REP 2006 was more effective as an anti-DHBV agent than REP 2031 when pre-treatment was applied to either DHBV or PDH as a single treatment at concentrations between 0.01-10 μM (Table 3.6). This finding agrees with observations found in the preliminary *in vitro* experiment in which both DHBV and PDH were treated with APDPs prior to the infection (Table 3.5). REP 2006 was effective against established DHBV infection whereas REP 2031 showed very low or no antiviral effect against established DHBV infection (Table 3.6; Figures 3.3c and 3.3f). SS of REP 2006, REP 2031 and REP 2055 were able to elicit antiviral activity against DHBV infection, which was similar to their respective ammonium salts (Table 3.7). An *in vivo* study using 14-day-old ducks was undertaken to further investigate the anti viral activity of SS of REP 2006 and REP 2031 against DHBV infection (Chapter 4).

Table 3.1: The chemical and antiviral properties of REP 2006, REP 2031, REP 2055, REP 2086, REP 2107, REP 2117 and REP 2118

Compound	Nucleotide/s of R Group	Chemical and antiviral properties
REP 2006	**[a]AGTC**	A 40mer, phosphorothioated molecule with enhanced amphipathicity and stability. Showed anti-DHBV activity.
REP 2031	**[b]C**	A 40mer, phosphorothioated molecule with enhanced amphipathicity and stability. Showed partial anti-DHBV activity.
REP 2055	**[c]AC**	A 40mer, phosphorothioated molecule with enhanced amphipathicity and stability. Showed anti-DHBV activity.
REP 2086	**[a]AGTC**	A 40mer, with 2' O-linked methyl group on the ribose and enhanced stability. Did not show anti-DHBV activity.
REP 2107	**[a]AGTC**	A 40mer, phosphorothioated molecule with 2' O-linked methyl group on the ribose and enhanced stability. Showed anti-DHBV activity.
REP 2117	**[d]Abasic**	A 40mer abasic phosphorothioated molecule with lowered hydrophilicity and enhanced stability. Did not show anti-DHBV activity.
REP 2118	**[d]Abasic**	A 40mer propane phosphorothioate with lowered hydrophilicity and enhanced stability. Did not show anti-DHBV activity.

[a] R = AGTC in random order (degenerate sequence) ;

[b] R = Poly C sequence;

[c] R = AC in alternate sequence;

[d] R = No base present.

Table 3.2: Experimental layout to test the cytotoxicity of REP 2006, REP 2031 and REP 2086

	REP 2006 Conc. (μM)				REP 2031 Conc. (μM)				REP 2086 Conc. (μM)			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	10	10	10	10	10	10	10	10	10	10	10	10
B	10	10	10	10	10	10	10	10	10	10	10	10
C	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
D	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
E	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
F	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
G	0	0	0	0	0	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0

REP 2006, REP 2031 and REP 2086 were tested in octruplicates for APDP concentration of 10 μM and untreated controls and in quadruplicates for the rest; A & B: 10 μM of REP 2006, REP 2031 and REP 2086; C: 1 μM of REP 2006, REP 2031 and REP 2086; D: 0.1 μM of REP 2006, REP 2031 and REP 2086; E: 0.01 μM of REP 2006, REP 2031and REP 2086; F: 0.001 μM of REP 2006, REP 2031 and REP 2086; G & H: Untreated PDH.

Table 3.3: Results of a cytotoxicity assay to measure the effect of REP 2006, REP 2031 & REP 2086 treatment to PDH

	1	2	3	4	5	6	7	8	9	10	11	12
	REP 2006 (OD Values)				**REP 2031 (OD Values)**				**REP 2086 (OD Values)**			
A	2.026	0.3718	0.3914	0.3301	0.5212	0.4176	0.5584	0.5831	1.2795	0.9585	1.5286	1.2318
B	0.6453	0.3602	0.3819	0.3535	0.4204	0.3821	0.6185	0.6476	1.1917	0.7499	0.9653	1.3269
C	0.4832	0.3666	0.3448	0.4004	0.4047	0.5489	0.4041	0.4325	1.0243	0.49	0.7524	1.3704
D	0.5529	0.3884	0.4065	0.4178	0.4032	0.4444	0.4068	0.4921	0.7259	0.373	0.6	1.2666
E	0.5705	0.4908	0.443	0.4755	0.3898	0.5273	0.4503	0.7064	0.8492	0.3886	0.4602	1.5553
F	0.4924	0.6744	0.4102	0.3664	0.4067	0.5683	0.395	0.6702	0.8451	0.7031	0.8269	2.2774
	Untreated control for REP 2006				**Untreated control for REP 2031**				**Untreated control for REP 2086**			
G	2.2008	0.4459	0.4945	0.3807	0.4111	0.587	0.4025	0.4761	1.2263	0.5149	1.2	1.6085
H	2.2216	0.4566	0.7938	0.4341	0.3928	0.6346	0.3321	0.5642	0.9477	0.3842	0.3434	1.9687

Optical density (OD) values at 450 nm are given in octruplicates for the APDP concentration of 10 μM and untreated controls and in quadruplicates for the rest; A & B: OD values at 10 μM of REP 2006, REP 2031 and REP 2086; C: OD values at 1 μM of REP 2006, REP 2031 and REP 2086; D: OD values at 0.1 μM of REP 2006, REP 2031 and REP 2086; E: OD values at 0.01 μM of REP 2006, REP 2031and REP 2086; F: OD values at 0.001 μM of REP 2006, REP 2031 and REP 2086; G & H: Untreated PDH.

Table 3.4: Results of a cytotoxicity assay to measure the effect of treatment with REP 2006, REP 2031 & REP 2086

APDP conc. (μM)[a]	Mean OD value ± SD		
	REP 2006	**REP 2031**	**REP 2086**
10	1.189[b]	0.619[b]	1.402[b]
1	0.460[c]	0.516[c]	1.286[c]
0.1	0.517[d]	0.478[d]	1.121[d]
0.01	0.549[e]	0.656[e]	1.348[e]
0.001	0.622[f]	0.643[f]	1.376[f]
Untreated PDH	1.730[g]	0.617[g]	1.615[g]

[a] APDP concentrations are given in μM;

[b] Mean OD value of well containing PDH treated with REP 2006, REP 2031 and REP 2086 at a concentration of 10 μM in octruplicates (Mean OD value ± SD);

Mean OD value of well containing PDH treated with REP 2006, REP 2031 and REP 2086 at concentrations of 1[c], 0.1[d] , 0.01[e] and 0.001[f] μM in quadruplicates (Mean OD value ± SD);

[g] Mean OD value of well containing untreated PDH in octruplicates (Mean OD value ± SD).

Table 3.5: Preliminary *in vitro* experiment: The antiviral effect of APDPs REP 2006 and REP 2031 against DHBV infection in comparison with non-APDP REP 2086

Compounds and MOI[b]	Mean percentage of DHBsAg-positive PDH					
	Concentration of APDPs (μM)[c]					
	10	**1**	**0.1**	**0.01**	**0.001**	**UC[d]**
REP 2006 and 100[b]	ND[e]	0.5	1	1	5.25	8.25
REP 2006 and 250[b]	0.75	1	1.3	1.5	3	30.75
REP 2031 and 100[b]	ND	ND	1.5	5	1.75	42.25
REP 2031 and 250[b]	0.25	1.5	1.25	2	8.5	16
REP 2086 and 100[b]	7.5	5.5	2.25	2.75	2.75	11.75
REP 2086 and 250[b]	10.25	3.75	5.25	7.75	3.75	7

[a] Amphipathic DNA polymers REP 2006 and REP 2031 and non-amphipathic DNA polymer REP 2086;

[b] The Multiplicity of infection (MOI) used to infect each well of PDH;

[c] Concentrations of APDP/non-APDP are given in μM;

[d] (UC)-Untreated control;

[e] No DHBsAg-positive PDH were detected;

DHBsAg-positive PDH were detected by IFA (Section 2.2.2).

Table 3.6: Larger *in vitro* experiment: The antiviral effect of APDPs REP 2006 and REP 2031 against DHBV infection

Compounds tested	Exp. Group	Treatment	Conc. (µM)[a]	%DHBsAg-positive PDH
REP 2006	A-2	Untreated control (UC)[c]	0	**12.18**
	A-3[b]	DHBV inoculum was pre-treated with REP 2006	0.01	0.62
	A-4[b]		0.1	ND
	A-5[b]		1	ND
	A-6[b]		10	0.31
REP 2006	B-2	Untreated control (UC)[c]	0	**10**
	B-3[d]	PDH were pre-treated with REP 2006 for 1 h prior to infection	0.01	ND
	B-4[d]		0.1	ND
	B-5[d]		1	ND
	B-6[d]		10	ND
REP 2006	C-2	Untreated control (UC)[c]	0	**11.56**
	C-3[e]	REP 2006 treatment of PDH 12 h p.i.	0.01	3.43
	C-4[e]		0.1	2.81
	C-5[e]		1	1.88
	C-6[e]		10	2.51
REP 2031	A-II	Untreated control (UC)[c]	0	**8.43**
	A-III[b]	DHBV inoculum was pre-treated with REP 2031	0.01	2.5
	A-IV[b]		0.1	3.12
	A-V[b]		1	6.56
	A-VI[b]		10	5.62
REP 2031	B-II	Untreated control (UC)[c]	0	**13.43**
	B-III[d]	PDH were pre-treated with REP 2031 for 1 h prior to infection	0.01	1.56
	B-IV[d]		0.1	0.31
	B-V[d]		1	0.44
	B-VI[d]		10	5.31
REP 2031	C-II	Untreated control (UC)[c]	0	**25.31**
	C-III[e]	REP 2031 treatment of PDH 12 h p.i.	0.01	52.18
	C-IV[e]		0.1	39.06
	C-V[e]		1	26.87
	C-VI[e]		10	18.75
Suramin	G[f]	Untreated control (UC)[c]	0	**24.68**
		PDH were treated with 12 h p.i. with suramin	100µg/mL	17.18
ETV	H[g]	PDH were treated with ETV 24 h prior to infection	0.001	ND
			0.01	ND
			0.1	ND

[a] Concentrations of APDP/NAPDP are given in µM;

[b] DHBV pre-treated with REP 2006 or REP 2031 prior to infecting the PDH;

[c] Untreated control;

[d] PDH were pre-treated with REP 2006 or REP 2031 prior to DHBV infection;

[e] REP 2006 or REP 2031 treatment was administered 12 h p.i. and at every second day during medium change;

[f] Suramin treatment was administered 12 h p.i. and at every second day during medium change;

[g] ETV treatment was administered 24 h prior to infection and at every second day during medium change.

Table 3.7: The antiviral effect of SS of REP 2006, SS of REP 2031 and SS of REP 2055 in comparison with ammonium salts of REP 2006 and REP 2031

APDPs[a]	Mean percentage of DHBsAg-positive PDH					
	Concentration of APDPs (µM)[b]				UC[c]	Uninfected PDH
	10	1	0.1	0.01		
SS of REP 2006	ND[d]	6.25	0.63	2.5	11.88	ND
REP 2006	1.25	1.88	1.25	3.13	11.88	ND
SS of REP 2031	ND	ND	3.75	2.5	8.13	ND
REP 2031	4.38	5.63	2.5	6.25	11	ND
SS of REP 2055	ND	ND	0.75	2.2	13.15	ND

[a] SS of APDPs REP 2006, REP 2031 and REP 2055 and ammonium salts of REP 2006 and REP 2031;

[b] Concentrations of APDP/non-APDP are given in µM;

[c] Untreated control;

[d] ND = No DHBsAg-positive PDH was detected.

DHBsAg-positive PDH were determined by IFA as described in Section 2.2.2.

Figure 3.1: Chemical structure of APDPs, REP 2006 (Panel A), REP 2031 (Panel B) and REP 2055 (Panel C).

Properties of REP 2006 (Panel A)

1. Polymer size (40mer)
2. R Group of REP 2006 is AGTC
3. Phosphorothioation
4. Enhanced stability
5. Enhanced hydrophobicity (amphipathic)
6. Polyanion

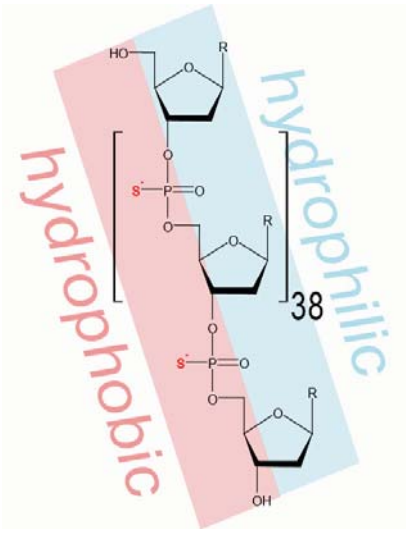
Properties of REP 2031 (Panel B)

1. Polymer size (40mer)
2. R Group of REP 2031 is poly C
3. Phosphorothioation
4. Enhanced stability
5. Enhanced hydrophobicity (amphipathic)
6. Polyanion

Properties of REP 2055 (Panel C)

1. Polymer size (40mer)
2. R Group of REP 2055 is alternative AC
3. Phosphorothioation
4. Enhanced stability
5. Enhanced hydrophobicity (amphipathic)
6. Polyanion

A



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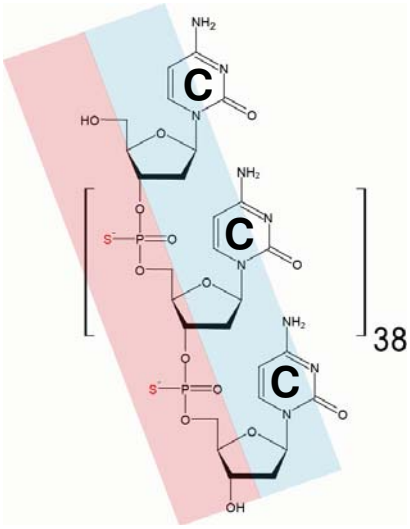


C

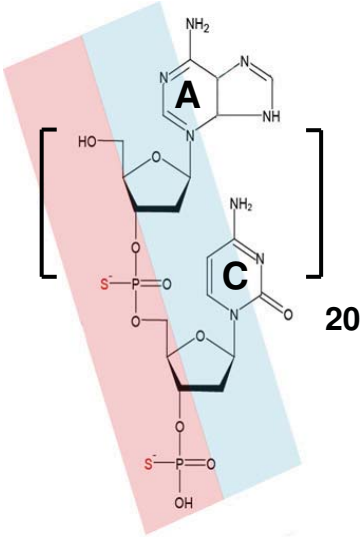


Figure 3.2: Chemical structure of REP 2086 (Panel A), REP 2107 (Panel B), REP 2117 (Panel C) and REP 2118 (Panel D).

Properties of REP 2086 (Panel A)

1. Polymer size (40mer)
2. R Group of REP 2086 is AGTC
3. 2' O-linked methyl group on the ribose lacks phosphorothioation
4. Enhanced stability but non-amphipathic (lowered hydrophobicity)
5. Polyanion

Properties of REP 2107 (Panel B)

1. Polymer size (40mer)
2. R Group of REP 2086 is AGTC
3. 2' O-linked methyl group on the ribose with phosphorothioation
4. Enhanced stability and amphipathic
5. Polyanion

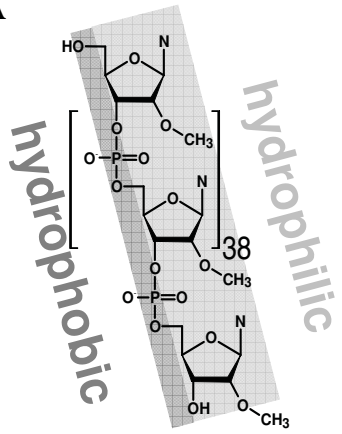
Properties of REP 2117 (Panel C)

1. Polymer size (40mer)
2. No R Group or abasic
3. Phosphorothioation
4. Enhanced stability and non-amphipathic (lowered hydrophilicity)
5. Polyanion

Properties of REP 2118 (Panel D)

1. Polymer size (40mer)
2. No R Group or abasic
3. Phosphorothioation
4. Enhanced stability and non-amphipathic (lowered hydrophilicity)
5. Polyanion

A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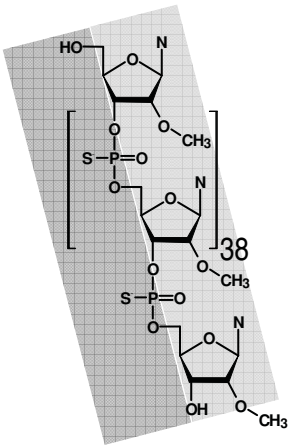
hydrophilic
HO
N
O
CH3
O
P=O
38
OH

B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N
O
CH3
S
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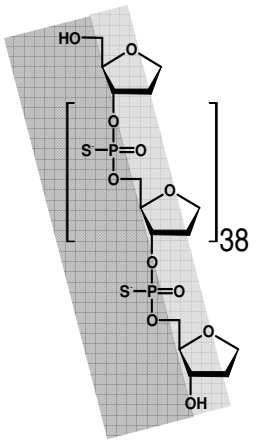
OH

C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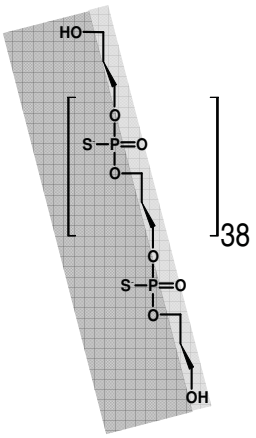
OH

D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38
OH

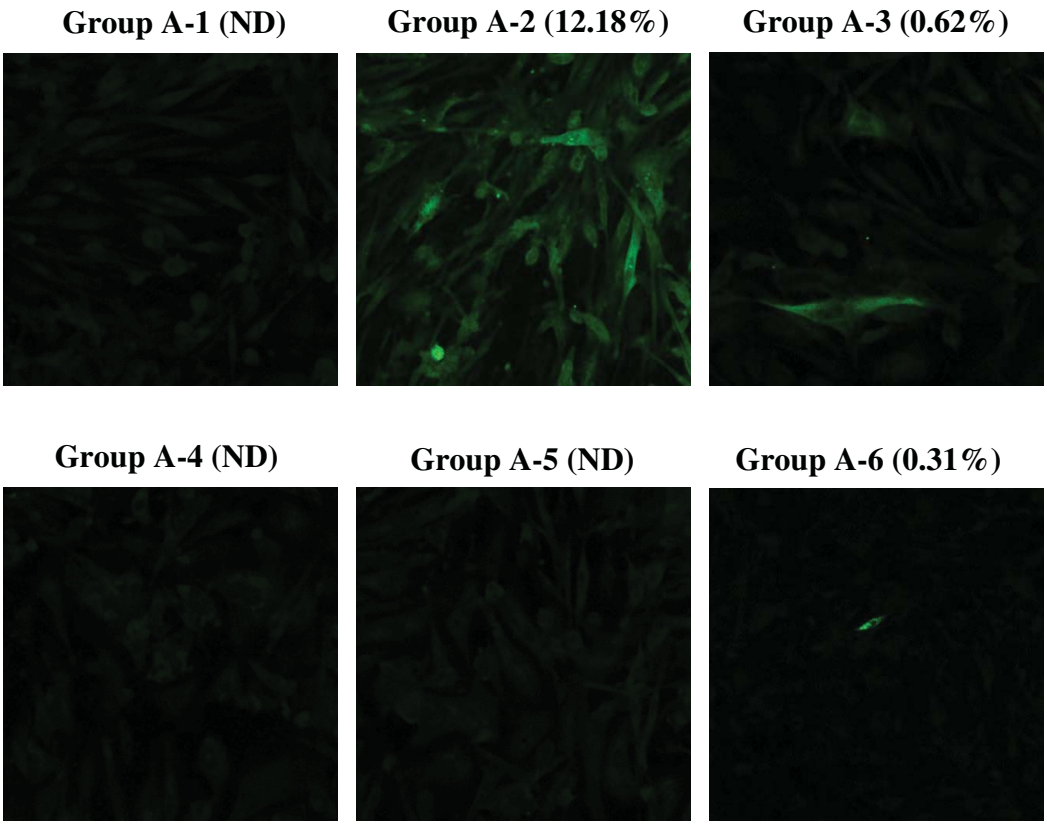


Figure 3.3a: The antiviral effect of REP 2006 pre-treatment of DHBV inoculum on the inhibition of DHBV infection. The DHBV inoculum was pre-treated with REP 2006 at concentrations of 0.01-10 μM for 1h (Test Group A3-A6).

DHBsAg-positive PDH were detected as described in Section 2.2.2 to measure the degree of inhibition of DHBV infection.

Green fluorescing PDH were positive for DHBsAg in the cytoplasm.

The mean percentage of DHBsAg-positive PDH in 5 camera fields of two wells as described in Section 3.2.4.1 is given in parenthesis.

ND: No DHBsAg-positive PDH were detected.

Group A-1: Uninfected and untreated; Group A-2: Infected and untreated;

Group A-3: DHBV pre-treated (0.01); Group A-4: DHBV pre-treated (0.1);

Group A-5: DHBV pre-treated (1); Group A-6: DHBV pre-treated (10).

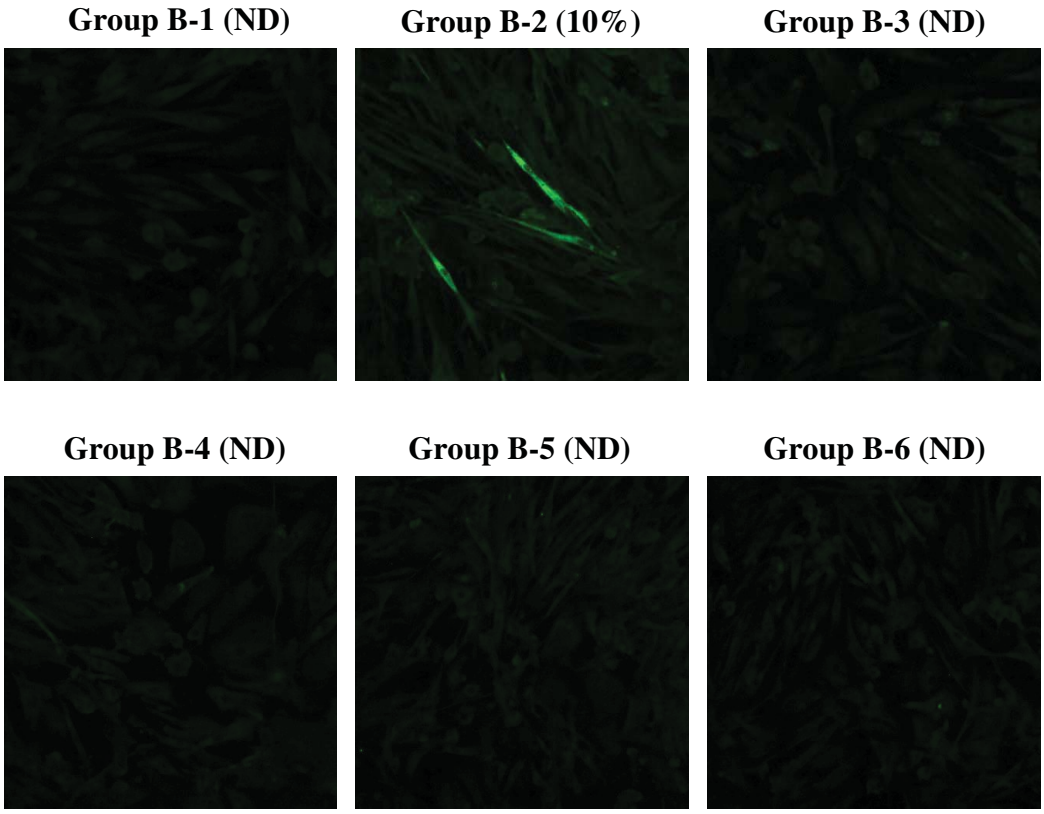


Figure 3.3b: The effect of REP 2006 pre-treatment of PDH on the inhibition of DHBV infection. The PDH were pre-treated with REP 2006 at concentrations of 0.01-10 μM for 1h (Test Group B3-B6).

DHBsAg-positive PDH were detected as described in Section 2.2.2 to measure the degree of inhibition of DHBV infection.

Green fluorescing PDH were positive for DHBsAg in the cytoplasm.

The mean percentage of DHBsAg-positive PDH in 5 camera fields of two wells as described in Section 3.2.4.1 is given in parenthesis.

ND: No DHBsAg-positive PDH were detected.

Group B-1: Uninfected and untreated; Group B-2: Infected and untreated;

Group B-3: PDH pre-treated (0.01); Group B-4: PDH pre-treated (0.1);

Group B-5: PDH pre-treated (1); Group B-6: PDH pre-treated (10).

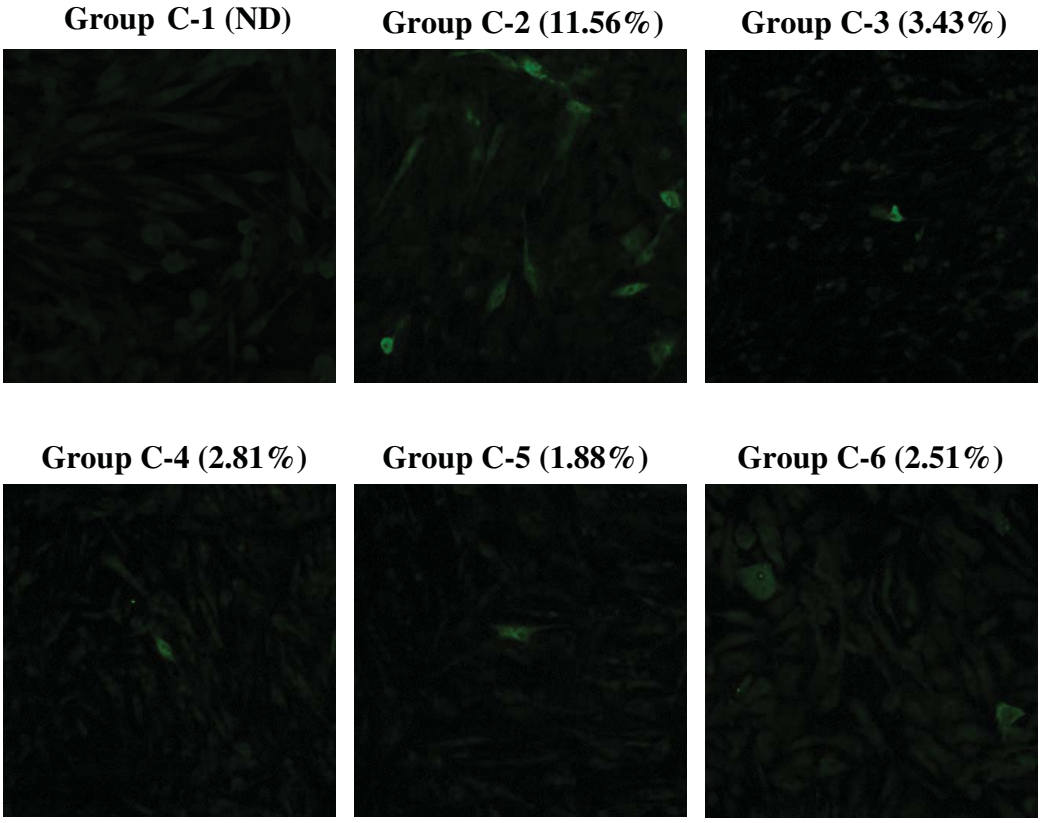


Figure 3.3c: The antiviral effect of REP 2006 against established DHBV infection. PDH were treated with REP 2006 12 h p.i. (Test Group C3-C6) at concentrations of 0.01-10 µM.

DHBsAg-positive PDH were detected as described in Section 2.2.2 to measure the degree of inhibition of DHBV infection.

Green fluorescing PDH were positive for DHBsAg in the cytoplasm.

The mean percentage of DHBsAg-positive PDH in 5 camera fields of two wells as described in Section 3.2.4.1 is given in parenthesis.

ND: No DHBsAg-positive PDH were detected.

Group C-1: Uninfected and untreated; Group C-2: Infected and untreated;

Group C-3: PDH treated (0.01); Group C-4: PDH treated (0.1);

Group C-5: PDH treated (1); Group C-6: PDH treated (10).

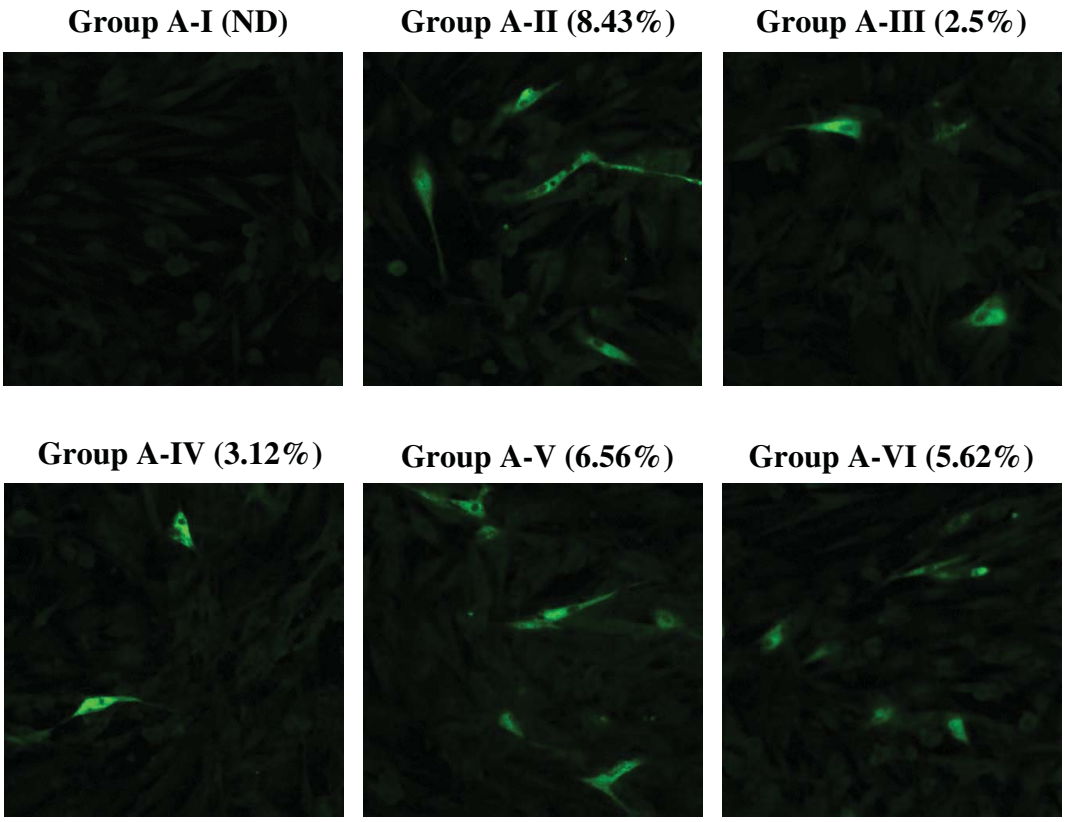


Figure 3.3d: The antiviral effect of REP 2031 pre-treatment on the inhibition of DHBV infection. The DHBV inoculum was pre-treated with REP 2031 at concentrations of 0.01-10 µM for 1h (PDH Group A-III to A-VI).

DHBsAg-positive PDH were detected as described in Section 2.2.2 to measure the degree of inhibition of DHBV infection.

Green fluorescing PDH were positive for DHBsAg in the cytoplasm.

The mean percentage of DHBsAg-positive PDH in 5 camera fields of two wells as described in Section 3.2.4.1 is given in parenthesis.

ND: No DHBsAg-positive PDH were detected.

Group A-I: Uninfected and untreated;

Group A-II: Infected and untreated;

Group A-III: DHBV pre-treated (0.01);

Group A-IV: DHBV pre-treated (0.1);

Group A-V: DHBV pre-treated (1);

Group A-VI: DHBV pre-treated (10).

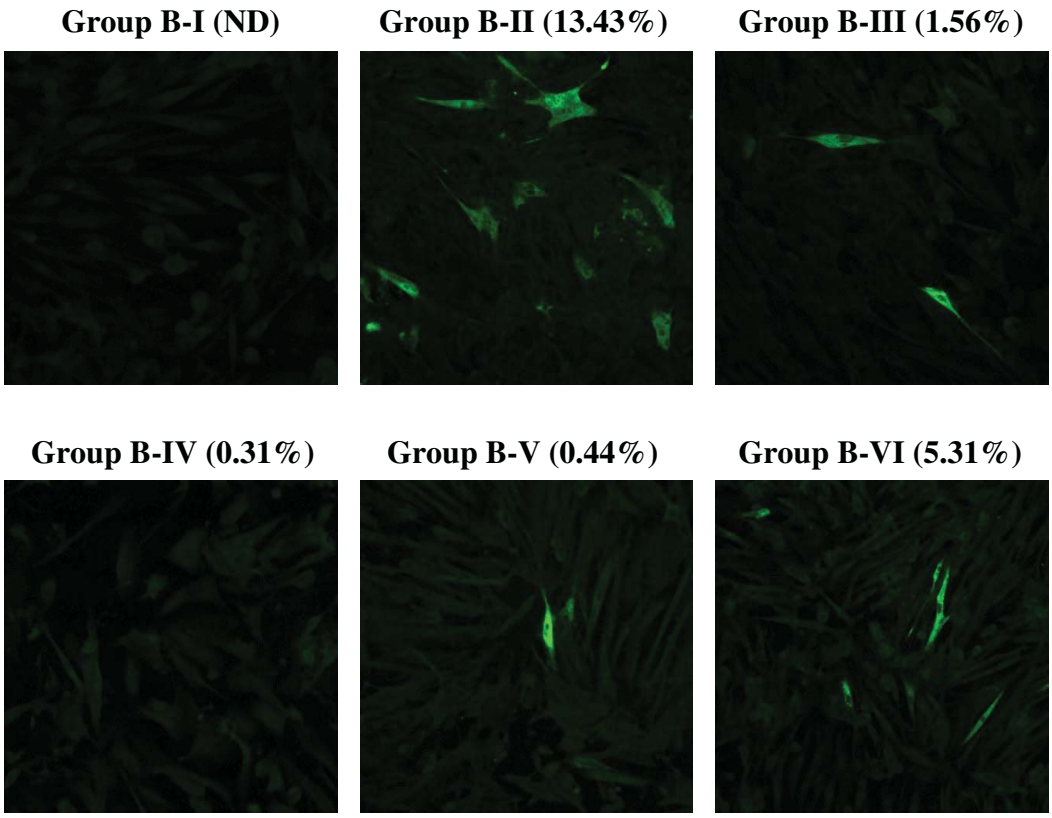


Figure 3.3e: The effect of REP 2031 pre-treatment of PDH on the inhibition of DHBV infection. The PDH were treated with REP 2031 at concentrations of 0.01-10 μM for 1h (Test Group B-III to B-VI).

DHBsAg-positive PDH were detected as described in Section 2.2.2 to measure the degree of inhibition of DHBV infection.

Green fluorescing PDH were positive for DHBsAg in the cytoplasm.

The mean percentage of DHBsAg-positive PDH in 5 camera fields of two wells as described in Section 3.2.4.1 is given in parenthesis.

ND: No DHBsAg-positive PDH were detected.

Group B-I: Uninfected and untreated;

Group B-II: Infected and untreated;

Group B-III: PDH pre-treated (0.01);

Group B-IV: PDH pre-treated (0.1);

Group B-V: PDH pre-treated (1);

Group B-VI: PDH pre-treated (10).

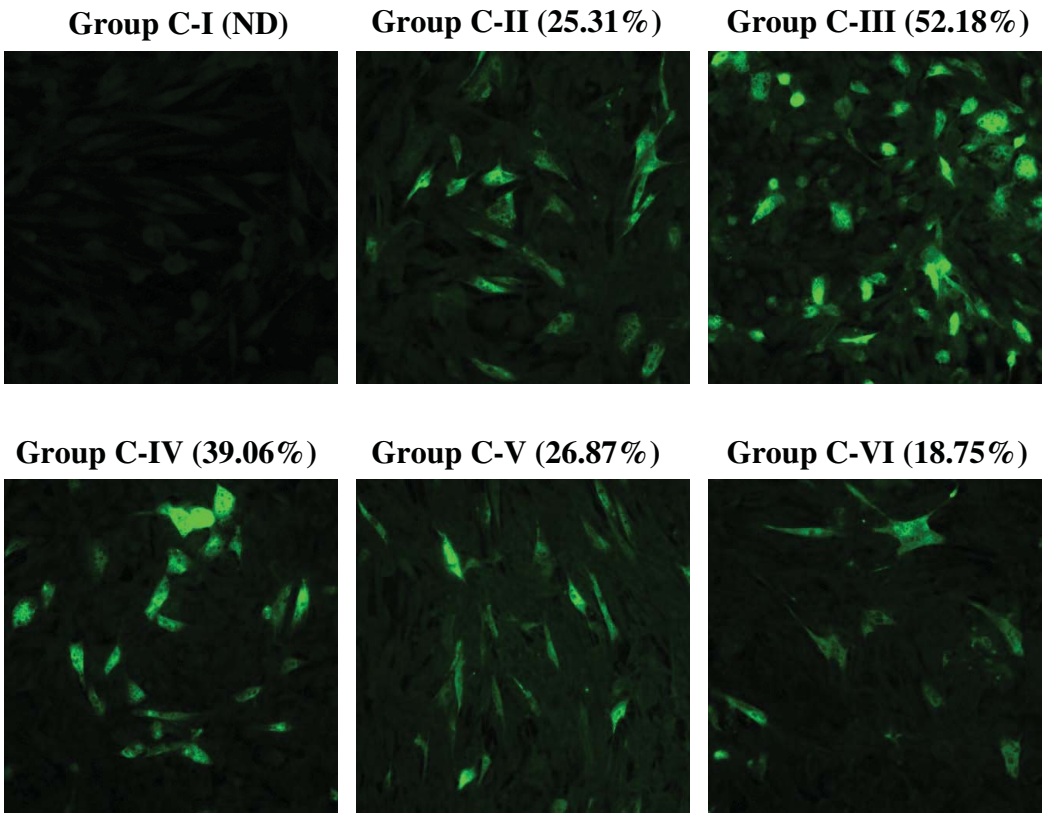


Figure 3.3f: The antiviral effect of REP 2031 against established DHBV infection. The REP 2031 treatment was applied 12 h p.i. at concentrations of 0.01-10 µM for 1h (Test Group C-III to C-VI).

DHBsAg-positive PDH were detected as described in Section 2.2.2 to measure the degree of inhibition of DHBV infection.

Green fluorescing PDH were positive for DHBsAg in the cytoplasm.

The mean percentage of DHBsAg-positive PDH in 5 camera fields of two wells as described in Section 3.2.4.1 is given in parenthesis.

ND: No DHBsAg-positive PDH were detected.

Group C-I: Uninfected and untreated; Group C-II: Infected and untreated;

Group C-III: PDH treated (0.01); Group C-IV: PDH treated (0.1);

Group C-V: PDH treated (1); Group C-VI: PDH treated (10).

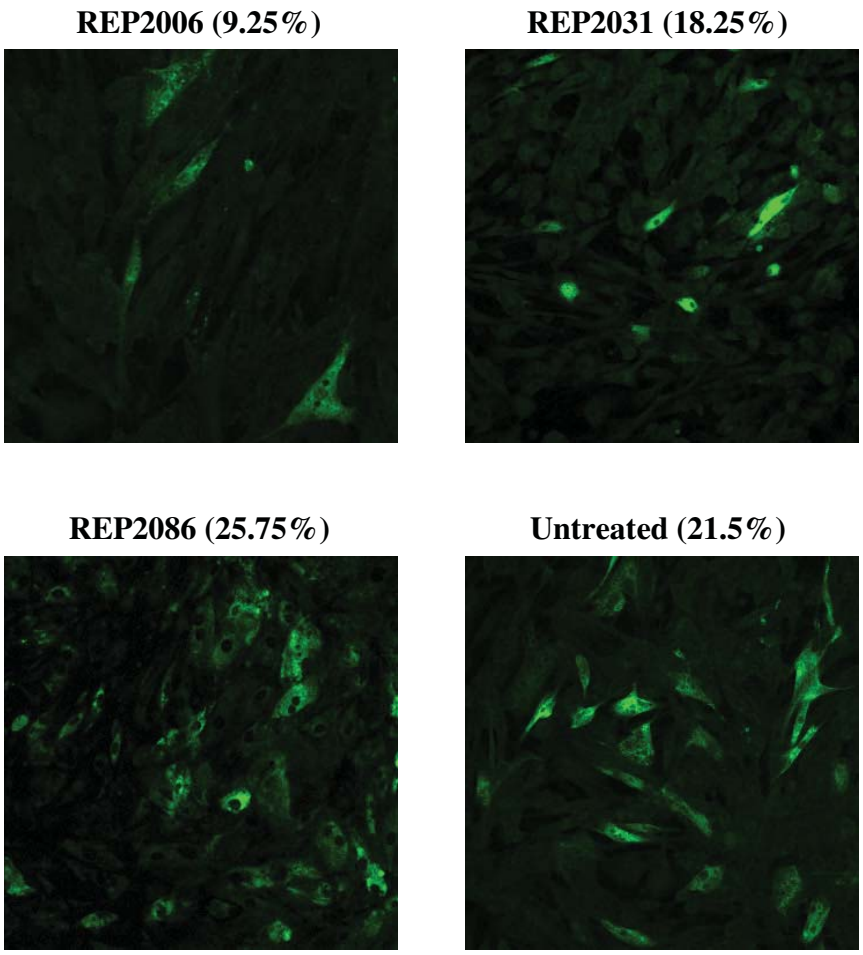


Figure 3.4: The effect of REP 2006, REP 2031 and REP 2086 on Alexa fluor 488 fluorescence signals.

REP 2006, REP 2031 and REP 2086 were added to DHBV infected PDH 24 h prior to harvest.

The mean percentage of DHBsAg-positive PDH in 5 camera fields of two wells as described in Section 3.2.4.1 is given in parenthesis.

Adding REP 2006, REP 2031 and REP 2086, 24 h prior to harvest reduced the intensity of the Alexa fluor 488 fluorescence signal for detection of DHBsAg to a lesser extent when compared to untreated PDH, however, it did not interfere the detection of DHBsAg-positive PDH (Section 3.4.1.5)

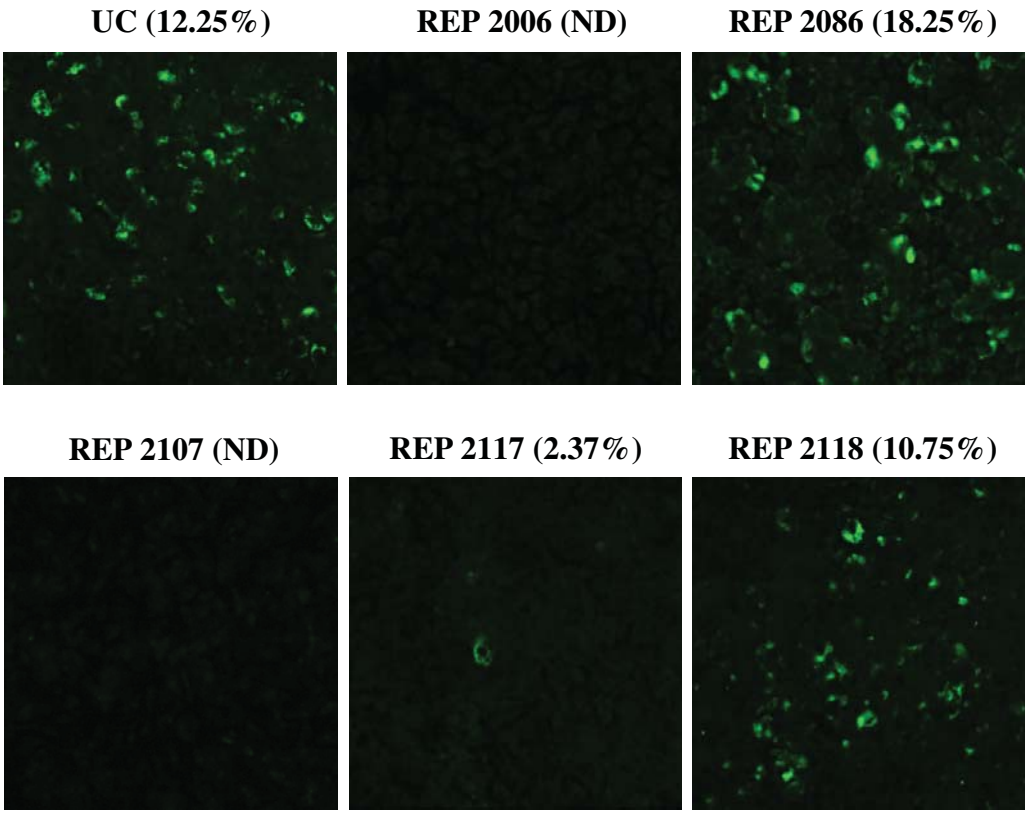


Figure 3.5: The effect of hydrophobicity on the antiviral activity as detected by IFA (Section 2.2.2). Green fluorescing PDH were positive for DHBsAg in the cytoplasm.

Chemical structure of REP 2006 (Figure 3.1), REP 2086, REP 2107, REP 2117 and REP 2118 (Figure 3.2) with their chemical properties are given in Table 3.1.

PDH were treated at the time of DHBV infection with REP 2006, REP 2086, REP 2107, REP 2117 and REP 2118 at a concentration of 10 μM.

The mean percentage of DHBsAg-positive PDH in 5 camera fields of two wells as described in Section 3.2.4.1 is given in parenthesis.

UC: Untreated control;

ND: No DHBsAg-positive PDH were detected.

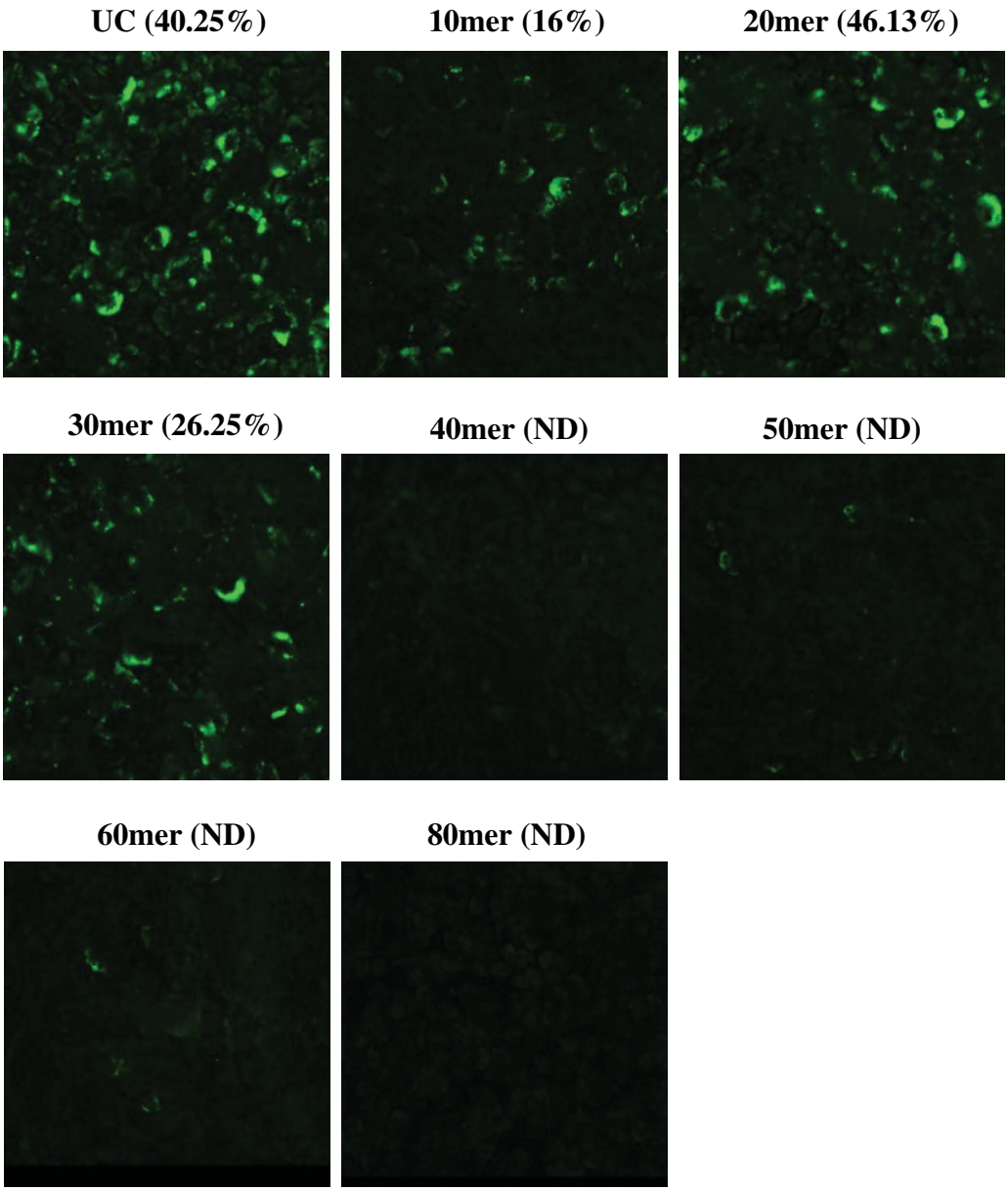


Figure 3.6: The role of polymer size on the antiviral activity as detected by IFA (Section 2.2.2). Green fluorescing PDH were positive for DHBsAg in the cytoplasm.

PDH were treated with 10mer, 20mer, 30mer, 40mer, 50mer, 60mer and 80mer at the time of DHBV infection at a concentration of 10 µM.

The mean percentage of DHBsAg-positive PDH of two wells is given in parenthesis.

UC: Untreated control; ND: No DHBsAg-positive PDH were detected.

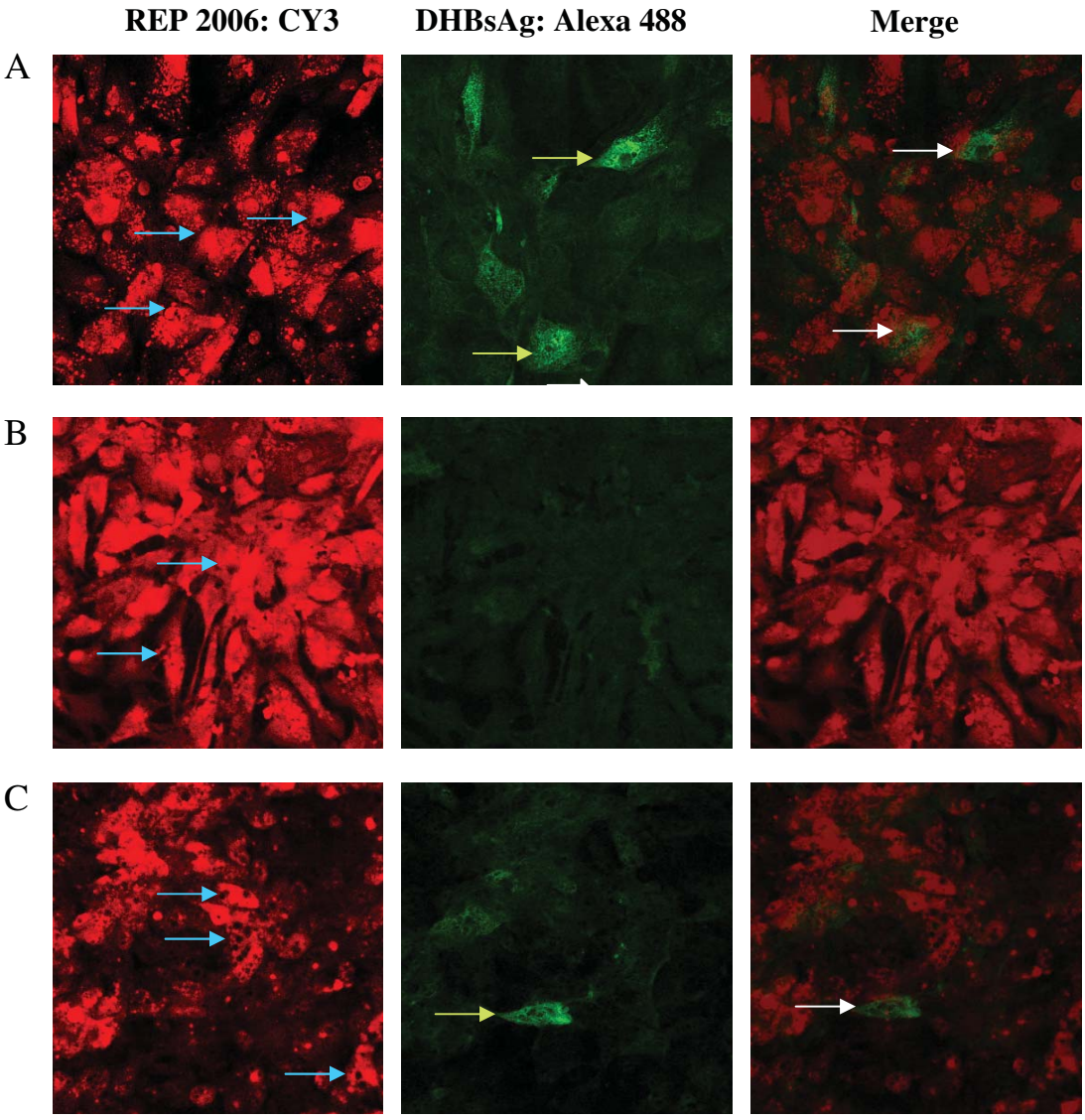


Figure 3.7a: Qualitative detection of colocalisation of CY3 labelled REP 2006 following pre-treatment of DHBV inoculum prior to infection. PDH were fixed on day 7 p.i. with 1% formalin (Panel A), EAA (Panel B) and 2% PFA (Panel C) as described in Section 3.2.11.

Green arrows show the DHBsAg-positive green fluorescing PDH.

CY3 labelled REP 2006 was diffusely distributed in the cytoplasm of infected (white arrows) and uninfected PDH (blue arrows) leaving the nuclei free of REP 2006.

No DHBsAg-positive PDH were detected in Panel B, however, low level of DHBV infected PDH were detected in Panels A and C when compared with untreated PDH (data not shown).

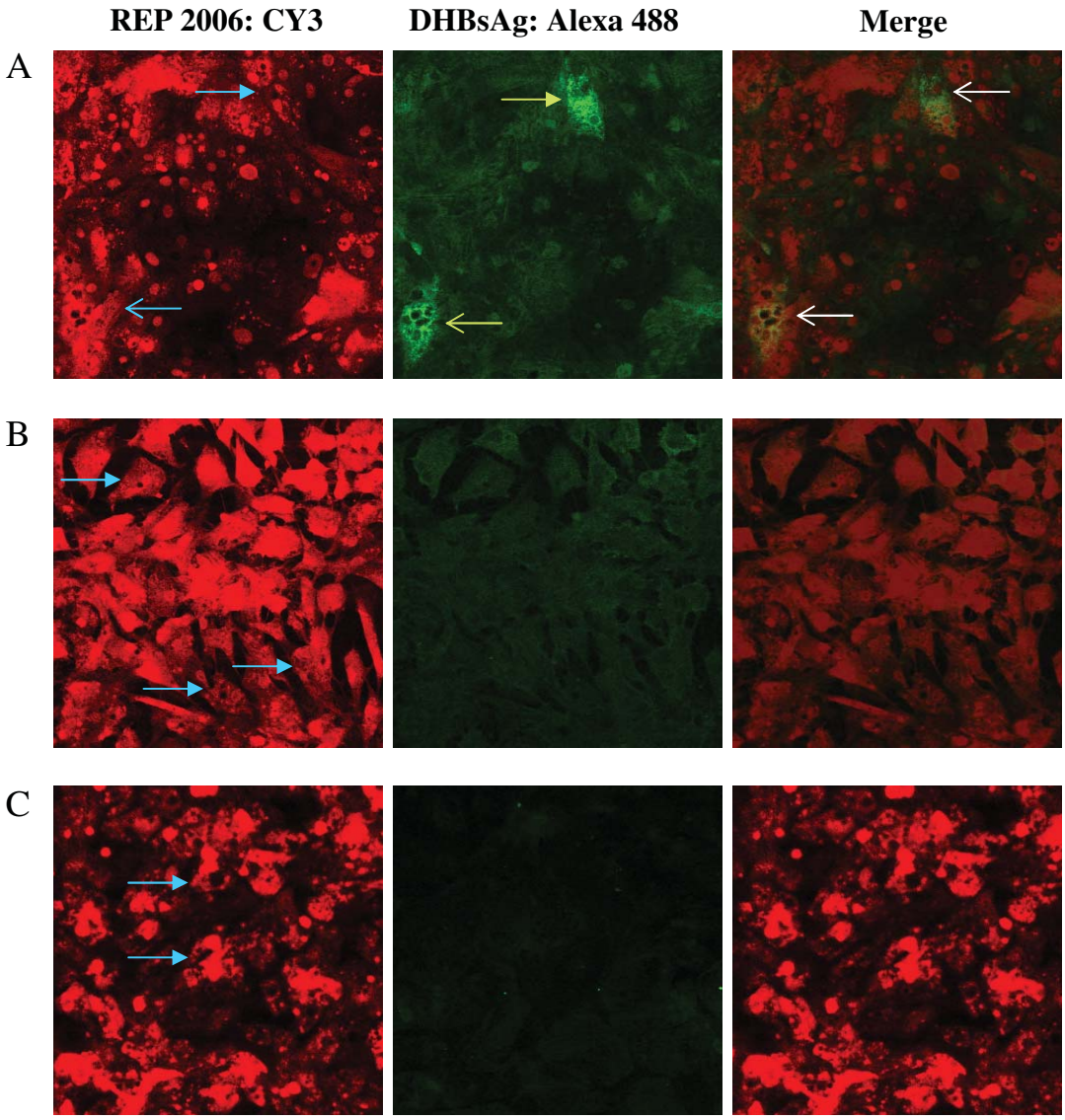


Figure 3.7b: Qualitative detection of colocalisation of CY3 labelled REP 2006 by PDH 12 h p.i. PDH were fixed on day 7 p.i. with 1% formalin (Panel A), EAA (Panel B) and 2% PFA (Panel C) as described in Section 3.2.11.

Green arrows show the DHBsAg-positive green fluorescing PDH.

REP 2006 was distributed diffusely in the cytoplasm in the infected (white arrows) and uninfected PDH (blue arrows) leaving the nuclei free of REP 2006.

No DHBsAg-positive PDH were detected in Panels B and C, however, low level of DHBV infected PDH were detected in Panel A when compared with untreated PDH (data not shown).

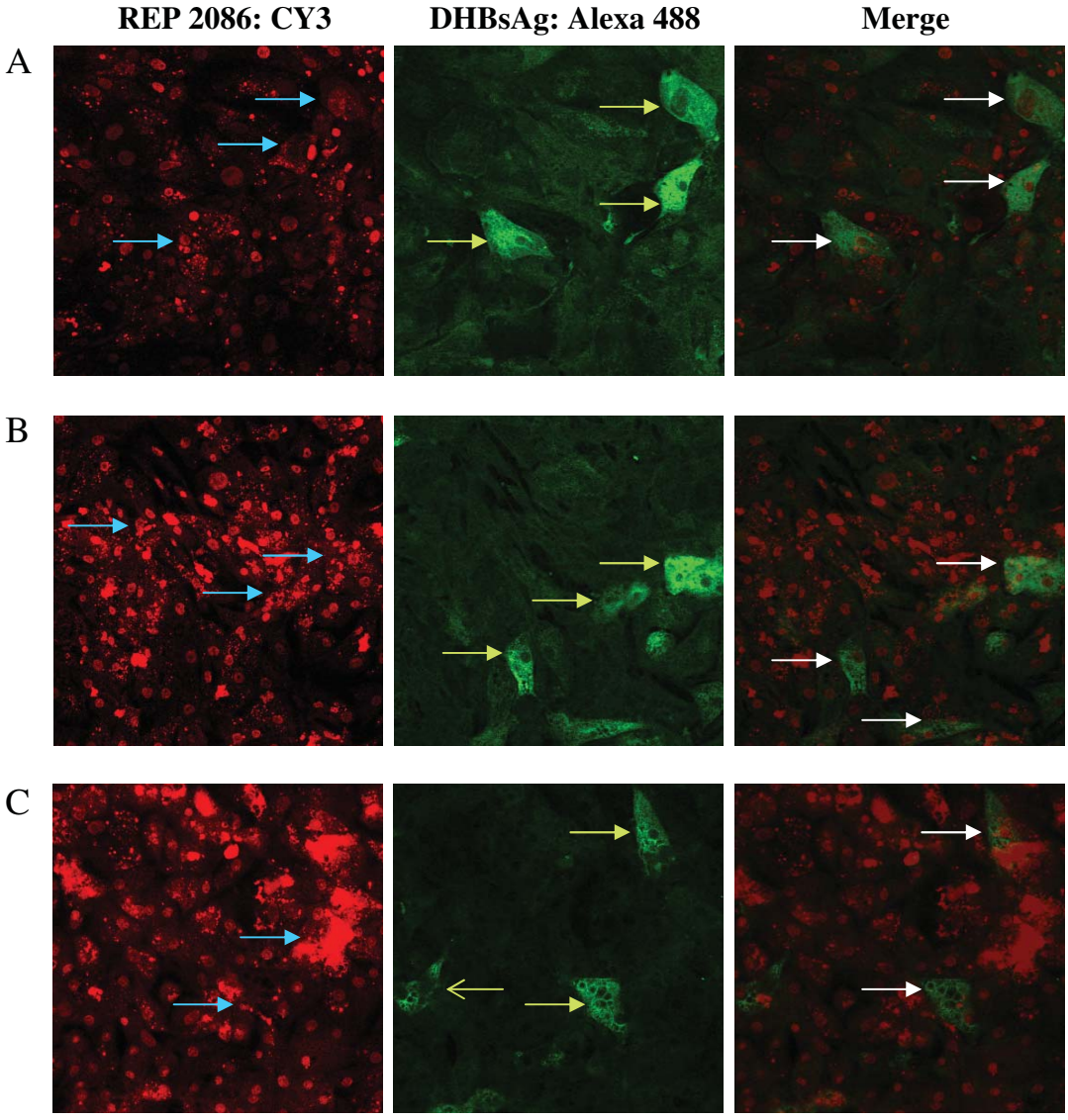


Figure 3.7c: Qualitative detection of colocalisation of CY3 labelled REP 2086 following pre-treatment of DHBV inoculum prior to infection. PDH were fixed on day 7 p.i. with 1% formalin (Panel A), EAA (Panel B) and 2% PFA (Panel C) as described in Section 3.2.11.

Green arrows show the DHBsAg-positive green fluorescing PDH.

REP 2086 was distributed less diffusely than REP 2006 in the cytoplasm infected (white arrows) and uninfected PDH (blue arrows).

White arrows show the nuclear (B) and cytoplasmic (C) co-localisation of REP 2086 in DHBV infected PDH. Nuclear distribution of REP 2086 appears to be an artefact as nuclear distribution of REP 2086 has not been demonstrated previously (Vaillant *et al.* unpublished).

DHBsAg-positive PDH were detected in 1% formalin Panels A, B and C similar to that found in untreated PDH (data not shown).

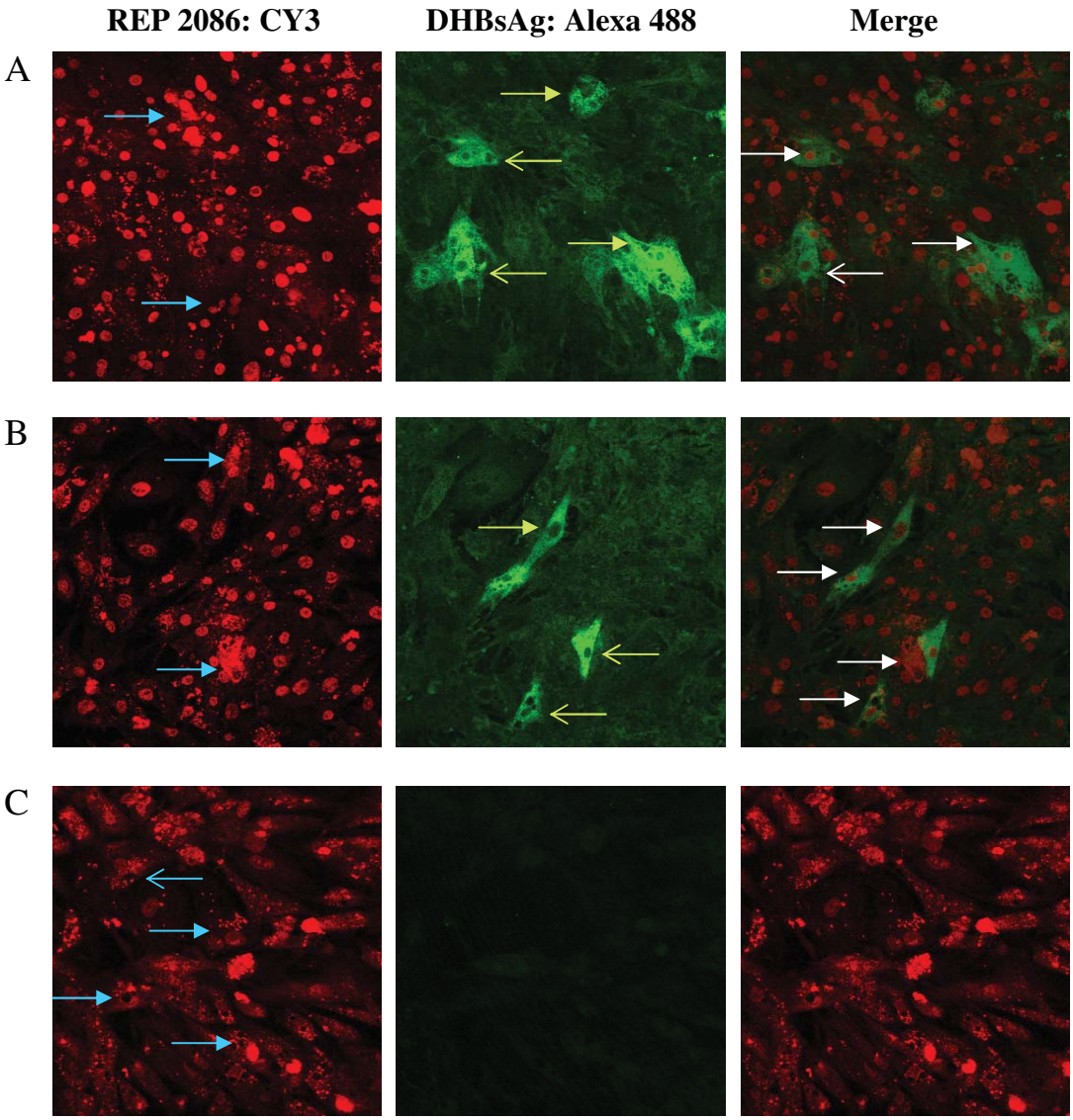


Figure 3.7d: Qualitative detection of colocalisation of CY3 labelled REP 2086 by PDH 12 h post DHBV infection. PDH were fixed on day 7 p.i. with 1% formalin (Panel A), EAA (Panel B) and 2% PFA (Panel C) as described in Section 3.2.11.

Green arrows show the DHBsAg-positive green fluorescing PDH.

REP 2086 was distributed less diffusely than REP 2006 in the cytoplasm infected (white arrows) and uninfected PDH (blue arrows)

White arrows show the nuclear (B) and cytoplasmic (C) co-localisation of REP 2086 in DHBV infected PDH. Nuclear distribution of REP 2086 appears to be an artefact as in some PDH no nuclear colocalisation of REP 2086 is present (B lower bottom white arrows). Furthermore, nuclear colocalisation of REP 2086 has not been demonstrated previously (Vaillant *et al.* unpublished).

DHBsAg-positive PDH were detected in Panels A, B and C, similar to that found in untreated PDH (data not shown).

Chapter 4: Studies testing the ability of REP 2006 and REP 2031 to inhibit DHBV infection *in vivo*

4.1 Introduction

Comprehensive data from Chapter 3 indicates that the ammonium salts of APDPs, REP 2006 and REP 2031, have the ability to prevent DHBV infection *in vitro*. These experiments have clearly shown the ability of REP 2006 to inhibit the DHBV infection either when REP treatment was given prior to or 12 h after DHBV infection. In contrast, REP 2031 was able to inhibit DHBV infection when the treatment was given prior to infection but not when the REP 2031 treatment was given 12 h after DHBV infection.

This project examined the antiviral effect of the APDPs, REP 2006 and REP 2031, in 14-day-old ducks. The REP 2006 and REP 2031 compounds used in the *in vivo* experiments were the sodium salts because ammonium salts cause systemic toxicity *in vivo* and are expensive to produce for large scale applications. The high purity sodium salts of APDPs were produced under GMP-like conditions making them more suitable for *in vivo* application. Conversely, PS-ONs that belong to the same class of APDPs are known to concentrate in the liver (>70% of total dose) (Yu *et al.* 2007; Yu *et al.* 2008), where the bulk of HBV and DHBV replication occurs. This supports the potential of APDPs to act as anti-HBV or anti-DHBV agents. Thus we hypothesised that the specific antiviral properties of REP 2006 and REP 2031 combined with their PK will result in a significant reduction in DHBV infection *in vivo*.

The only animal species that can be infected with the human HBV are humans and higher primates. Practical, ethical and financial problems limit the use of primate models in many laboratories. Hence, HBV-like viruses and their natural hosts are widely used for experimental studies. DHBV in its natural host, the Pekin duck *(Anas domesticus platyrhynchos)* can be used as an animal model to study treatment outcomes of antiviral drugs at the pre-clinical level (Zoulim *et al.* 2008). Extended information about viral replication and infection outcomes of hepadnavirus infection has been identified from studies of the DHBV-duck system (Schultz *et al.* 2004; Zoulim *et al.* 2008). Furthermore,

this model is less expensive than the primate model, reagents are available in many laboratories and the virus is less hazardous to laboratory staff.

Moreover, experimental manipulation of infection outcome has been intensively studied in the duck model by adjusting the infection dose of DHBV and age of the ducks (Jilbert *et al.* 1996; Jilbert *et al.* 1998; Foster *et al.* 2005). For example, newly hatched ducks (7-14 days) inoculated with 4 x 10^4 DHBV DNA genomes produced transient DHBV infection. When the inoculum size was increased 25-fold (1 x 10^6 DHBV DNA genomes) the infection became persistent. When four month-old ducks were inoculated with different doses of DHBV, only the ducks receiving the highest dose (2 x 10^{11} DHBV DNA genomes) showed viraemia and extensive viral replication in the liver (Jilbert *et al.* 1998). The ability to produce varying infection outcomes by adjusting the DHBV inoculum size and age of the host has immensely contributed to the pre-clinical investigation of anti-viral agents and vaccines against DHBV infection.

Several immunotherapeutic and antiviral studies have been performed in this model (Foster *et al.* 2003; Foster *et al.* 2005; Miller *et al.* 2006a; Miller *et al.* 2006b; Miller *et al.* 2008; Thermet *et al.* 2008). The BMS NA ETV, a guanosine analogue and a potent selective inhibitor of HBV Pol, has been thoroughly investigated for its antiviral activity against DHBV infection in young ducks infected with a dose of DHBV (5 x 10^8 DHBV DNA genomes). It has been shown to produce chronic infection in 14-day-old ducks. Other studies (Foster *et al.* 2003; Miller *et al.* 2006a; Miller *et al.* 2006b; Miller *et al.* 2008) have also used ETV but these studies investigated the efficacy of immunotherapeutic strategies in combination with different forms of DNA vaccines. These studies have established a platform for future treatment trials using novel therapeutic antiviral agents for HBV infection using the DHBV-duck system.

Hence, the current study was designed to investigate the antiviral efficacy of APDPs, REP 2006 and REP 2031 against DHBV infection using 14-day-old ducks.

4.2 Experimental design

Approval to conduct the studies in Pekin Ayelsbury ducks *(Anas domesticus platyrhynchos)* was obtained from the Animal Ethics Committees of the IMVS, Adelaide, South Australia and The University of Adelaide, South Australia.

Ducks that were 14 days old were divided into 4 groups of 5, and infected IV with 5 x 10^8 DHBV DNA genomes. Group 4.A ducks were treated with REP 2006 (10 mg/kg, IP), Group 4.B ducks were treated with REP 2031 (10 mg/kg, IP), Group 4.C ducks were treated with ETV (1.0 mg/kg, oral) and Group 4.D ducks were treated with NS (IP) (Table 4.1). Ducks in all experimental Groups received daily treatment with respective drugs or NS starting from one day prior to DHBV infection for 15 days. A minor amendment in REP 2006 treatment was made to the ducks in Group 4.A in that the REP 2006 was given every other day from day 4 p.i. (from the time of the liver biopsy).

The source and dose of DHBV, the candidate drugs used and their concentrations, the collection of blood samples, IP injection, biopsy and autopsy procedures, tissue samples for future analysis, analysis of blood and serum for CBE and liver function tests, ELISA to detect serum DHBsAg, immuno-staining to detect hepatocellular DHBsAg and detection of DHBV DNA by Southern blot hybridisation are described in Section 2.10.3. CBE with special reference to total RBC and WBC counts was performed at the Division of Haematology of Diagnostic Clinical Pathology Unit at the IMVS. Blood samples collected at autopsy (day 14 p.i.) from all ducks in Groups 4.A–4.D were tested using an automated complete blood evaluation system (Sysmex Cooperation 2000, Sysmex, model XE2100).

Sera from blood samples were also tested for levels of liver enzymes to elucidate possible changes in liver function due to the treatment with REP 2006 and REP 2031. Sera of ETV- and NS-treated ducks were used as comparators for liver function analysis.

Blood samples collected on days 0, 5, 10 and 14 p.i. were tested for DHBsAg using a qualitative ELISA. Liver tissues on days 4 and 14 p.i. were examined for histological changes, percentage of DHBsAg-positive hepatocytes by immuno-staining and DHBV DNA by Southern blot hybridisation.

Ducks were assessed every day for any abnormalities in feed and water intake, weight changes, gait and changes in behaviour during the study period. Ducks were clinically

examined through a gentle palpation of the abdomen for signs of pain in the abdominal area. The IP injection was given just below the tip of the sternum, which permits an easy access to the peritoneal cavity. Furthermore, ducks were examined for signs of pain on IP injection.

Statistical analysis: Differences in mean body weights, WBC counts, liver enzyme levels and the percentage of DHBsAg-positive hepatocyte counts in liver tissues of ducks in Groups 4.A, 4.B, 4.C and 4.D were statistically analysed using multiple ANOVA followed by *Post hoc* analysis. All the analyses were performed using the analytical software Graph Pad Prism Version 5. Differences were considered statistically significant when the *p* values were <0.05.

4.3 Results

Previous *in vitro* data (Chapter 3) from experiments in DHBV-infected PDH suggested that REP 2006 has an excellent anti-DHBV activity. The current study aimed to test the anti-DHBV activity of this compound *in vivo* in 14-day-old ducks. Although REP 2031 could only partially block DHBV infection *in vitro*, the anti-DHBV activity of this compound was also tested to see whether it could elicit a better anti-DHBV activity *in vivo* than *in vitro*.

4.3.1 Group 4.A: The effect of REP 2006 treatment on clinicopathological, haematological, biochemical and virological markers

Ducks treated with REP 2006 (Group 4.A) showed abdominal tenderness on palpation and signs of abdominal pain on IP injection compared with ducks treated with ETV (Group 4.C) or NS (Group 4.D), that had neither abdominal tenderness on gentle abdominal palpation nor abdominal pain on IP injection. At biopsy, ducks in Group 4.A, bled more at the surgical incision site than ducks in the other three Groups. These findings were reported to the IMVS Animal Ethics Committee. However, bleeding at biopsy did not lead to any interference with surgery or post-surgical mortality. *In situ* examination of internal organs at autopsy did not reveal any gross pathological changes in all 4 Groups of ducks. Following the biopsy on day 4 p.i., REP 2006 treatment was administered every other day to avoid any possible aggravation of side effects experienced by the ducks treated with REP 2006 (Group 4.A).

A moderate rise in total WBC count was observed in Ducks 553 and 554 from REP 2006 treated Group 4.A when compared to ducks treated with ETV or NS. Only 3 blood samples

from Group 4.A could be analysed because 2 samples were not accepted by the automated analyser due to sample quality issues. The mean WBC count of the Group 4A REP 2006-treated ducks did not show statistically significant elevation when compared with the mean WBC count of ducks treated with either ETV or NS ($p>0.05$) (Table 4.2). There was no statistically significant difference in liver enzymes, GGT, ALT and AST in ducks treated with REP 2006 when compared with ducks treated with ETV or NS ($p>0.05$). This suggests that the liver function was not affected by treatment with REP 2006 (Table 4.3).

Treatment of ducks with REP 2006 prevented the development of detectable levels of serum DHBsAg in 5/5 ducks when sera from these ducks were tested using a qualitative DHBsAg ELISA (Figure 4.1 Panel A). Liver tissue from these ducks on day 4 p.i. had DHBV infection in 0.005–0.008% of hepatocytes (mean=0.006%) as shown by immuno-staining (Table 4.4; Figure 4.2). On day 14 p.i., these ducks had <0.001 % (mean=<0.001%) of DHBsAg-positive hepatocytes (Table 4.4; Figure 4.2). REP 2006 treatment has significantly reduced the percentage of DHBsAg-positive hepatocytes in REP 2006-treated ducks ($p<0.05$) when compared with the mean of DHBsAg-positive hepatocyte counts for Group 4.B REP 2031- or Group 4.D NS-treated ducks on day 4 and 14 p.i. (Table 4.4; Figure 4.2). Furthermore, no DHBV DNA was detected in liver samples from the Group 4.A ducks treated with REP 2006 on day 4 and 14 p.i. In contrast, DHBV DNA was detected in liver samples derived from the Groups 4.B and 4.D ducks treated with either REP 2031 or NS on day 4 and 14 p.i. (Figure 4.3). Hepatic DHBV DNA profiles agreed with the levels of DHBsAg-positive hepatocytes detected by immuno-staining.

In summary, REP 2006 treatment was able to prevent the development of detectable levels of serum and hepatic DHBsAg and hepatic DHBV DNA in 5/5 Group 4.A ducks (Figure 4.1; Figure 4.2; Figure 4.3). All five Group 4.A REP 2006-treated ducks had no detectable DHBsAg-positive hepatocytes (mean= <0.001%) in the liver by immuno-staining.

4.3.2 Group 4.B: The effect of REP 2031 treatment on clinicopathological, haematological, biochemical and virological markers

Ducks treated with REP 2031 did not show signs of abdominal tenderness on palpation and abdominal pain on IP injection. The surgical incision site at biopsy of ducks in this Group did not bleed like the ducks treated with REP 2006 and examination of internal organs at autopsy revealed no gross pathological changes.

A moderate rise in total WBC count was observed in Duck 560 from this Group when compared with Groups 4.C and 4.D treated with ETV or NS. Three blood samples could be analysed from the Group treated with REP 2031 as 2 samples were not accepted by the automated analyser due to sample quality issues. The mean WBC count of this Group of ducks did not show any significant elevation when compared with the mean WBC count of ducks treated with ETV or NS ($p>0.05$) (Table 4.2). There were no significant differences in liver enzymes GGT, ALT and AST in ducks treated with REP 2031 when compared with ETV or NS-treated ducks ($p>0.05$). This suggests that the liver function was not altered by treatment with REP 2031 (Table 4.3).

Treatment of ducks with REP 2031 could not prevent the development of serum DHBsAg in 3/5 ducks when sera from these ducks were tested using a qualitative DHBsAg ELISA (Figure 4.1). Ducks treated with REP 2031 had a mean of 0.208% DHBsAg-positive hepatocytes on day 4 p.i. At this point NS-treated ducks had a mean of 1.537% of DHBsAg-positive hepatocytes, suggesting that REP 2031 showed an anti-DHBV activity at an early phase of the infection. This compared noticeably with the levels of hepatic DHBV infection in NS-treated ducks. However, this anti-DHBV activity was much inferior to that of REP 2006 or ETV on day 4 p.i. and the liver infection increased to >95% DHBsAg-positive hepatocytes on day 14 p.i. as a result of widespread infection in the liver (Table 4.4; Figure 4.2). On day 4, DHBV DNA was not detected in liver samples of 5/5 ducks treated with REP 2031 whereas 3/5 NS-treated ducks had DHBV DNA in the liver at this point (Figure 4.3). The intensity of DHBV DNA signal detected in liver samples of ducks treated with REP 2031 was less than that detected in the livers of NS-treated ducks (Figure 4.3). Hepatic DHBV DNA profiles concurred with the levels of DHBsAg-positive hepatocytes detected by immuno-staining.

In summary, REP 2031 treatment was unable to prevent the development of serum DHBsAg levels in 3/5 ducks and hepatic DHBsAg levels and hepatic DHBV DNA levels in 5/5 treated ducks (Figure 4.1; Figure 4.2; Figure 4.3).

4.3.3 Group 4.C: The effect of ETV treatment on clinicopathological, haematological, biochemical and virological markers

Ducks treated with ETV did not show signs of abdominal tenderness on palpation. Ducks in this Group did not bleed at biopsy like the ducks treated with REP 2006 and had no gross pathological changes on examination of internal organs at autopsy.

The mean WBC count of this Group of ducks did not show any significant elevation when compared with the mean WBC count of ducks treated with NS ($p>0.05$) (Table 4.2). There were no significant differences in liver enzymes GGT, ALT and AST in ducks treated with ETV when compared with NS-treated ducks ($p>0.05$). It suggests that the liver function was unaltered by ETV treatment (Table 4.3).

Treatment of ducks with ETV did prevent the development of serum DHBsAg in 5/5 ducks when sera from these ducks were tested (Figure 4.1 Panel B). Ducks treated with ETV had a mean of 0.023% and 0.048 % DHBsAg-positive hepatocytes on day 4 and 14 p.i. (Table 4.4; Figure 4.2). DHBV DNA was not detected by Southern blot hybridisation in liver samples from ETV treated ducks with on day 4 and 14 p.i. (Figure 4.3). The percentage of DHBsAg-positive hepatocytes detected by immuno-staining suggests that more sensitive assays such as PCR may be needed to detect the DHBV DNA in the liver of ducks (Figure 4.1 Panel B; Figure 4.2; Figure 4.3).

In summary, ETV treatment prevented the development of detectable levels of serum DHBsAg levels and hepatic DHBV DNA levels in 5/5 treated ducks (Figure 4.1 Panel B; Figure 4.2; Figure 4.3). However, detection of low level of DHBsAg-positive hepatocytes in all 5 ducks (mean=0.048%) by immuno-staining suggested the presence of DHBV infection in the liver.

4.3.4 Group 4.D: The effect of NS treatment on clinicopathological, haematological, biochemical and virological markers

NS treatment in Group 4.D ducks was used as a control to compare any possible toxic effects of candidate drugs on the health of the ducks and drug induced changes in haematological and biochemical markers. On the other hand the positive effect of the candidate drugs on virological markers in the serum and liver was compared with those parameters in NS-treated ducks.

Ducks treated with NS did not show signs of abdominal tenderness on palpation and pain on IP injection. Ducks in this Group had only a mild to moderate bleeding at biopsy and no gross pathological changes were noted on examination of internal organs at autopsy. The mean WBC count of this Group of ducks was in agreement with published reference values for ducks (Table 4.2). Liver enzymes, GGT, ALT and AST in NS-treated ducks also agreed with reference values published for ducks (Table 4.3).

As expected, treatment of ducks with NS was unable to prevent the development of serum DHBsAg in 3/5 ducks when sera from these ducks were tested using a qualitative DHBsAg ELISA (Figure 4.1 Panel D). Ducks treated with NS had a mean of 1.537% of DHBsAg-positive hepatocytes on 4 p.i. and the liver infection increased to >95% DHBsAg-positive hepatocytes on day 14 p.i. as a result of widespread infection in the liver (Table 4.4; Figure 4.2). DHBV DNA was detected in liver samples from ducks treated with NS on day 4 and 14 p.i. (Figure 4.3). Hepatic DHBV DNA profiles were in agreement with the levels of DHBsAg-positive hepatocytes detected by immuno-staining.

In summary, NS treatment was unable to prevent the development of serum DHBsAg levels in 3/5 ducks and hepatic DHBsAg levels and hepatic DHBV DNA levels in 5/5 treated ducks (Figure 4.1 Panel D; Figure 4.2; Figure 4.3).

4.4 Discussion

NS treatment of ducks was used as a placebo to evaluate the safety profile and the antiviral effect of REP 2006 and REP 2031. As DHBV infection in ducks is asymptomatic the Group 4.D ducks were also used to evaluate the drug induced changes in clinicopathological, haematological and biochemical markers of the Groups 4.A, 4.B and 4.C drug-treated ducks.

As anticipated, NS treatment was well tolerated by the ducks and did not alter any of the clinicopathological, haematological and biochemical markers, indicating the inertness of NS. This made NS treatment suitable for assessing the safety of REP 2006 and REP 2031 as antiviral agents. This study produced an expected DHBV infection outcome of widespread DHBV infection when the 14-day-old ducks were inoculated with a dose of 5 x 10^8 DHBV DNA genomes (Foster *et al.* 2003; Foster *et al.* 2005; Miller *et al.* 2006a; Miller *et al.* 2006b; Miller *et al.* 2008). All ducks inoculated with this dose and treated with NS developed widespread DHBV infection in the liver with more than 95% infected hepatocytes by day 14 p.i. This infection outcome provided a valuable comparator to evaluate the antiviral effects of REP 2006 and REP 2031.

Previous work performed in the DHBV model shows that in the absence of antiviral treatment the levels of serum DHBsAg are proportional to the percentage of infected hepatocytes in the DHBV infected ducks at an early phase of infection (Jilbert *et al.* 1996; Triyatni *et al.* 2001; Meier *et al.* 2003). This finding was true to some extent in the current study in which the levels of DHBsAg present in the sera of the majority of NS-treated ducks (3/5 ducks treated with NS) had detectable levels of DHBsAg on day 5 and 10 p.i. All the ducks treated with NS had more than 95% infected hepatocytes in their liver at this time point (Table 4.4; Figures 4.2 and 4.3). Two out of 5 ducks in this Group had no detectable levels of DHBsAg in the serum and this may be due to the complexing of DHBsAg with antibodies in the serum and that prevented the detection of DHBsAg in the serum. This finding has been reported in other studies (Foster *et al.* 2003; Foster *et al.* 2005; Miller *et al.* 2006a; Miller *et al.* 2006b; Miller *et al.* 2008). Furthermore, varying levels of DHBV replication in the liver following inoculation with DHBV might explain the undetectable level of serum DHBsAg in 2/5 NS-treated ducks.

ETV treatment of ducks was used as a positive comparison for measurable antiviral activity because ETV is a known inhibitor of DHBV. The mechanism of the anti-DHBV activity of ETV is through inhibiting the reverse transcription step in the DHBV replication (Marion *et al.* 2002). Furthermore, effective anti-DHBV activity of ETV has been reported against DHBV infection in ducks (Foster *et al.* 2003; Foster *et al.* 2005; Miller *et al.* 2008). In the current study, on day 14 p.i. (at treatment endpoint), the mean percentage of DHBsAg-positive hepatocytes in the livers of Group 4.A REP 2006-treated ducks was less

than that of the Group 4.C ETV-treated ducks. This indicated the superior anti-DHBV activity of REP 2006. However, DHBV DNA detection in the cellular and viral DNA extract was unable to differentiate the different levels of hepatic DHBV DNA in Groups 4.A and 4.C ducks and this may due to low sensitivity of Southern blot hybridization used to detect the DHBV DNA. More sensitive PCR or qPCR assays could be used to quantify the DHBV DNA. However, cations such as Mg^{2+} used in the PCR amplification can be partially or fully inhibited by APDPs (Vaillant and Liang unpublished) as the livers were collected at the treatment endpoint at which time the APDP concentration in the liver would be high. Any possible interference in the PCR amplification process can give rise to false negative results for DHBV DNA and this can be interpreted as the true antiviral effect of REP 2006. Therefore, to avoid confusion Southern blot hybridization was used to detect the hepatic DHBV DNA.

The antiviral mechanism of action of REP 2006 against DHBV infection is different to that of ETV. REP 2006 appears to inhibit DHBV through its ability to interact with the amphipathic alpha helical interactions between DHBV and duck hepatocytes. The ability of REP 2006 to interact with these amphipathic alpha helical interactions between the virus and the hepatocyte is through a mechanism that is sequence independent but chemistry dependent (Noordeen *et al.* unpublished: Chapter 3). Hence, ETV treatment in ducks was only used to evaluate the measurable antiviral activity despite having a different mechanism of action for inhibiting DHBV infection compared to that of REP 2006.

Treatment of ducks with REP 2006 or ETV (Figure 4.1) prevented the development of serum DHBsAg in 5/5 ducks. Moreover, these livers had undetectable or very low levels of infected hepatocytes as detected by immuno-staining (Table 4.4; Figures 4.2 and 4.3). This appears to be due to the anti-DHBV activity elicited by REP 2006 and ETV in the respective Groups of ducks.

REP 2006 is a fully degenerate 40mer and showed an excellent anti-DHBV activity *in vivo* and inhibited DHBV infection. REP 2031 is also a 40mer but has a poly cytosine sequence and this compound demonstrated anti-DHBV activity only at the early phase of the infection. This agrees with the results of *in vitro* anti-DHBV activity of REP 2031 especially against established DHBV infection. However, in the *in vivo* experiment the treatment started 1 day prior to DHBV infection. This finding strengthens the hypothesis made in

Chapter 3 explaining the inefficient *in vitro* anti-DHBV activity of REP 2031. That is, the activity of these compounds must occur at least in part in a low pH environment where REP 2031 undergoes some conformational changes that neutralize its ability to interfere with the amphipathic interaction between the DHBV and the hepatocyte. Hence, REP 2031 becomes ineffective in inhibiting DHBV infection. These low-pH dependent conformational changes do not occur with REP 2006 and so it remains fully potent.

In conclusion, REP 2006 and ETV as previously reported showed excellent anti-DHBV activity *in vivo*. REP 2006-treated ducks experienced some adverse effects such as abdominal discomfort on IP injection, more bleeding at the incision site during biopsy than the NS-treated ducks and marginal increases in WBC counts that were not statistically significant. The abnormalities experienced during REP 2006 treatment may be due to the presence of CpG motifs in the sequence of REP 2006. CpG motifs are known to stimulate the proinflammatory immune activation in humans and mice (Krieg 2000; Agrawal and Kandimalla 2001; Shen *et al.* 2002; Wilson *et al.* 2006). Thus CpG-related proinflammatory response may have contributed to the side effects which appear to be restricted to the periphery because liver enzyme analysis showed unaltered levels of liver enzymes when compared with the liver enzyme levels of Groups 4.B-4.D. Moreover, the values observed for liver enzymes GGT, AST and ALT were within the range of previously published values for these enzymes for ducks (Foster *et al.* 2003).

REP 2006 has also been used to treat other species such as mice and this APDP did not cause any detectable proinflammatory response in this species with changes in clinicopathological parameters when given to mice that were not infected with murine CMV (Cardin *et al.* 2009). However, treatment with REP 2006 to mice infected with murine CMV produced splenomegaly which is consistent with CpG-realated activity (Cardin *et al.* 2009).

REP 2031 only partially blocked DHBV infection at an early phase and was unable to block the DHBV infection at the late phase. REP 2031 was well tolerated by ducks during the trial period with no adverse reactions (no abdominal discomfort, no gross pathological lesions and no changes in WBC count) observed during clinical monitoring. In agreement with our finding of REP 2031 treatment in ducks, mice treated with REP 2031 against murine CMV infection also did not show any detectable proinflammatory changes (Cardin

et al. 2009) as REP 2031 is a homopolymeric cytosine compound which lacks CpG in its sequence.

Based on these results, it was decided to use a different APDP, REP 2055, which combines the active features of REP 2006 and the lack of CpG activity present in REP 2031. The antiviral efficacy and dose optimization of REP 2055 against DHBV infection are discussed in Chapter 5.

Table 4.1: The effect of REP 2006, REP 2031, ETV and NS treatment on duck weights

Treatment Groups	Body weights (g)					
	Age (days)					
	Duck No	**14**	**17**	**20**	**23**	**27**
4.A REP 2006 IP[a] Daily only until 4 days p.i. and every other day thereafter	551[b]	395	470	579	975	1025
	552[b]	395	445	569	745	979
	553[b]	385	415	450	490	550
	554	405	489	569	798	950
	555	400	497	595	785	1015
Mean body weight		***396[c]***	***432.2[d]***	***552.4[e]***	***758.6[f]***	***903.8[g]***
4.B REP 2031 IP[a] Daily	556	395	475	595	895	1009
	557	415	495	598	795	1229
	558	425	537	695	958	1300
	559	405	475	595	755	950
	560	415	487	695	938	1220
Mean body weight		***411[c]***	***493.8[d]***	***635.6[e]***	***868.2[f]***	***1141.6[g]***
4.C ETV Orally Daily	561	375	495	685	895	955
	562	405	500	628	895	1129
	563	415	530	695	958	1250
	564	405	515	635	755	950
	565	400	487	695	938	1220
Mean body weight		***400[c]***	***505.4[d]***	***667.6[e]***	***888.2[f]***	***1100.8[g]***
4.D NS IP[a] Daily	566	395	505	785	995	1335
	567	405	515	728	895	1125
	568	425	539	795	998	1350
	569	405	525	645	765	995
	570	400	587	675	958	1120
Mean body weight		***406[c]***	***534.2[d]***	***725.6[e]***	***922.2[f]***	***1185[g]***

[a] IP-Intraperitoneal injection;

[b] Abdominal discomfort on IP injection;

Mean body weight at 14[c], 17[d], 20[e], 23[f] and 27[g] days of age;

Differences in the mean body weight among Groups 4.A, 4.B, 4.C and 4.D on 14, 17, 20, 23 and 27 days of age were statistically analysed and differences were not significant ($p>0.05$).

Table 4.2: The effect of REP 2006, REP 2031, ETV and NS treatment on blood cells

Treatment Groups	Duck No	Total RBC[a] and WBC[b] counts/L	
		Total RBC[a]	**Total WBC[b]**
4.A REP 2006	553	0.7×10^{12}	25.38×10^{9}
	554	1.82×10^{12}	27.06×10^{9}
	555	1.24×10^{12}	18.31×10^{9}
Mean		***1.25×10^{12}*** **c**	***23.59×10^{9}*** **d**
4.B REP 2031	556	2.39×10^{12}	20.69×10^{9}
	558	2.02×10^{12}	13.22×10^{9}
	560	2.43×10^{12}	26.02×10^{9}
Mean		***2.28×10^{12}*** **e**	***19.95×10^{9}*** **f**
4.C ETV	562	1.82×10^{12}	17.02×10^{9}
	563	1.69×10^{12}	12.18×10^{9}
	564	1.70×10^{12}	13.71×10^{9}
	565	1.99×10^{12}	Not corrected for NRBCs[g]
Mean		***1.8×10^{12}*** **h**	***10.73×10^{9}*** **i**
4.D NS	566	2.45×10^{12}	17.79×10^{9}
	567	2.39×10^{12}	Not corrected for NRBCs[g]
	568	2.81×10^{12}	16.83×10^{9}
	569	2.49×10^{12}	16.25×10^{9}
	570	2.37×10^{12}	15.67×10^{9}
Mean		***2.5×10^{12}*** **j**	***13.31×10^{9}*** **k**

[a] Red blood cells expressed as per litre (RBC/L);

[b] White blood cells expressed as per litre (WBC/L);

[c] Mean RBC and [d] WBC count of REP 2006 treated ducks;

[e] Mean RBC and [f] WBC count of REP 2031 treated ducks;

[g] NRBCs: Nucleated red blood cells;

[h] Mean RBC and [I] WBC count of ETV treated ducks;

[j] Mean RBC and [k] WBC count of NS treated ducks;

Mean WBC counts among Groups 4.A, 4.B, 4.C and 4.D at autopsy were analysed and differences were not statistically significant ($p>0.05$).

Table 4.3: The effect of REP 2006, REP 2031, ETV and NS treatment of ducks on liver enzymes

Treatment Groups	Duck No	Liver enzymes GGT[a], ALT[b] and AST[c] (U/L)					
		GGT[a]	Mean GGT[a]	ALT[b]	Mean ALT[b]	AST[c]	Mean AST[c]
4.A REP 2006	551	3	**5[d]**	31	**28.6[e]**	26	**38.3[f]**
	553	7		27		76	
	554	5		28		13	
4.B REP 2031	556	5	**5.2[d]**	42	**43.6[e]**	12	**45.2[f]**
	557	3		53		108	
	558	7		35		26	
	559	4		45		61	
	560	7		43		19	
4.C ETV	561	4	**4.3[d]**	86	**49[e]**	320	**119[f]**
	562	4		26		18	
	563	5		35		19	
4.D NS	566	5	**5[d]**	40	**39.4[e]**	14	**32.4[f]**
	567	5		28		31	
	568	5		48		38	
	569	5		31		17	
	570	5		50		62	

[a] GGT: γ Glutamyl transferase;

[b] ALT: Alanine amino transferase;

[c] AST: Aspartate transferase;

[d] Mean levels of GGT;

[e] Mean levels of ALT;

[f] Mean levels of ALT;

Normal range (mean± standard deviation) for duck liver enzymes, GGT = 2.3 ± 1.2, ALT = 26.6 ± 7.7 and AST = 15.9 ± 5.9 U/L U/L; Units per litre (Foster *et al.* 2003);

Mean liver enzyme levels among Groups 4.A, 4.B, 4.C and 4.D were analysed and differences were not statistically significant ($p>0.05$).

Table 4.4: The antiviral effect of REP 2006, REP 2031, ETV and NS treatment on DHBsAg-positive hepatocytes

Treatment Groups	Duck No	Path No	% DHBsAg-positive hepatocytes on day 4 p.i.	% DHBsAg-positive hepatocytes on day 14 p.i.
4.A REP 2006	551	5019/5059	0.008	<0.001[a]
	552	5021/5061	0.005	<0.001[a]
	553	5023/5063	0.005	<0.001[a]
	554	5025/5065	0.005	<0.001[a]
	555	5027/5067	0.007	<0.001[a]
Mean % DHBsAg positive hepatocytes			***0.006***[b]	***0.001***[c]
4.B REP 2031	556	5029/5069	0.193	>95
	557	5031/5071	0.291	>95
	558	5033/5073	0.429	>95
	559	5035/5075	0.085	>95
	560	5037/5077	0.042	>95
Mean % DHBsAg positive hepatocytes			***0.209***[b]	***>95***[c]
4.C ETV	561	5049/5089	0.005	0.005
	562	5051/5091	0.004	0.004
	563	5053/5093	0.025	0.005
	564	5055/5095	0.019	0.005
	565	5057/5097	0.062	0.005
Mean % DHBsAg positive hepatocytes			***0.023***[b]	***0.046***[c]
4.D NS	566	5039/5089	2.728	>95
	567	5041/5091	No liver tissue collected	>95
	568	5043/5093	0.432	>95
	569	5045/5095	1.289	>95
	570	5047/5097	1.698	>95
Mean % DHBsAg positive hepatocytes			***1.537***[b]	***>95***[c]

[a] Lower limit of detection of DHBsAg-positive hepatocytes is 0.001%;

Mean DHBsAg-positive hepatocytes on day [b]4 and [c]14 p.i.;

Mean % DHBsAg-positive hepatocytes among Groups 4.A, 4.B, 4.C and 4.D were analysed and differences in mean % DHBsAg-positive hepatocytes of Groups 4.A and 4.C, were statistically significant to that of Groups 4.B and 4.D ($p < 0.05$).

Figure 4.1: DHBsAg levels in the sera of REP 2006 (Panel A), REP 2031 (Panel B), ETV (Panel C) and NS (Panel D) treated ducks. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with REP 2006 or REP 2031 or ETV or NS from 1 day prior to DHBV infection for 15 days.

Serum samples were tested for DHBsAg levels using a qualitative enzyme linked immunosorbent assay (ELISA) described in Section 2.8.1.

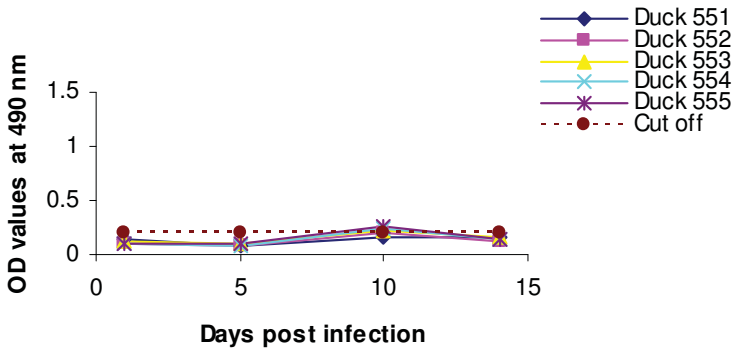
Panel A: Group 4.A - REP 2006, 10 mg/kg, IP only until 4 days p.i. and every other day thereafter;

Panel B: Group 4.B - REP 2031, 10 mg/kg, IP daily;

Panel C: Group 4.C - ETV, 1 mg/kg, orally daily;

Panel D: Group 4.D - NS, IP daily.

A


Duck 551
Duck 552
Duck 553
Duck 554
Duck 555
Cut off
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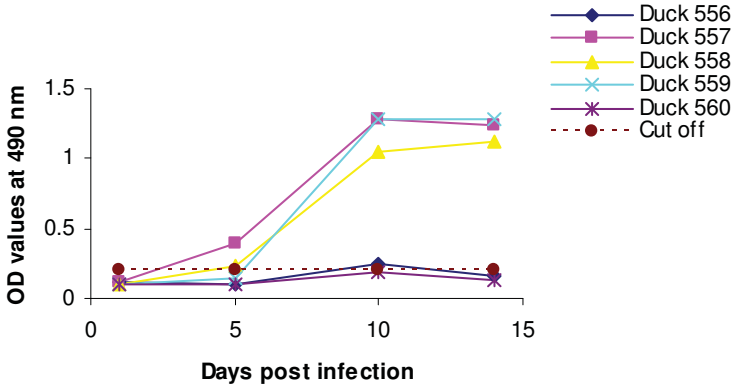
1.5
1
0.5
0
0
5
10
15
Days post infection

B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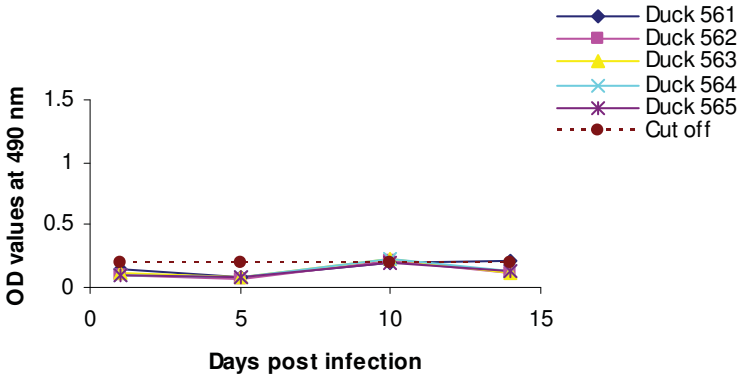
Duck 557
Duck 558
Duck 559
Duck 560
Cut off
OD values at 490 nm
1.5
1
0.5
0
0
5
10
15
Days post infection

C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Duck 562
Duck 563
Duck 564
Duck 565
Cut off
OD values at 490 nm
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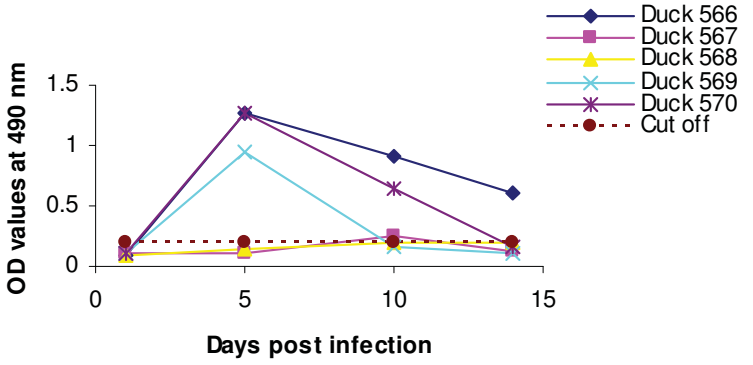
1
0.5
0
0
5
10
15
Days post infection

D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Duck 567
Duck 568
Duck 569
Duck 570
Cut off
OD values at 490 nm
1.5
1
0.5
0
0
5
10
15
Days post infection

Figure 4.2: The percentage of DHBsAg in the liver of REP 2006 (Panel A), REP 2031 (Panel B), ETV (Panel C) and NS (Panel D) treated ducks on day 4 and 14 p.i. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with REP 2006 or ETV or REP 2031 or NS from 1 day prior to DHBV infection for 15 days.

Panel A: Group 4.A - REP 2006, 10 mg/kg, IP daily only until 4 days p.i. and every other day thereafter; ;

Panel B: Group 4.B - REP 2031, 10 mg/kg, IP daily;

Panel C: Group 4.C - ETV, 1 mg/kg, orally daily;

Panel D: Group 4.D - NS, IP daily.

Liver samples were tested for DHBsAg-positive hepatocytes using immuno-staining methods described in Section 2.9.2.

All the sections were photographed using 400x magnification and sections were counter stained with haematoxylin. Arrows indicate the DHBsAg-positive hepatocytes (brown) in liver sections.

The sensitivity of detection of cytoplasmic DHBsAg by immuno-staining is <0.001. This level of sensitivity was calculated by counting a total of 100,000 hepatocytes in the liver tissue sections as explained in Section 2.9.2.

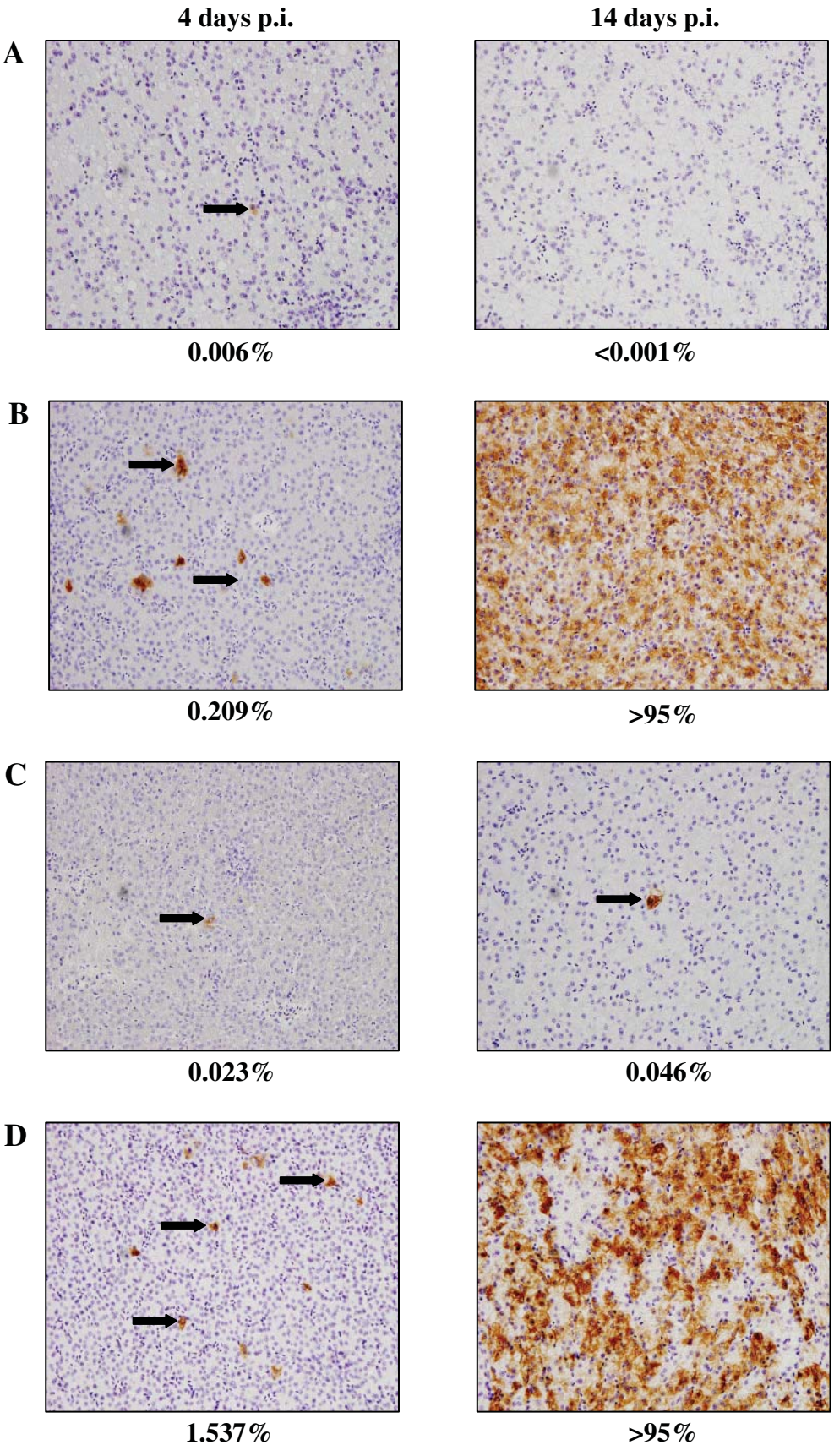

4 days p.i.
14 days p.i.
A
0.006%
<0.001%
B
0.209%
>95%
C
0.023%
0.046%
D
1.537%
>95%

Figure 4.3: DHBV DNA levels in the liver of REP 2006 (Panels A and E), REP 2031 (Panels B and F), ETV (Panels C and G) and NS (Panels D and H) treated ducks on day 4 and 14 p.i. 14-day-old ducks were inoculated with 5 x 10^8 DNA DHBV genomes and treated with REP 2006 or ETV or REP 2031 or NS from 1 day prior to DHBV infection for 15 days.

Cellular and viral DNA extracts were tested for DHBV DNA by Southern blot hybridisation using methods described in Sections 2.10.2 and 2.10.3 (Radiographic exposure time: 24 hours).

Lane I: DHBV plasmid pBL4.8 X 2 (100 pg).

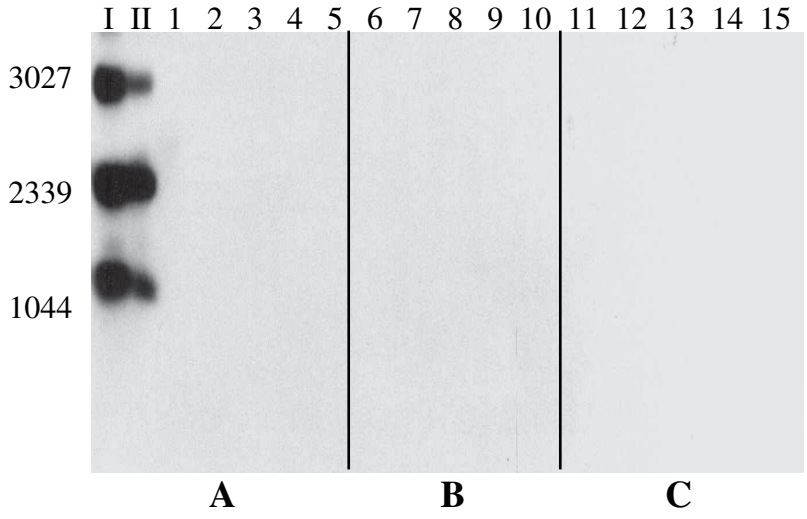
Lane II: DHBV plasmid pBL4.8 X 2 (10 pg).

Panels A and E: Group 4.A - REP 2006, 10 mg/kg, IP daily only until 4 days p.i. and every other day thereafter; Lanes 1-5 (day 4 p.i.) and lanes 21-25 (day 14 p.i.).

Panels B and F: Group 4.B - REP 2031, 10 mg/kg, IP daily; Lanes 6-10 (day 4 p.i.) and lanes 26-30 (day 14 p.i.).

Panels C and G: Group 4.C - ETV, 1 mg/kg, orally daily; Lanes 11-15 (day 4 p.i.) and lanes 31-35 (day 14 p.i.).

Panels D and H: Group 4.D - NS, IP daily; Lanes 16-20 (day 4 p.i.) and lanes 36-40 (day 14 p.i.).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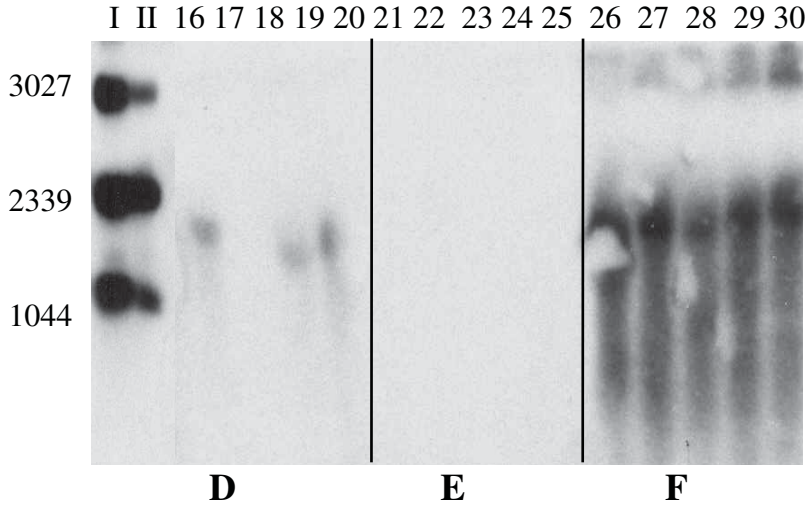
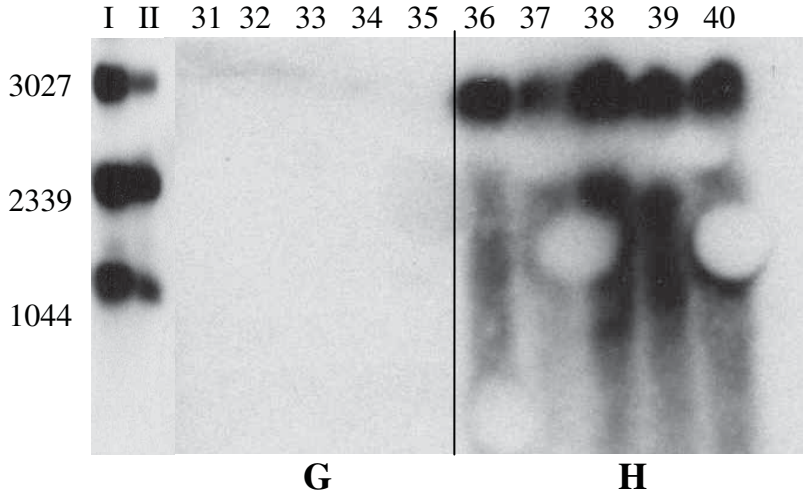
3027
2339
1044
A
B
C


I II 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
3027
2339
1044
D
E
F


I II 31 32 33 34 35 36 37 38 39 40
3027
2339
1044
G
H