Development of antiviral therapies for chronic hepatitis B virus infection

Faseeha Noordeen

B.VSc., M.Phil.

A thesis submitted for the Degree of Doctor of Philosophy

School of Molecular and Biomedical Science

The University of Adelaide

Australia

July 2009

Chapter 5: *In vivo* studies on antiviral efficacy and the effective dose range of REP 2055 against DHBV infection

5.1 Introduction

Present studies were based on the results of experiments discussed in Chapters 3 and 4 in which the APDP, REP 2006, a degenerate population of 40mer PS-ONs, was shown to be effective in inhibiting the DHBV infection *in vitro* and *in vivo*. REP 2031, a homopolymeric 40mer PS-ON with a poly cytosine sequence, was also investigated for its antiviral activity against DHBV infection (Noordeen *et al.* unpublished, Chapters 3 and 4). It was hypothesised that REP 2031 loses its therapeutically active structure under low pH conditions and becomes ineffective in inhibiting DHBV infection (Noordeen *et al.* unpublished, Chapter 4).

On the other hand, when the safety of a therapeutic agent is concerned, APDP REP 2031 was well tolerated by the ducks during the study period. There were no detectable drug induced side effects or adverse reactions observed in ducks treated with REP 2031 when ducks were clinically assessed for any abnormalities in feed and water intake, weight loss, gait or changes in behaviour and abdominal palpation for signs of pain. In contrast, as described in Chapter 4, ducks treated with REP 2006 had abdominal tenderness and signs of pain on abdominal palpation and during IP injections. Furthermore, ducks treated with REP 2006, bled more than those treated with NS or REP 2031 at biopsy. It was hypothesised that these adverse effects may be due to the possible influence of CpG motifs present in the REP 2006 sequence. CpG motifs are able to induce TLR 9, which triggers proinflammatory activities (Krieg 2000; Agrawal and Kandimalla 2001; Shen *et al.* 2002; Wilson *et al.* 2006; Plitas *et al.* 2008; Wang *et al.* 2008) whereas REP 2031, being a poly-C compound, does not have CpG motifs that can trigger potential proinflammatory activities. Based on these data, it was decided to test the efficacy and safety of REP 2055, a heteropolymeric APDP with the sequence $(AC)_{20}$. This compound had been previously shown to have comparable antiviral activity against other viruses while lacking the CpG-mediated reactivity (Vaillant *et al.* unpublished).

For *in vivo* experiments, REP 2055 was prepared as a SS under GMP-like conditions. Like all PS-ONs that were tested in clinical trials (Yu *et al.* 2007; Yu *et al.* 2008), it was anticipated that REP 2055 would be non-toxic and non-carcinogenic

Furthermore, PS-ON compounds that are similar to REP 2055 are known to concentrate in the liver of mammalian species (Henry *et al.* 1999; Geary *et al.* 2003; Yu *et al.* 2007; Yu *et al.* 2008). Liver cells are the target cells for HBV or DHBV infection in their natural hosts and it is in the liver in which the bulk of HBV (Mast *et al.* 2005; McMahon 2005; Yuen and Lai 2008) and DHBV (Le Mire *et al.* 2005; Jilbert *et al.* 2008; Zoulim *et al.* 2008b) replication occurs. These anticipated characteristics of REP 2055 are expected to make this compound an effective antiviral agent against DHBV infection without producing adverse reactions.

The current project aimed to test the antiviral activity of REP 2055 against DHBV infection *in vivo*. It also examines the effective dose range for treating DHBV infection using 5 different doses from 0.5 to 10 mg/kg body weight, administered to 14-day-old ducks.

5.2 Experimental design

Approval to conduct the *in vivo* studies was obtained from the Animal Ethics Committees of the IMVS, and University of Adelaide. Chapter 2 provides details on the following experimental requirements: source and the dose of DHBV; preparation and reconstitution of REP 2055 for IP injection; collection of blood samples; autopsy and specimen collection procedures; analysis of blood and serum for virological (DHBsAg); and biochemical markers (liver enzymes) to assess the level of DHBV infection.

14-day-old ducks were divided into 3 Groups of 5 (Groups 5.A, 5.B and 5.C) for Experiment I (Table 5.1) and 6 Groups of 5 (Groups 5.D, 5.E, 5.F, 5.G, 5.H and 5.I) for Experiment II (Table 5.2). Ducks were infected with 5 x 10^8 DHBV DNA genomes via jugular vein inoculation. The ducks in Experiment I received daily treatment of REP 2055 (10 mg/kg, IP) or ETV (1 mg/kg, oral) or NS (IP) from 1 day prior to DHBV infection for 15 days. The ducks in Experiment II received daily treatment of REP 2055 in five dose regimens (0.5, 2, 3 and 5 mg/kg, twice daily, IP and 10 mg/kg, once daily, IP) and NS (twice daily, IP) from 3 days prior to DHBV infection for 17 days. The effectiveness of a dose

range was tested after confirming that APDPs, REP 2006 and REP 2055 work effectively as anti-DHBV agents at a standard dose of 10 mg/kg. It is also true that other DNA-based pharmacological agents (such as an 2' ribose modified antisense PS-ON directed against the TNF-α mRNA) have been shown to be safe between a range of doses of 0.1-10 mg/kg among a wide range of animal species (Geary *et al.* 2003). Hence, identifying an effective dose for various prophylactic or therapeutic applications is necessary in order to avoid overdosing and its implications.

Ducks were examined every day for any abnormalities in feed and water intake, weight loss or changes in gait and behaviour. Ducks were also clinically examined through a gentle palpation of the abdomen for signs of pain in the abdominal area. IP injection was given just below the sternal end where the access to the peritoneal cavity is easy and ducks were examined for signs of pain at the time of IP injection.

WBC counts were performed at the Division of Haematology of the Diagnostic Clinical Pathology unit at the IMVS using whole blood samples collected at autopsy from all ducks in Groups 5.A-5.C. Sera separated from blood samples were also analysed for levels of liver enzymes (or transaminases) to elucidate the liver function in the presence of REP 2055. Sera of ETV- and NS-treated ducks were used as comparators for liver enzyme analysis. Methods used for these analyses are discussed in detail in Chapter 2.

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

Statistical analysis: Differences in mean body weights, WBC counts, liver enzyme levels and percentage of DHBsAg-positive hepatocytes in liver tissues among Groups 5.A, 5.B and 5.C in Experiment I and Groups 5.D, 5.E, 5.F, 5.G, 5.H and 5.I in Experiment II 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 to be statistically significant when the *p* values were <0.05.

5.3 Results

Previous data from experiments using 14-day-old ducks treated with REP 2006 showed an excellent anti-DHBV activity, however three ducks experienced some adverse reactions to REP 2006 treatment. The CpG motifs present REP 2006 was thought to be responsible for those adverse effects and were absent in REP 2055.

The current project was conducted to test: firstly, the anti-DHBV activity of the new APDP, REP 2055 *in vivo* (Experiment I); and secondly, to study the effective range of dose of REP 2055 against DHBV infection (Experiment II).

5.3.1 Experiment I: Testing the antiviral efficacy of REP 2055 against DHBV infection using a standard dose (10 mg/kg) and once daily administration

5.3.1.1 Group 5.A: The effect of REP 2055 treatment on clinicopathological, haematological, biochemical and virological markers

REP 2055 treatment in ducks did not produce any observable changes in duck health or weight (Table 5.1). Ducks treated with REP 2055 showed neither abdominal tenderness nor abdominal pain on IP injection and no abnormalities were noted during the clinical examination of the abdomen. Furthermore, *in situ* examination of internal organs at autopsy did not reveal any gross pathological changes in ducks in both experiments.

Moreover, no significant differences were noted in the mean total WBC count in ducks ($p>0.05$) receiving REP 2055 when compared to ducks receiving ETV or NS when tested at the end of 15 days of treatment (Table 5.3).

There were no significant differences in the mean levels of liver enzymes in ducks treated with REP 2055 in comparison to ducks treated with ETV or NS. This was in terms of changes in transaminases GGT, ALT and AST when tested at the end of 15 days of treatment ($p>0.05$) (Table 5.4).

Treatment of ducks with REP 2055 (Group 5.A) prevented the development of serum DHBsAg in 5/5 ducks when sera from these ducks were tested using a qualitative DHBsAg ELISA (Figure 5.1).

On day 4 p.i., liver tissues from ducks treated with REP 2055 had DHBV infection in 0.004–0.0126% of hepatocytes (mean=0.007%) when tested by immuno-staining (Table 5.6; Figure 5.3). On day 14 p.i., these ducks had a mean of <0.001% of DHBsAg-positive hepatocytes (Table 5.6; Figure 5.3). Treatment with REP 2055 reduced the mean percentage of DHBsAg-positive hepatocytes from 0.007% (Table 5.6; Figure 5.3) to <0.001% on day 14 p.i. The mean differences of DHBsAg-positive hepatocytes in the liver of REP 2055-treated ducks when compared with that of NS-treated ducks at biopsy and autopsy were statistically significant ($p<0.05$).

DHBV DNA in liver as detected by Southern blot hybridisation agreed with the results of assessing the DHBV infection by immuno-staining of liver tissue sections (Figures 5.3 and 5.5).

In summary, REP 2055 treatment prevented the DHBV infection in the serum and the liver without producing observable drug induced side effects (clinico-pathological, haematological and biochemical changes) in treated ducks.

5.3.1.2 Group 5.B: The effect of ETV treatment on clinicopathological, haematological, biochemical and virological markers

Ducks treated with ETV had no observable changes in general health and weight during the clinical monitoring period. *In situ* examination of internal organs at autopsy revealed no gross pathological changes in ducks treated with ETV. No significant differences were noted in the mean total WBC count in ducks ($p>0.05$) receiving ETV when compared to ducks receiving REP 2055 or NS (Table 5.3). There were no significant differences in the mean levels of liver enzymes among the ducks treated with ETV when compared with ducks treated with REP 2055 and NS in terms of changes in transaminases GGT, ALT and AST ($p>0.05$) (Table 5.4).

Treatment of ducks with ETV prevented the development of serum DHBsAg in 5/5 ducks when sera from these ducks were tested using a qualitative DHBsAg ELISA (Figure 5.1).

Treatment with ETV reduced the percentage of DHBsAg-positive hepatocytes on days 4 and 14 p.i. from 0.009% to 0.006% (Table 5.6; Figure 5.3). In contrast, ducks treated with NS had 1.412% of DHBsAg-positive hepatocytes on day 4 p.i. and this increased to >95% on day 14 p.i. (Table 5.6; Figures 5.3). The mean differences of DHBsAg-positive hepatocytes of ETV-treated ducks when compared with DHBsAg-positive hepatocytes of NS-treated ducks at biopsy and autopsy liver samples were statistically significant ($p<0.05$).

In the ETV-treated ducks, the levels of DHBV DNA in the liver tested by Southern blot hybridisation were consistent with the levels of DHBV infection tested by immuno-staining (Figures 5.3 and 5.5).

In summary ETV treatment was able to prevent the development of DHBV infection in the serum and in the liver without producing observable drug induced side effects in ducks in terms of clinicopathological, haematological and biochemical changes.

5.3.1.3 Group 5.C: The effect of NS treatment on clinicopathological, haematological, biochemical and virological markers

Ducks treated with NS recorded no observable changes in general health and weight during the clinical monitoring period. *In situ* examination of internal organs at autopsy revealed no gross pathological changes in ducks treated with NS. The mean total WBC count and the mean levels of liver enzymes of ducks treated with NS provided a good internal comparison of these parameters in ducks treated with REP 2055 (Tables 5.3 and 5.4).

In contrast to REP 2055 or ETV-treated ducks, 5/5 ducks treated with NS had detectable levels of serum DHBsAg on days 5, 10 and 14 p.i. (Figure 5.1). As expected, ducks treated with NS had 1.412% of DHBsAg-positive hepatocytes on day 4 p.i. and this increased to >95% on day 14 p.i. demonstrating the widespread nature of DHBV infection (Table 5.6; Figure 5.3). In this respect, the antiviral effect of REP 2055 became very obvious when compared to the levels of DHBV infection in the serum and liver of NS-treated ducks.

5.3.2 Experiment II: Testing the antiviral efficacy of REP 2055 against DHBV infection using a range of dose (0.5-10mg/kg) regimens

5.3.2.1 Groups 5.D-5.H: The effect of REP 2055 treatment on clinicopathological, biochemical and virological markers between the dose range of 0.5-10 mg/kg

REP 2055 treatment in ducks did not produce any observable changes in duck health or weight during the clinical monitoring period (Table 5.2). Furthermore, ducks treated with REP 2055 in any of the 5 dose regimens showed neither abdominal tenderness nor pain on IP injection. Neither abnormalities during the clinical examination of the abdomen nor gross pathological changes on *in situ* examination of internal organs at autopsy of REP 2055-treated ducks.

A CBE was not performed for Experiment II as there were no significant changes observed when ducks were treated with REP 2055 using a standard dose of 10 mg/kg body weight in Experiment I. However, liver enzymes were analysed to ascertain any possible changes in liver enzymes as the bulk of PS-ONs concentrate in the liver (Geary *et al.* 2003; Yu *et al.* 2009a; Yu *et al.* 2009b). Treatment with REP 2055 resulted in no significant changes in transaminases GGT, ALT and AST in Groups 5.D-5.I, treated with five different doses of REP 2055 and Group 5.I, treated with NS ($p>0.05$) (Table 5.5). Furthermore, liver enzyme values noted in the present studies were within the normal values reported for ducks by Foster *et al*. (2003).

Treatment of ducks with REP 2055 in 5 different dose regimens (Groups 5.D-5.H) prevented the development of serum DHBsAg in 5/5 ducks (Figure 5.2).

In Experiment II, liver tissues collected from ducks in Groups 5.D and 5.E on day 4 p.i., DHBV infection was detected in 0.33-1.52% (mean=0.87%) and 0.21-0.72% of hepatocytes (mean=0.45%) by immuno-staining (Table 5.7; Figure 5.4). Groups 5.F, 5.G and 5.H had 0.1-0.46 (mean=0.32%), 0.001-0.07 (mean=0.02%) and 0.07-0.4 (mean=0.17%) of DHBsAg-positive hepatocytes on day 4 p.i. (Table 5.7; Figure 5.4).

On day 14 p.i., ducks in Groups 5.D-5.H receiving 5 different doses of REP 2055 treatment had <0.001% of DHBsAg-positive hepatocytes except Duck 503 in Group 5.D. This particular duck showed an increase of 4.3% of DHBsAg-positive hepatocytes (Table 5.7; Figure 5.4).

In Experiment II, the mean differences of DHBsAg-positive hepatocytes of REP 2055-treated ducks (5 different doses) when compared with DHBsAg-positive hepatocytes of NS-treated ducks at biopsy and autopsy livers were statistically significant ($p<0.05$). Moreover, in Experiment II, DHBV DNA detected by Southern blot hybridisation agreed with DHBs-positive hepatocytes present in the liver as shown by the immuno-staining (Figures 5.4 and 5.6).

However, Duck 503, which was treated with 0.5 mg/kg dose of REP 2055 had 1.52% and 4.3% of DHBsAg-positive hepatocytes in the liver on days 4 and 14 p.i., respectively. On days 4 and 14 p.i., the mean percentage of DHBsAg-positive hepatocytes in the liver of Duck 503 was higher than that of other 3 ducks in Group 5.D. However, the level of infection in Duck 503 on days 14 p.i. (4.3% of DHBsAg-positive hepatocytes) was significantly less than the mean percentage of DHBsAg-positive hepatocytes of NS-treated ducks ($p<0.05$). This could be explained in terms of the dosage of REP 2055; *i.e* 0.5 mg/kg dose of REP 2055 twice daily dosing was enough to inhibit DHBV replication in the liver to a level that was significantly superior to NS. However, this level of inhibition was not superior to REP 2055 treatments with 2-5 mg/kg twice daily dosing (Groups 5.E, 5.F and 5.G) or 10 mg/kg once daily dosing (Group 5H).

5.3.2.2 Group 5.I: The effect of NS treatment on clinicopathological, biochemical and DHBV markers

NS treatment in ducks did not produce any observable changes in the health of ducks or weight (Table 5.2) during the clinical monitoring period. No gross pathological changes were noted on *in situ* examination of internal organs at autopsy. The mean total WBC count and the mean levels of liver enzymes of NS-treated ducks provided a good internal comparison of these parameters for ducks treated with REP 2055 (Table 5.5).

In contrast to REP 2055 treated ducks, 5/5 ducks treated with NS had detectable levels of DHBsAg in the serum on days 5, 10 and 14 p.i. (Figure 5.2). As expected, ducks treated with NS had 13.56% of DHBsAg-positive hepatocytes on day 4 p.i. and this increased to >95% on day 14 p.i., indicating that DHBV infection was widespread (Table 5.7; Figure 5.4). In this respect, the antiviral effect of REP 2055 became very clear in all 5 dose

regimens when compared to the levels of DHBV infection in the serum and liver of NS-treated ducks.

5.4 Discussion

Studies explained in Experiments I and II produced an anticipated DHBV infection outcome when inoculated with 5 x 10^8 DHBV DNA genomes in 14-day-old ducks (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 virus dose and treated with NS developed widespread DHBV infection in the liver with more than 95% infected hepatocytes by day 14 p.i. (Tables 5.6 and 5.7; Figures 5.3 and 5.4). The levels of DHBsAg present in the sera of NS-treated ducks were proportional to the DHBV infection in the liver in the current study. This finding is in complete agreement with previous studies (Jilbert *et al.* 1996; Triyatni *et al.* 1998; Meier *et al.* 2003) in which the serum DHBsAg levels are proportional to the percentage of infected hepatocytes in the liver at the early phase of infection.

In Experiment I, treatment of ducks in Groups 5.A and 5.B with REP 2055 or ETV (Figure 5.1) prevented the development of serum DHBsAg in 5/5 ducks. Moreover, livers of these ducks had undetectable or a very low levels of DHBsAg-positive hepatocytes (Table 5.6; Figure 5.3). REP 2055 appeared to accomplish the predicted anti-DHBV activity of this APDP, similar to its parent compound REP 2006. In Experiment I, results from the Southern blot hybridisation were in complete agreement with results obtained from the immuno-staining when assessing the percentage of infected hepatocytes in the liver on days 4 and 14 p.i. DHBV DNA were not detected in liver samples from the Groups 5.A-5.C ducks that were treated with REP 2055, ETV and NS on day 4 p.i. The amount of DHBV DNA in liver tissues at this stage of the infection might be below the lower limit of the assay. However, on day 14 p.i., no DHBV DNA was detected in liver samples from the Groups 5.A and 5.B ducks treated with REP 2055 or ETV showing the ability of REP 2055 and ETV to suppress the DHBV infection to an undetectable level. In this respect, measurable anti-DHBV activity of REP 2055 might be similar or superior to ETV, which is a known suppressor of DHBV (Marion *et al.* 2002; Foster *et al.* 2003; Foster *et al.* 2005; Miller *et al.*2006a; Miller *et al.* 2006b; Miller *et al.* 2008). DHBV DNA was detected in all liver samples from the Group

5.C ducks treated with NS indicating the occurrence of widespread DHBV infection in the absence of an effective antiviral treatment (Figure 5.5).

In Experiment II, treatment of ducks with REP 2055 in 5 different doses prevented the development of serum DHBsAg in 5/5 ducks (Figure 5.2). Livers of these ducks had undetectable or a very low percentage of DHBsAg-positive hepatocytes as detected by immuno-staining (Table 5.7; Figure 5.4) supporting the anti-DHBV activity of REP 2055 at all 5 doses. The antiviral activity of REP 2055 between the doses of 2-10 mg/kg in Experiment II was similar to what was observed in Experiment I, when investigating the anti-DHBV activity of REP 2055 with a standard dose of 10 mg/kg daily dosing for 15 days starting from 1 day prior to DHBV infection. Similar to Experiment I, the levels of DHBV DNA detected in the liver by Southern blot hybridisation agreed with results obtained from the immuno-staining, when assessing the percentage of infected hepatocytes in the liver of ducks on day 4 and 14 p.i. in Experiment II. No DHBV DNA was detected in liver samples of ducks treated with any of the five doses of REP 2055 or NS on day 4 p.i. On day 14 p.i., no DHBV DNA was detected in any liver samples from the Group 5.D-5.H ducks treated with REP 2055, indicating the anti-DHBV activity of REP 2055 under a range of doses. In contrast, DHBV DNA was detected in all liver samples from Group 5.I ducks treated with NS on day 14 p.i. (Figure 5.6).

The relatively higher levels of DHBsAg-positive hepatocytes on days 4 and 14 p.i. in Duck 503 than other REP 2055-treated ducks of (Groups 5.E, 5.F, 5.G, 5.H and 5.I) can be explained in terms of 0.5 mg/kg dose of REP 2055 twice daily dosing being sufficient to inhibit the DHBV replication in the liver to a level that was significantly superior to NS. However, this level of inhibition was not superior to REP 2055 treatments with 2-5 mg/kg twice daily dosing (Groups 5.E, 5.F and 5.G) or 10 mg/kg once daily dosing (Group 5.H). In this respect, a 0.5 mg/kg twice daily dosing appeared weaker in achieving optimal plasma concentration that was effective in suppressing DHBV and then its spread in the liver. Hence, continuous suboptimal dosing of the drug would have failed to build a therapeutic maintenance drug level in the plasma and liver in order to elicit an effective antiviral response that had been shown by REP 2055 in other regimens (Groups 5.E, 5.F, 5.G and 5.H). The suboptimal plasma concentrations at 0.5 mg/kg dose of REP 2055 twice daily dosing appears to be associated with short plasma half life of PS-ONs in ducks (Soni *et al.*

1998). Hence, a higher dose of REP 2055 in divided or single daily regimens is expected to achieve drug levels that are effective in eliciting a better antiviral activity (until the next dosing) than lower doses such as 0.5 mg/kg regimen.

REP 2055, a 40mer poly AC phosphorothioate oligonucleotide, showed excellent anti-DHBV activity in Experiment I with a standard dose of 10 mg/kg and in all 5 doses tested in Experiment II. This finding reinforced the conclusion that this compound could inhibit DHBV infection in a sequence independent manner similar to that of its parent compound REP 2006 in terms of its antiviral activity. REP 2055-treated ducks did not elicit any adverse effects such as abdominal discomfort on IP injection, except mild bleeding at the incision site during biopsy in both Experiments I and II. However, the WBC count and liver enzyme analysis in Experiment I and liver enzyme analysis in Experiment II showed no alterations in liver function leading to changes in liver enzymes.

In conclusion, REP 2055 showed an excellent anti-DHBV activity and was well tolerated *in vivo* in all 5 different doses when the treatment started 1 day or 3 days prior to infection. However, the autopsy was performed at the treatment end point to test for virological markers and this did not provide the opportunity to see what might have happened if the treatment was stopped and the ducks were subsequently monitored in a drug-free period. This strategy would have tested whether or not REP 2055 is able to prevent the rebound of DHBV infection. Furthermore, REP 2055 treatment was started one day or 3 days prior to DHBV infection in order to test the inhibitory effect or the anti-DHBV activity of the agent when the infection was given in the presence of the drug. It was, however, unknown what would have happened to the treatment outcome if ducks were treated at an early stage of DHBV infection or a full-blown DHBV infection and monitored after the treatment end point. These questions are clinically relevant for the treatment of chronic HBV infections in humans. The ability of REP 2055 to prevent the rebound of DHBV infection when the treatment is administered 1 day prior to or 4 and 12 days after DHBV infection was then studied and is discussed in the Chapter 6.

Table 5.1: Experiment I-The effect of REP 2055 and ETV treatment on body weight

Treatment Groups	Body weight (g)					
	Age (days)					
	Duck No.	**14**	**17**	**20**	**23**	**27**
5.A REP 2055 10 mg/kg IP[a] Daily	401	400	465	590	885	1315
	402	405	485	575	790	1220
	403	395	530	625	950	1370
	404	410	475	585	750	1155
	405	405	480	685	935	1320
Mean body weight		***403***[b]	***487***[c]	***612***[d]	***862***[e]	***1276***[f]
5.B ETV 1 mg/kg Oral Daily	406	385	460	675	885	1075
	407	425	500	620	875	1105
	408	415	510	685	955	1290
	409	415	505	625	855	1125
	410	400	480	690	945	1320
Mean body weight		***408***[b]	***491***[c]	***659***[d]	***903***[e]	***1183***[f]
5.C NS IP[a] Daily	411	390	465	755	1005	1300
	412	415	495	715	995	1225
	413	420	515	755	1095	1295
	414	425	500	655	995	1340
	415	405	505	665	1025	1320
Mean body weight		***411***[b]	***496***[c]	***709***[d]	***1023***[e]	***1296***[f]

[a] IP - Intraperitoneal injection;

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

Mean body weight of Groups 5.A, 5.B and 5.C on 14, 17, 20, 23 and 27 days of age were statistically analysed and differences were not significant ($p > 0.05$).

Table 5.2: Experiment II-The effect of REP 2055 in 5 doses on body weight of ducks

Treatment Groups	Body weight (g)						
	Age (days)						
	Duck No	**12**	**14**	**17**	**20**	**23**	**27**
5.D REP 2055 0.5 mg/kg IP[a] Twice Daily	501	300	400	495	600	985	1285
	502	305	Didn't recover from anaesthesia				
	503	295	390	495	605	780	1280
	504	310	415	495	595	800	1255
	505	305	425	515	635	925	1310
Mean body weight		***303***[b]	***408***[c]	***500***[d]	***609***[e]	[f]***873***	***1283***[g]
5.E REP 2055 2 mg/kg IP[a] Twice Daily	506	285	395	490	575	895	1175
	507	325	405	505	610	975	1135
	508	315	425	515	620	895	1280
	509	315	405	525	615	885	1195
	510	300	395	485	590	955	1315
Mean body weight		***308***[b]	***405***[c]	***504***[d]	***602***[e]	[f]***921***	***1220***[g]
5.F REP 2055 3 mg/kg IP[a] Twice Daily	511	290	385	495	655	990	1310
	512	315	405	505	645	995	1220
	513	320	410	515	650	1000	1390
	514	325	415	505	650	990	Died
	515	305	400	515	660	1015	1325
Mean body weight		***311***[b]	***403***[c]	***507***[d]	***652***[e]	[f]***998***	***1311***[g]
5.G REP 2055 5 mg/kg IP[a] Twice Daily	516	310	420	515	595	985	1295
	517	295	400	495	595	795	1120
	518	285	390	490	600	930	1290
	519	300	Didn't recover from anaesthesia				
	520	315	415	520	635	955	1310
Mean body weight		***301***[b]	***406***[c]	***505***[d]	***606***[e]	[f]***916***	***1254***[g]
5.H REP 2055 10 mg/kg IP[a] Once Daily	521	295	390	480	605	895	1195
	522	315	425	520	640	885	1125
	523	325	435	530	645	950	1295
	524	305	405	505	615	875	1145
	525	285	390	490	595	925	1310
Mean body weight		***305***[b]	***409***[c]	***505***[d]	***620***[e]	[f]***906***	***1212***[g]
5.I REP 2055 NS IP[a] Twice Daily	526	295	395	485	655	1000	1305
	527	305	400	515	625	990	1235
	528	310	415	525	655	1105	1265
	529	305	405	500	615	995	1270
	530	315	410	515	645	1000	1310
Mean body weights		***306***[b]	***405***[c]	***508***[d]	***639***[e]	***1018***[f]	***1277***[g]

[a] IP - Intraperitoneal injection; Mean body weight on 12[b], 14[c], 17[d], 20[e], 23[f] and 27[g] days of age;

Mean body weight of Groups 5.D, 5.E, 5.F, 5.G, 5.H and 5.I on 12, 14, 17, 20, 23 and 27 days of age were statistically analysed and differences were not significant ($p>0.05$).

Table 5.3: Experiment I-The effect of REP 2055 and ETV treatment on total WBCs

Treatment Groups	Duck No	Total WBC[b] counts/L	
		Total WBC[b]	Mean WBC[b]
5.A REP 2055 10 mg/kg IP[a] Daily	401	9.12×10^9	**9.08×10^{9c}**
	402	8.82×10^9	
	403	10.60×10^9	
	404	8.89×10^9	
	405	7.90×10^9	
5.B ETV 1 mg/kg Oral Daily	406	7.60×10^9	**7.95×10^{9d}**
	407	8.39×10^9	
	408	7.11×10^9	
	409	8.55×10^9	
	410	8.47×10^9	
5.C NS IP[a] Daily	411	9.42×10^9	**9.44×10^{9e}**
	412	9.16×10^9	
	413	8.66×10^9	
	414	8.58×10^9	
	415	11.40×10^9	

[a] IP - Intraperitoneal injection;

[b] WBC/L - WBC counts per litre;

Mean WBC count of REP 2055-[c], ETV-[d] and NS-[e] treated ducks;

Mean WBC count of Groups 5.A, 5.B and 5.C at autopsy were analysed and differences were statistically not significant ($p>0.05$).

Table 5.4: Experiment I: Liver enzyme levels at autopsy in REP 2055-, ETV- and NS-treated ducks

Treatment Groups	Duck No	Liver enzymes GGT[a], ALT[b] and AST[c] (U/L)[d]					
		GGT[a]	Mean GGT[a]	ALT[b]	Mean ALT[b]	AST[c]	Mean AST[c]
5.A REP 2055 10 mg/kg IP[e] Daily	401	4	**3.66[f]**	28	**25.70[g]**	15	**16.53[h]**
	402	2		38		22	
	403	5		39		17	
	404	4		28		17	
	405	3		37		18	
5.B ETV 1 mg/kg Oral Daily	406	5	**3.60[f]**	21	**27[g]**	14	**14.06[h]**
	407	3		18		10	
	408	3		27		6	
	409	3		18		16	
	410	4		51		24	
5.C NS IP[e] Daily	411	4	**2.80[f]**	28	**22.60[g]**	19	**15.64[h]**
	412	3		24		19	
	413	3		19		10	
	414	2		22		19	
	415	2		20		13	

[a] GGT: γ Glutamyl transferase;

[b] ALT: Alanine amino transferase;

[c] AST: Aspartate transferase;

[d] Units per litre

[e] IP - Intraperitoneal injection;

Mean levels of GGT [f], ALT [g] and AST [h];

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

Mean liver enzyme levels of Groups 5.A, 5.B and 5.C were statistically analysed and differences were not significant ($p>0.05$).

Table 5.5: Experiment II-Liver enzyme levels at autopsy in REP 2055- (5 doses) and NS-treated ducks

Treatment Groups	Duck No	Liver enzymes GGT[a], ALT[b] and AST[c] (U/L)[d]					
		GGT[a]	Mean GGT[a]	ALT[b]	Mean ALT[b]	AST[c]	Mean AST[c]
5.D 0.5 mg/kg IP[e] Twice Daily	501	6	**4.25[f]**	41	**38.5[g]**	13	**26.75[h]**
	503	5		22		24	
	504	2		55		37	
	505	4		36		33	
5.E 2 mg/kg IP[e] Twice Daily	506	5	**7.2[f]**	41	**34.6[g]**	25	**27.4[h]**
	507	4		32		23	
	508	19		48		57	
	509	5		26		9	
	510	3		26		23	
5.F 3 mg/kg IP[e] Twice Daily	511	5	**5[f]**	21	**34.25[g]**	35	**89.75[h]**
	512	5		31		15	
	513	3		54		251	
	515	7		31		58	
5.G 5 mg/kg IP[e] Twice Daily	516	5	**5.75[f]**	32	**31.5[g]**	15	**14.25[h]**
	517	5		37		16	
	518	8		33		8	
	520	5		24		18	
5.H 10 mg/kg IP[e] Once Daily	521	7	**6[f]**	27	**28.6[g]**	31	**19.6[h]**
	522	7		32		11	
	523	6		22		14	
	524	6		30		7	
	525	4		32		35	
5.I NS IP[e] Twice Daily	526	4	**3.6[f]**	39	**37.2[g]**	33	**79.2[h]**
	527	5		38		14	
	528	1		34		20	
	529	4		31		12	
	530	4		44		317	

[a] GGT: γ Glutamyl transferase; [b] ALT: Alanine amino transferase;

[c] AST: Aspartate transferase; [d] Units per litre

[e] IP - Intraperitoneal injection; Mean levels of GGT [f], ALT [g] and AST [h];

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

Mean liver enzyme levels of Groups 5.D, 5.E, 5.F, 5.G, 5.H and 5.I were statistically analysed and differences were not significant ($p>0.05$).

Table 5.6: Experiment I-Percentage of DHBsAg-positive hepatocytes in the liver at biopsy and autopsy

Treatment Groups	Duck No	Path No	% DHBsAg positive hepatocytes	
			Days 4 p.i.	Days 14 p.i.
5.A REP 2055 10 mg/kg IP[a] Daily	401	5113/5143	0.013	<0.001
	402	5115/5145	0.004	<0.001
	403	5117/5147	0.004	<0.001
	404	5119/5149	0.008	<0.001
	405	5121/5151	0.008	<0.001
Mean %DHBsAg-positive hepatocytes			***0.007***[b]	***<0.001***[c]
5.B ETV 1 mg/kg Oral Daily	406	5123/5153	0.013	0.011
	407	5125/5155	0.004	0.007
	408	5127/5157	0.004	0.011
	409	5129/5159	0.021	<0.001
	410	5131/5161	0.004	<0.001
Mean %DHBsAg-positive hepatocytes			***0.009***[b]	***0.006***[c]
5.C NS IP[a] Daily	411	5133/5163	0.308	>95
	412	5135/5165	0.962	>95
	413	5137/5167	3.426	>95
	414	5139/5169	1.278	>95
	415	5141/5171	1.088	>95
Mean %DHBsAg-positive hepatocytes			***1.412***[b]	***>95***[c]

[a] IP - Intraperitoneal administration;

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

Mean %DHBsAg-positive hepatocytes among Groups 5.A, 5.B and 5.C were analysed and differences of mean %DHBsAg-positive hepatocytes of Groups 5.A and 5.B were statistically significant to the mean %DHBsAg-positive hepatocytes of Group 5.C ($p<0.05$).

Table 5.7: Experiment II-Percentage of DHBsAg-positive hepatocytes in the liver

Treatment Groups	Duck No	Path No	% DHBsAg-positive hepatocytes	
			Days 4 p.i.	Days 14 p.i.
5.D REP 2055 0.5 mg/kg IP[a] Twice Daily	501	5249/5309	0.98	<0.001
	503	5251/5311	1.52	4.3
	504	5253/5313	0.38	<0.001
	502	5255/Died	0.33	Died
	505	5257/5315	1.12	<0.001
Mean %DHBsAg-positive hepatocytes			***0.87***[b]	***1.07***[c]
5.E REP 2055 2 mg/kg IP[a] Twice Daily	506	5259/5317	0.21	<0.001
	507	5261/5319	0.72	<0.001
	508	5263/5321	0.55	<0.001
	509	5265/5323	0.33	<0.001
	510	5267/5325	0.44	<0.001
Mean %DHBsAg-positive hepatocytes			***0.45***[b]	***<0.001***[c]
5.F REP 2055 3 mg/kg IP[a] Twice Daily	511	5269/5327	0.46	<0.001
	512	5271/5329	0.24	<0.001
	513	5273/5331	0.40	<0.001
	514	5275/Died	0.40	Died
	515	5277/5333	0.10	<0.0001
Mean %DHBsAg-positive hepatocytes			***0.32***[b]	***<0.001***[c]
5.G REP 2055 5 mg/kg IP[a] Twice Daily	516	5279/5335	0.07	<0.001
	517	5281/5337	<0.001	<0.001
	518	5283/5339	<0.001	<0.001
	519	5285/Died	0.03	Died
	520	5287/5341	<0.001	<0.001
Mean %DHBsAg-positive hepatocytes			***0.02***[b]	***<0.001***[c]
5.H REP 2055 10 mg/kg IP[a] Once Daily	525	5289/5343	0.4	<0.001
	522	5291/5345	0.12	<0.001
	523	5293/5347	0.13	<0.001
	524	5295/5349	0.07	<0.001
	521	5297/5351	0.13	<0.001
Mean %DHBsAg-positive hepatocytes			***0.17***[b]	***<0.001***[c]
5.I NS IP[a] Twice Daily	526	5299/5353	11.07	>95
	527	5301/5355	11.53	>95
	528	5303/5357	16.46	>95
	529	5305/5359	16.76	>95
	530	5307/5361	12	>95
Mean %DHBsAg-positive hepatocytes			***13.56***[b]	***>95***[c]

[a] IP - Intraperitoneal administration; Mean %DHBsAg-positive hepatocytes on days 4[b] and 14[c] p.i.; Mean %DHBsAg-positive hepatocytes among Groups 5.D, 5.E, 5.F, 5.G, 5.H and 5.I were analysed and differences between Groups 5.D, 5.E, 5.F, 5.G and 5.H were statistically significant to 5.I ($p<0.05$).

Figure 5.1: DHBsAg levels in the sera of REP 2055 (Panel A), ETV (Panel B), and NS (Panel C) treated ducks. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with REP 2055 or ETV or NS from 1 day prior to DHBV infection for 15 days.

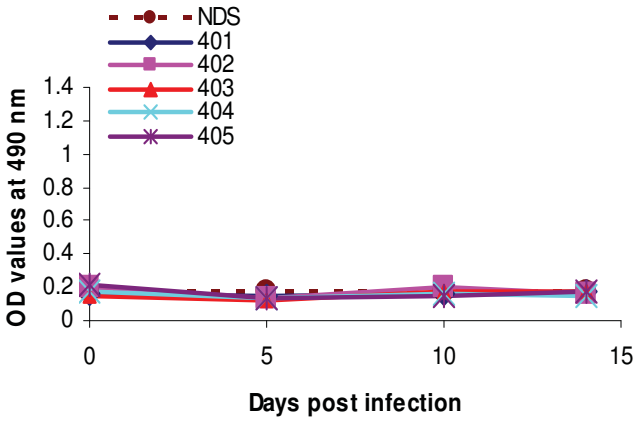
Serum samples were tested for DHBsAg levels using a qualitative enzyme linked immunosorbent assay (ELISA) described in Section 2.8.1.

Panel A: Group 5.A. Treated with REP 2055, 10 mg/kg, IP daily;

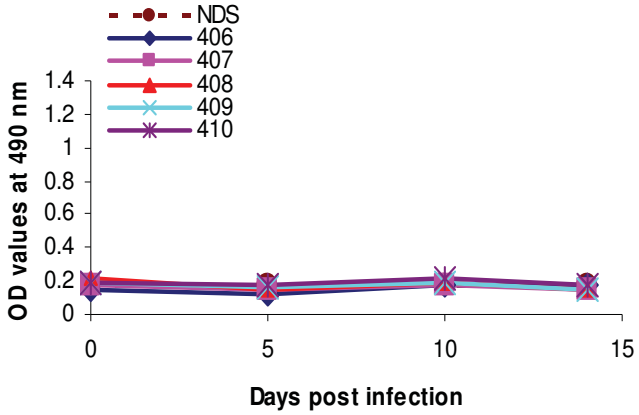
Panel B: Group 5.B. Treated with ETV, 1 mg/kg, orally daily;

Panel C: Group 5.C. Treated with NS, IP daily.

A


NDS
401
402
403
404
405
OD values at 490 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
Days post infection

B


NDS
406
407
408
409
410
OD values at 490 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
Days post infection

C

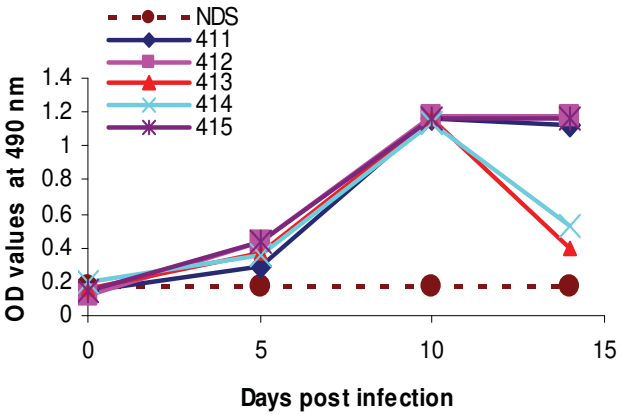

NDS
411
412
413
414
415
OD values at 490 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
Days post infection

Figure 5.2: DHBsAg levels in the sera of REP 2055 and NS treated ducks. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with REP 2055 with 5 different dose regimens as shown below (Panels D-H) or NS (Panel I) from 3 days prior to DHBV infection for 17 days.

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

Panel D: Group 5.D. Treated with 0.5 mg/kg, IP twice daily;

Panel E: Group 5.E. Treated with 2 mg/kg, IP twice daily;

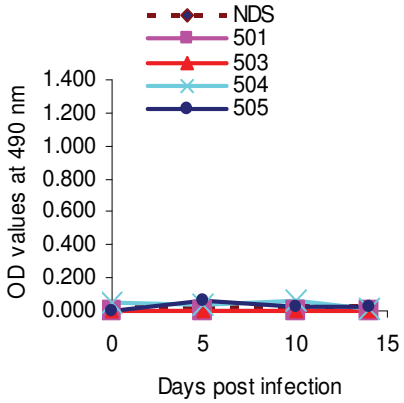
Panel F: Group 5.F. Treated with 3 mg/kg, IP twice daily;

Panel G: Group 5.G. Treated with 5 mg/kg, IP twice daily;

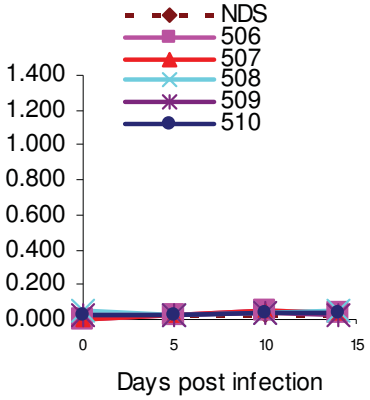
Panel H: Group 5.H. Treated with 10 mg/kg, IP once daily;

Panel I: Group 5.I. Treated with NS, IP twice daily.

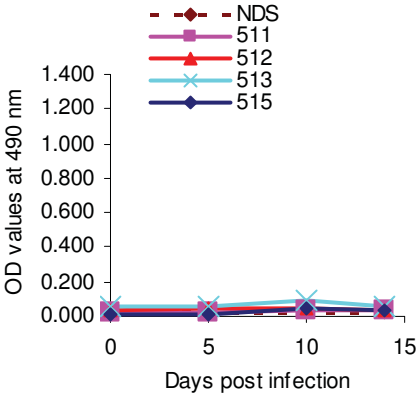
D


NDS
501
503
504
505
OD values at 490 nm
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
5
10
15
Days post infection

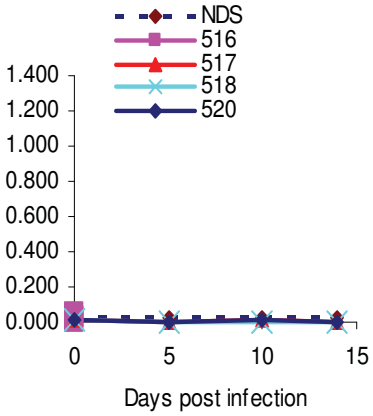
E


NDS
506
507
508
509
510
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
5
10
15
Days post infection

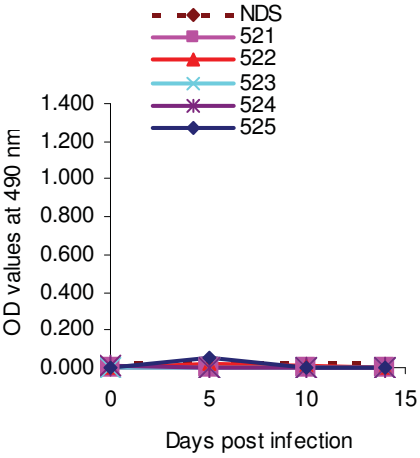
F


NDS
511
512
513
515
OD values at 490 nm
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
5
10
15
Days post infection

G


NDS
516
517
518
520
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
5
10
15
Days post infection

H


NDS
521
522
523
524
525
OD values at 490 nm
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
5
10
15
Days post infection

I

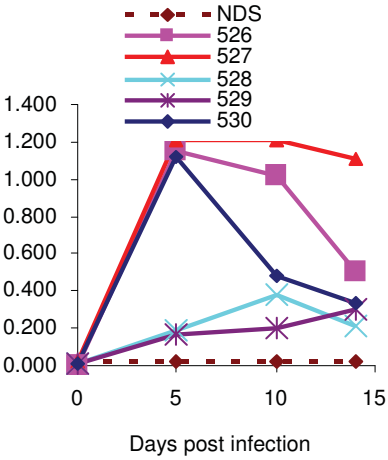

NDS
526
527
528
529
530
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
5
10
15
Days post infection

Figure 5.3: DHBsAg levels in the liver of REP 2055 (Panel A), ETV (Panel B) and NS (Panel C) treated ducks on day 4 at biopsy and day 14 p.i. at autopsy. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with REP 2055 or ETV or NS from 1 day prior to DHBV infection for 15 days.

Panel A: Group 5.A. Treated with REP 2055, 10 mg/kg, IP daily; Days 4 and 14 p.i.

Panel B: Group 5.B. Treated with ETV, 1 mg/kg, orally daily; Days 4 and 14 p.i.

Panel C: Group 5.C. Treated with NS, IP daily; Days 4 and 14 p.i.

.

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.

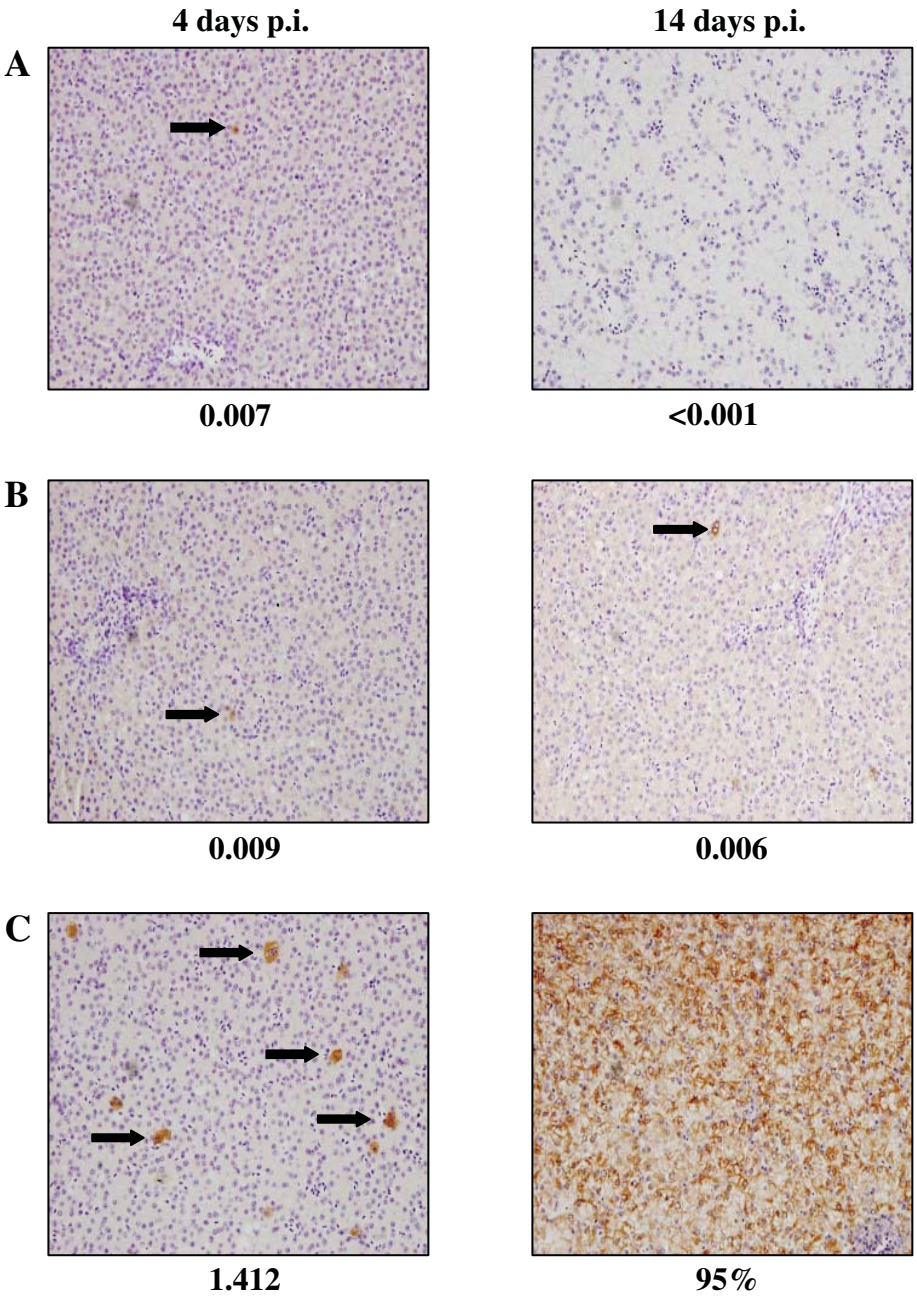

4 days p.i.
14 days p.i.
A
0.007
<0.001
B
0.009
0.006
C
1.412
95%

Figure 5.4: DHBsAg levels in the liver of REP 2055 and NS treated ducks. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with REP 2055 using 5 different dose regimens or NS from 3 days prior to DHBV infection for 17 days.

Percentage DHBsAg-positive hepatocytes present in liver sections of ducks treated with REP 2055 (Panels D-H) and NS (Panel I) on day 4 and 14 p.i.

Panel D: Group 5.D. Treated with REP 2055, 0.5 mg/kg, IP twice daily; Days 4 and 14 p.i.;

Panel E: Group 5.E. Treated with REP 2055, 2 mg/kg, IP twice daily; Days 4 and 14 p.i.;

Panel F: Group 5.F. Treated with REP 2055, 3 mg/kg, IP twice daily; Days 4 and 14 p.i.;

Panel G: Group 5.G. Treated with REP 2055, 5 mg/kg, IP twice daily; Days 4 and 14 p.i.;

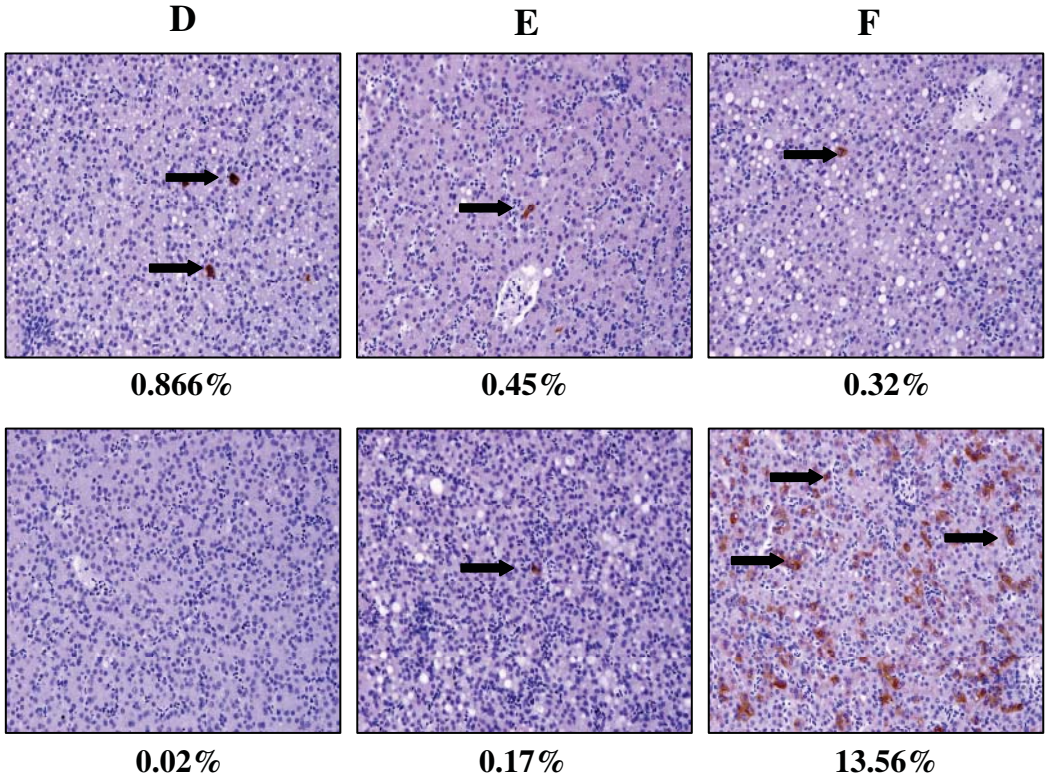
Panel H: Group 5.H. Treated with REP 2055, 10 mg/kg, IP once daily; Days 4 and 14 p.i.;

Panel I: Group 5.I. Treated with NS, IP twice daily; Days 4 and 14 p.i.
.

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.


D
E
F
0.866%
0.45%
0.32%
0.02%
0.17%
13.56%

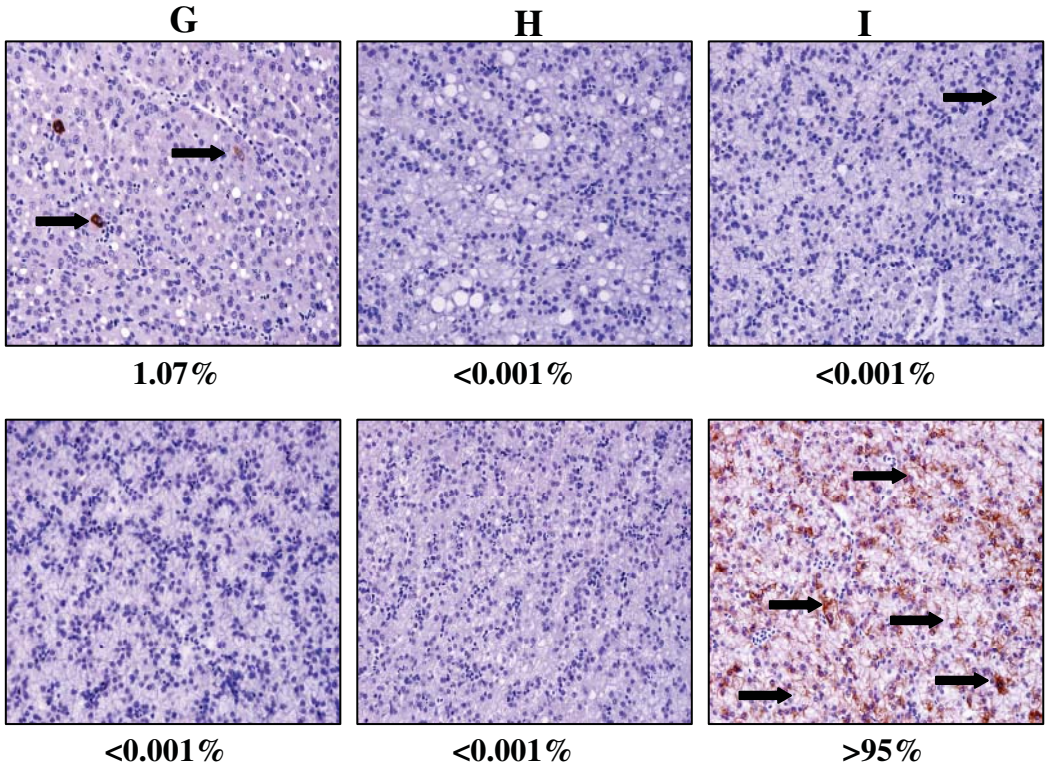

G
H
I
1.07%
<0.001%
<0.001%
<0.001%
<0.001%
>95%

Figure 5.5: DHBV DNA levels in the liver of REP 2055 (Panels A and D), ETV (Panels B and E) and NS (Panels C and F) treated ducks on day 4 and day 14 p.i. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with REP 2055 (10 mg/kg) or ETV (1 mg/kg) or NS from 1 day prior to DHBV infection for 15 days.

Hepatocellular and viral DNA extracts were tested for DHBV DNA using the Southern blot hybridisation methods described in Section 2.10.4. (Radiographic exposure time: 24 hours).

Lanes I and II:	DHBV plasmid pBL4.8 X 2 (10 and 100 pg)
Panels A and D:	Group 5.A. Treated with REP 2055; Lanes 1-5 (day 4 p.i.) and Lanes 16-20 (day 14 p.i.).
Panels B and E:	Group 5.B. Treated with ETV; Lanes 6-10 (day 4 p.i.) and Lanes 21-25 (day 14 p.i.).
Panels C and F:	Group 5.C. Treated with NS; Lanes 11-15 (day 4 p.i.) and Lanes 26-30 (day 14 p.i.).

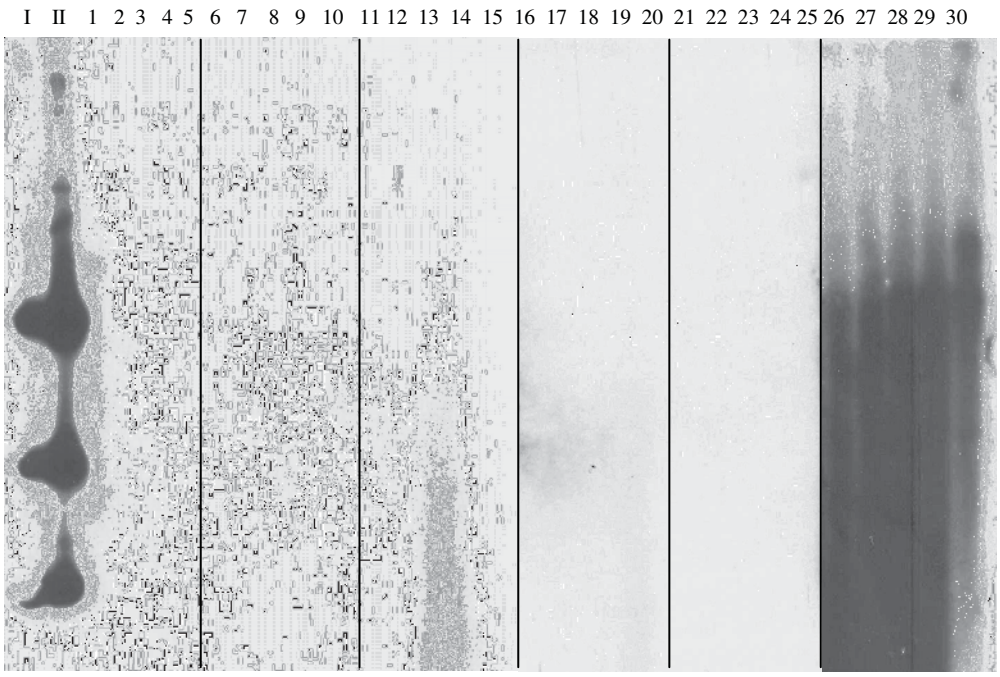

I II 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
A
B
C
D
E
F

Figure 5.6: DHBV DNA levels in the liver of REP 2055 (Panels D to H and D' to H') and NS (I and I') treated ducks on days 4 and 14 p.i. 14-day-old ducks were inoculated with 5 x 10^8 DHBV genomes and treated with REP 2055 or NS from 3 days prior to DHBV infection for 17 days.

Hepatocellular and viral DNA extracts were tested for DHBV DNA using the Southern blot hybridisation methods described in Section 2.10.4. (Radiographic exposure time: 24 hours).

Lanes I and II: DHBV plasmid pBL4.8 X 2 (100 and 10 pg).

Panels A – F: DHBV DNA detected on day 4 p.i

Panels A' - F': DHBV DNA detected on day 14 p.i

Panels A and A': Group 5.D. 0.5 mg/kg, IP twice daily;
Lanes 1-4.

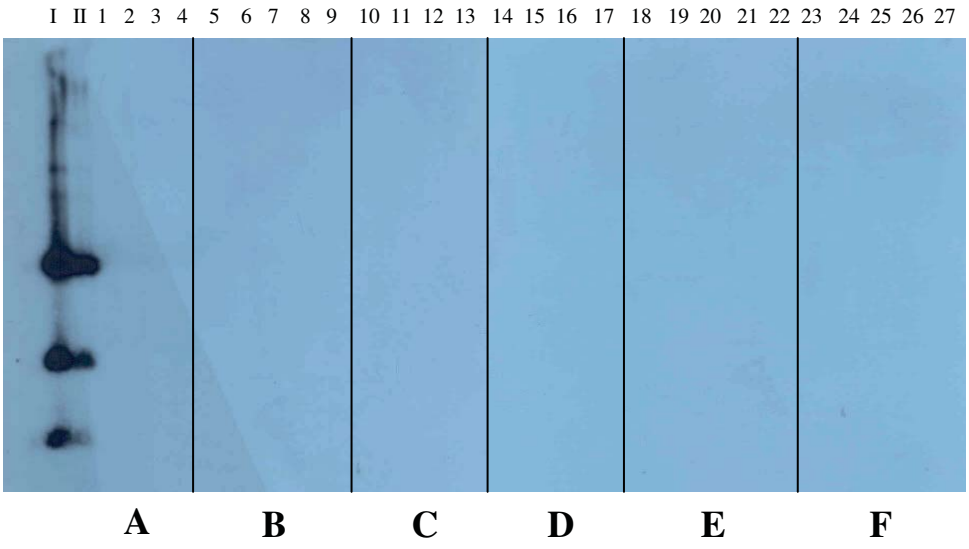
Panels B and B': Group 5.E. 2 mg/kg, IP twice daily;
Lanes 5-9.

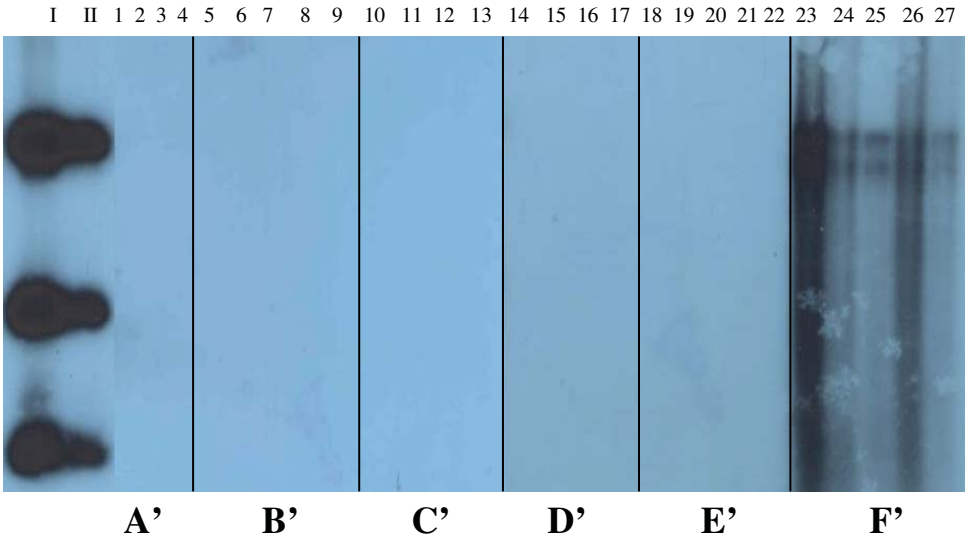
Panels C and C': Group 5.F. 3 mg/kg, IP twice daily;
Lanes 10-13.

Panels D and D': Group 5.G. 5 mg/kg, IP twice daily;
Lanes 14-17.

Panels E and E': Group 5.H. 10 mg/kg, IP once daily;
Lanes 18-22.

Panels F and F': Group 5.I. NS, IP daily;
Lanes 23-27.


I II 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
A
B
C
D
E
F


I II 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
A’
B’
C’
D’
E’
F’

Chapter 6: Testing the prophylactic and therapeutic activity of REP 2055 against DHBV infection *in vivo*

6.1 Introduction

This study was carried out to test the potential of REP 2055 as an anti-viral agent in a therapeutic HBV infection experimental model. Studies described in Chapter 5 showed that the drug could inhibit DHBV infection when treatment began 1 or 3 days prior to DHBV inoculation. No side effects were detected and no changes in clinicopathological, haematological and biochemical parameters were observed (Chapter 5).

It was therefore hypothesised that REP 2055 might also be an effective antiviral agent when therapy began during early and late phases of persistent DHBV infections; that is, when infection occurred under conditions that normally lead to a persistent infection. Experiments described in this chapter tested the hypothesis. To do this, REP 2055 treatment was started 4 days p.i. at a dose of either 10 mg/kg or 2 mg/kg. A dose ranging from 2-10 mg/kg of REP 2055 proved to be effective in the prophylactic experiments described in Chapter 5. It was therefore decided to use 2 and 10 mg/kg against established DHBV infection (Noordeen *et al.* unpublished, Chapter 5). Experiments in this chapter also investigated the ability of REP 2055 treatment to achieve a SVR by monitoring the REP 2055 treated ducks for up to ~50 days after treatment endpoint. Finally, REP 2055 treatment (10 mg/kg/day) was administered to ducks with persistent DHBV infection from 12 days p.i. for 7 days, followed by 7 weekly doses. The daily treatment regimen was expected to provide an effective antiviral response against the ongoing replication that occurs in persistent DHBV infection. It was hoped that a lower dose would prove effective once virus replication was suppressed by the early higher dose.

6.2 Experimental design

Approval to conduct the *in vivo* studies was obtained from the Animal Ethics Committees of the IMVS and University of Adelaide. The source and dose of DHBV, preparation and reconstitution of REP 2055, collection of blood samples, autopsy and specimen collection procedures, analysis of serum and liver for DHBsAg and DHBV DNA to assess the level of DHBV infection, are presented in Chapter 2.

Twenty 14-day-old ducks, divided into 4 groups of 5 (Table 6.1), were infected IV with 5 x 10^8 DHBV DNA genomes. Group 6.A ducks were treated with REP 2055 (10 mg/kg/day) from one day prior to DHBV infection to 14 days p.i. Group 6.B ducks were treated with REP 2055 (10 mg/kg/day) from 4 days p.i. to 18 days p.i. Delaying the REP 2055 treatment allowed time for DHBV infection to spread throughout the liver prior to treatment. Ducks in Group 6.C were treated similarly to Group 6.B ducks but the dose of REP 2055 used to treat Group 6.C ducks was 2 mg/kg/day. Ducks in Groups 6.A, 6.B and 6.C were monitored until 67 days p.i. to test the ability of REP 2055 to prevent the rebound of DHBV infection after REP 2055 treatment had ceased. Ducks in Group 6.D were treated with REP 2055 (10 mg/kg/day) from 12 to 19 days p.i. with 7 daily doses, followed by 7 weekly doses. Delaying REP 2055 treatment for 12 days allowed time for DHBV to spread through the liver to infect >95% of hepatocytes prior to treatment.

Ducks in all the experimental Groups 6.A-6.D were assessed each day for any changes in feed and water intake, weight changes and gait or changes in behaviour. Ducks were also clinically examined through a gentle palpation of the abdomen for signs of pain. Ducks were also examined for signs of pain on IP injection. IP injection was given just below the sternal end of the abdomen, which permits easy access to the peritoneal cavity. Haematological and biochemical parameters such as WBC counts or liver enzyme analysis were not performed in this pilot study. These parameters were not altered by REP 2055 treatment in the first two *in vivo* studies discussed in Chapter 5, in which the REP 2055 treatment (10 mg/kg) was administered for 15 or 17 days.

Blood samples collected on days 0, 2, 7, 12, 17, 22, 27, 32, 37, 42, 47, 52, 57, 62, and 67 p.i. were tested for levels of DHBsAg using a qualitative ELISA as described in Section 2.8.1 and for DHBV DNA using qPCR described in Section 2.10.4. Liver tissues collected at biopsy (12 or 16 days p.i.) and autopsy (67 days p.i.) were sectioned and examined for

evidence of pathological changes. The percentage of DHBsAg-positive hepatocytes was determined by immuno-staining of tissue sections and the levels of DHBV DNA were assessed using Southern blot hybridisation.

In Chapters 4 and 5, PCR was not performed to quantify viral DNA. This was because PS-ONs, which co-purify with nucleic acids, inhibit PCR reactions, probably through interaction with the divalent cation, Mg, as explained in Sections 3.2.4 and 4.4. However, serum PS-ONs have a short half-life; thus DHBV DNA detection by qPCR was used in this chapter as a method of quantifying DHBV DNA levels in the serum. Serum is expected to have low levels of REP 2055 when compared to the liver in which the PS-ONs are known to concentrate in large amounts (Yu *et al.* 2007; Yu *et al.* 2008). It has been reported that serum PS-ON concentration in ducks reaches undetectable levels within 4 to 8 h of IP injection (Soni *et al.* 1998). Hence, blood samples were collected just prior to IP injection during the treatment period, a time at which serum levels should be at their lowest.

6.3 Results

Previous *in vivo* data from experiments discussed in Chapter 5 showed that treatment of ducks with 10 mg/kg/day of REP 2055 completely prevented development of persistent infection when treatment was initiated 3 days prior to DHBV inoculation. This protective effect was complete with daily doses as low as 2 mg/kg. The current study aimed to test the potential of REP 2055 to act as an effective antiviral agent against experimental DHBV infection by administering REP 2055 starting 4 or 12 days after DHBV infection. We also tested the effect of starting treatment 1 rather than 3 days prior to DHBV infection. Moreover, ducks were monitored for 50 days or more after stopping treatment to determine if REP 2055 therapy led to a SVR after treatment was stopped. Ducks that failed to exhibit viral rebound were judged to have achieved SVR.

6.3.1 Clinical signs during the study period and changes at autopsy

Ducks in Group 6.A-6.D, treated with REP 2055 using 4 different treatment protocols, showed neither abdominal tenderness nor abdominal pain on IP injection. No abnormalities were noted during clinical examination of the abdomen. Furthermore, *in situ* examination of internal organs at autopsy did not reveal any gross pathological changes.

Duck 289 of Group 6.B was found dead in its cage within a week of treatment and the post-mortem examination revealed a perihepatic abscess indicating possible septicaemia and shock resulting in death. Perihepatic abscess could have resulted from introduction of pyogenic bacteria during repeated IP injections for the 13 days prior to the time of death. Pyogenic bacteria gain access to the circulation and cause septicaemia and shock, conditions which are often fatal.

Inhalational isoflurane anaesthesia was administered to induce and maintain anesthesia and this mode of inhalational isoflurane anaesthesia has been shown to be safe in chickens, geese and ducks (Olkowski and Classen 1998). Isoflurane has cardio protective properties that minimise cardiac arrhythmias. However, some ducks are more stressed than others during restraint for anaesthesia and this could contribute to the development of cardiac arrhythmias leading to failure to recover from anaesthesia (Olkowski and Classen 1998). Duck 294 of Group 6.B failed to recover from anaesthesia after biopsy; this has been documented in the laboratory as occurring sporadically during other experiments.

6.3.2 Group 6.A: The ability of REP 2055 (10 mg/kg) to prevent the rebound of DHBV infection following 14 days of prophylactic treatment

Prophylactic treatment with REP 2055 (10 mg/kg) for 15 days starting from 1 day prior to DHBV infection protected 5 out of 5 ducks from the development of detectable serum DHBsAg levels until the end of the follow-up period of 67 days p.i. (Figure 6.1). Furthermore, 4 out of 5 Group 6.A ducks were protected from the development of serum DHBV DNA until the end of follow-up (Figure 6.2).

Biopsy liver tissues collected on 12 days p.i. from the Group 6.A ducks had <0.001-0.002% (mean=0.001%) of DHBsAg-positive hepatocytes as detected by immuno-staining (Table 6.3; Figure 6.3a). At autopsy on 67 days p.i., 4 out of 5 ducks in Group 6.A (Ducks 281, 282, 283 and 581) showed no evidence of DHBsAg-positive hepatocytes in their liver (Table 6.3; Figure 6.3a). Furthermore, the 4 ducks that achieved SVR did not have detectable levels of DHBV DNA in serum (Figure 6.2) and liver (Figure 6.4).

Duck 285 had a higher level of serum DHBV DNA by qPCR (Figure 6.2) although a difference between this and other ducks in the group was not evident using an ELISA for serum DHBsAg (Figure 6.1). Undetectable levels of DHBsAg by ELISA can be due to the lower sensitivity of ELISA when compared with qPCR or to complexing of DHBsAg with antibodies to form immune complexes; hence, they were not available for detection (Foster *et al.* 2005; Miller *et al.* 2008). Duck 285 also had detectable levels of DHBV DNA in the liver when tested for DHBV DNA using Southern blot hybridisation (Figure 6.4), indicating persistent DHBV infection.

In summary, REP 2055 prophylactic treatment protected 4 out of 5 Group 6.A ducks from persistent DHBV infection.

6.3.3 Group 6.B: The ability of REP 2055 (10 mg/kg) to prevent the rebound of DHBV infection following 14 days of treatment starting from 4 days p.i.

In Group 6.B, all ducks had elevated levels of serum and hepatic DHBsAg and DHBV DNA prior to REP 2055 treatment and these virological markers became undetectable in the serum by the end of treatment in 4/4 ducks. After cessation of REP 2055 treatment, 3 out of 4 of these ducks (Ducks 286, 287 and 290) continued to have undetectable levels of serum DHBsAg and DHBV DNA until 67 days p.i (Figures 6.1 and 6.2).

At biopsy on 16 days p.i. (2 days prior to cessation of treatment), one duck (Duck 287) had undetectable levels of DHBsAg-positive hepatocytes (<0.001%) and DHBV DNA at biopsy (Table 6.3; Figures 6.3b and 6.4). In contrast, 3 out of 4 ducks (Ducks 286, 288 and 290) had >75% DHBsAg-positive hepatocytes in the liver (Table 6.3) but no detectable DHBsAg (Figure 6.1) or DHBV DNA (Figure 6.2) in the serum. By the end of follow-up at 49 days after stopping REP 2055 therapy 3 ducks, including two ducks (Ducks 286 and 290) that had >75% DHBsAg-positive hepatocytes, showed no evidence of hepatic DHBV infection. DHBsAg positive hepatocytes (mean=<0.001) were not detected by immuno-staining and DHBV DNA was not detected by Southern blot hybridisation (Table 6.3; Figures 6.3b and 6.4).

Thus, REP 2055 is able to control infection leading to viral clearance when therapy is started prior to complete infection of the hepatocyte population. The next section asks if a lower dose also achieves this level of control.

6.3.4 Group 6.C: The ability of REP 2055 (2 mg/kg) to prevent the rebound of DHBV infection following 14 days of treatment starting from 4 days p.i.

In Group 6.C, all ducks had elevated levels of serum and hepatic DHBsAg and DHBV DNA prior to REP 2055 treatment and these virological markers became moderately suppressed in the serum at the end of treatment. After cessation of REP 2055 treatment, one duck (Duck 291) continued to have undetectable levels of serum DHBsAg and DHBV DNA until 67 days p.i (Figures 6.1 and 6.2). In 3 out of 4 ducks (Ducks 292, 593 and 594) serum DHBsAg and DHBV DNA rebounded to the pre-treatment levels.

At biopsy (16 days p.i), Duck 291 had undetectable levels of DHBsAg-positive hepatocytes (<0.001%) while the 3 other Group 6.C ducks had >75-95 of DHBsAg positive hepatocytes (Table 6.3; Figure 6.3c). As noted, only Duck 291 achieved SVR at the end of follow-up at 67 days p.i. or 49 days after stopping the REP 2055 treatment (Figures 6.1, 6.2, 6.3c and 6.4; Table 6.3). Three ducks out of 4 (Ducks 292, 593 and 594) had persistent infection (Figures 6.1, 6.2, 6.3c and 6.4; Table 6.3). All Group 6.C ducks had detectable levels of DHBV DNA as assayed by Southern blot hybridisation except Duck 291 (Figure 6.4).

Based on serum and hepatic virological markers, 1 out of 4 ducks in Group 6.C achieved SVR by the end of follow-up at 67 days p.i. or 49 days after stopping the REP 2055 treatment (Figures 6.1, 6.2, 6.3c and 6.4; Table 6.3). REP 2055 treatment (2 mg/kg) of 14 days duration starting from 4 days p.i. until 18 days p.i. protected only 1 out of 4 ducks from developing persistent and rebounding DHBV infection.

6.3.5 Group 6.D: The ability of REP 2055 to act as a therapeutic antiviral agent against DHBV infection with 7 daily doses, followed by 7 weekly doses

All 5 ducks indicated a substantial virological response to REP 2055 for the initial 7 days of daily treatment. In other words, DHBV viraemia was suppressed by 3 logs. Duck 297 continued to have low levels of DHBsAg after the change to weekly dosing. DHBV DNA spiked between 32 and 47 days p.i., after which levels reached the lower limit of detection of the assay. In contrast, weekly treatment did not sustain the initial virological response in the remaining 4 ducks, as the serum DHBsAg and DHBV DNA levels became moderately

elevated immediately after stopping the daily dosing of REP 2055 and their levels fluctuated until 67 days p.i. (Figures 6.1 and 6.2).

All ducks in Group 6.D had >95% DHBsAg positive hepatocytes (Table 6.3; Figure 6.3) by immuno-staining of liver sections and detectable levels of DHBV DNA (Figure 6.4) using Southern blot hybridisation at biopsy on 12 days p.i. At autopsy (67 days p.i), Duck 297 had undetectable levels of DHBsAg-positive hepatocytes (<0.001%). However, the remaining ducks had >55-95% DHBsAg-positive hepatocytes at autopsy on 67 days p.i (Figures 6.3d and 6.4; Table 6.3).

In brief, 1 out of 5 ducks was protected from the development of persistent infection (Figure 6.1 and 6.2). Duck 297 achieved SVR (Figures 6.1, 6.2, 6.3d and 6.4; Table 6.3) with undetectable levels of DHBV infection in the serum and liver.

6.4 Discussion

In the duck model, REP 2055 treatment has shown promising results in terms of its ability to inhibit DHBV infection with prophylactic treatments that started 1or 3 days prior to DHBV infection with no detectable side effects (Noordeen *et al.* unpublished, Chapter 5).

Firstly, the present study tested the ability of REP 2055 to act as a prophylactic antiviral agent against DHBV infection in an experimental Group (Group 6.A) to which REP 2055 was administered from 1 day prior to DHBV infection for 14 days, using a standard dose of 10 mg/kg. In this experiment, the ducks were followed until 67 days p.i., 51 days after cessation of REP 2055 treatment. As it had shown previously, REP 2055 was effective as a prophylactic antiviral agent and prevented the rebound of DHBV infection after cessation treatment in 4 out of 5 ducks. This is a favourable response when compared to the findings of a recent study by (Miller *et al.* 2008). In this study 14-day-old ducks infected with the same dose of DHBV (5 x 10^8 DHBV genome equivalents) with simultaneous ETV treatment for 14 days showed rebound of DHBV infection 14-22 days after ceasing the ETV treatment. Hence, compared to ETV, REP 2055 proved to be superior in preventing the rebound of DHBV infection in most ducks (4/5 ducks) 53 days after stopping the treatment. This may be because REP 2055, unlike most NAs, is effective in blocking initiation of infection leading to cccDNA formation. One duck (Duck 285) in Group 6.A showed evidence of rebound 14 days after stopping treatment, based on serum DHBV DNA

detection (Figure 6.2), but the rebound was not evident when serum DHBsAg ELISA (Figure 6.1) was used. These results suggest that longer prophylactic dosing may be required to improve the efficacy of prophylactic therapy in 5/5 ducks.

Secondly, the present study tested the ability of REP 2055 to act as a therapeutic antiviral agent against established DHBV infection (Group 6.B). REP 2055 was administered from 4 days p.i. for 14 days with a standard dose of 10 mg/kg. Treatment was delayed for 4 days p.i. to allow a low level of DHBV infection in the liver. According to previous experiments discussed in Chapters 4 and 5, approximately 5-10% DHBsAg-positive hepatocytes are expected in the liver by 4 days p.i. Thus, delaying REP 2055 treatment tested the therapeutic activity of REP 2055. With this treatment approach, REP 2055 was effective and reduced the viraemia to an undetectable level within 7 days of treatment in 4 out of 4 ducks. Moreover, REP 2055 was able to prevent rebound of DHBV infection in 3 out of 4 ducks, demonstrating an effective therapeutic efficacy against an established DHBV infection. One duck experienced rebound of DHBV infection 2-3 days after stopping REP 2055 treatment. It may be that a longer treatment duration would have prevented rebound in this duck. In comparison, in one study (Trahair *et al.* unpublished) in which ETV treatment was given for 14 days from 4 days p.i., all 5 ducks rebounded with infection 1 to 4 days after stopping ETV treatment. This indicated the better therapeutic efficacy of REP 2055 than ETV against an established DHBV infection. Furthermore, a significant level of DHBV infection in the liver of 3 out of 4 ducks on 16 days p.i. in the absence of detectable level of viraemia (DHBsAg and DHBV DNA) in the serum reveals the ability of REP 2055 to inhibit the release of DHBV from the liver into the circulation.

Thirdly, the present study tested the ability of REP 2055 to act as a therapeutic antiviral agent against established DHBV infection (Group 6.C) when given at a lower dose of 2 mg/kg, again from 4 days p.i. for 14 days. The dose of 2 mg/kg was only able to prevent rebound of DHBV infection in 1 out of 4 ducks leaving the other 3 ducks with fluctuating levels of DHBsAg and DHBV DNA in the serum. REP 2055 treatment did, however, reduce viraemia by 2-5 logs by 7 days after starting treatment, even though it was unable to achieve SVR in 3 out of 4 ducks. This suggests that the 2 mg/kg dose is unable to achieve therapeutically effective levels in the liver, as evidenced by the poor DHBsAg clearance in these ducks compared to that observed in ducks treated with 10 mg/kg (Group 6.B). In this

respect, 2 mg/kg was unable to achieve intracellular concentrations that are therapeutically effective to suppress ongoing DHBV replication although 2 mg/kg dose was prophylactically effective in our previous experiments (Noordeen *et al.* unpublished, Chapter 5). Moreover, continuous suboptimal dosing of the drug might fail to build a therapeutic maintenance drug level intracellularly to elicit an effective antiviral response. This could have contributed to the ineffectiveness of REP 2055 at the dose rate of 2 mg/kg against an established DHBV infection. This may be due to the fact that in prophylactic dosing the drug action is needed both outside the cell or possibly in endocytic vesicles to block the entry of DHBV into the hepatocytes as well as inside the cell to block virus release. It appears that 2 mg/kg of REP 2055 was able to achieve an extracellular concentration that was sufficient to block virus entry during the prophylactic dosing. However, 2 mg/kg of REP 2055 was unable to achieve the effective concentration in the intracellular compartment to block the release of DHBV from the hepatocytes during the therapeutic dosing.

Fourthly, the present study tested the ability of REP 2055 to act as a therapeutic antiviral agent against established DHBV infection (Group 6.D) in that the REP 2055 was administered from 12 days p.i. for 7 days, followed by 7 weekly doses with a standard dose of 10 mg/kg. In Group 6.D, the REP 2055 treatment was delayed by 12 days p.i. to test the ability of REP 2055 against widespread DHBV infection with >95% DHBsAg-positive hepatocytes. This treatment was able to produce a robust antiviral effect by reducing the viraemia by 3-4 logs in 5 out of 5 ducks after 7 days of daily therapy; however, only one duck achieved SVR. Four out of 5 ducks had fluctuating levels of DHBsAg and DHBV DNA in the serum with >55-95% DHBsAg-positive hepatocytes and detectable levels of DHBV DNA in the liver, indicating failure of this treatment protocol in the majority of the ducks. This finding appears to be due to a suboptimal treatment regimen with REP 2055 against persistent DHBV infection especially during the period of 7 weekly doses. During the weekly dosing, the interval between the drug doses appears to be too long to allow the build-up of an intracellular therapeutic maintenance drug level that is adequate for an effective antiviral response. PS-ONs that are similar to REP 2055 have a short intrahepatic half-life which may contribute to a low intracellular level of REP 2055 (Soni *et al.* 1998).

Ducks 291 and 297 of Groups 6.C and 6.D, respectively had a lower serum DHBsAg set point prior to and after treatment. This may be due to an altered immunocompetence in the host supported by studies on CHB patients in whom the removal of HBsAg from the serum improves the host's ability to seroconvert and control the infection (Fattovich *et al.* 2008; Yuen and Lai 2008; Marcellin *et al.* 2008). The DHBsAg and the qPCR data suggested that Duck 297 of Group 6.D had a small spike in serum DNA during weekly therapy that was correlated with a small spike in the DHBsAg levels.

The inability of REP 2055 to prevent rebound of DHBV infection in 1 out of 5 ducks in Groups 6.A and 6.B may be due to either anti-viral resistance or variation in extracellular and intracellular APDP concentrations between individual ducks. Anti-viral resistance seems unlikely as it appears that the effective anti-DHBV activity of APDPs, REP 2006 and REP 2055 is sequence independent and involves the interaction with structurally conserved amphipathic domains. The sequence independent mode of action of APDPs makes the point mutations at multiple sites which are responsible for anti-viral resistance less likely to occur. Conversely, the structural conservation of amphipathic domains in enveloped viruses is evident from the fact that APDPs have shown effective antiviral activity against a broad spectrum of enveloped viruses (Vaillant *et al.* 2006; Guzman *et al.* 2007; Bernstein *et al.* 2008; Matsumura *et al.* 2009) including HBV (Vaillant *et al.* unpublished) and DHBV (Noordeen *et al.* unpublished, Chapters 3, 4 and 5). On the other hand, variation in extracellular and intracellular APDP concentrations between individual ducks is a possibility due to biological variation in metabolism. Thus ducks that had lower extracellular and intracellular APDP concentrations may not have been were not able to achieve a SVR.

In conclusion, REP 2055 (10 mg/kg) was able to prevent the rebound of DHBV infection in 4 out of 5 Group 6.A and 3 out of 4 Group 6.B ducks when treatment was given starting from 1 day prior (Group 6.A) to or 4 days p.i. (Group 6.B) for 14 days with a follow-up of 51 days after stopping REP 2055 treatment. A reduction in REP 2055 dose rate to 2 mg/kg failed to prevent the rebound of DHBV infection in 3 out of 4 ducks with treatment starting from 4 days p.i. (Group 6.C). REP 2055 treatment for 7 days, followed by 7 weekly doses against widespread DHBV infection (Group 6.D), starting from 12 days p.i. produced a 3-4 log reduction in viraemia but failed to produce SVR in 4 out of 5 ducks.

The next experiment was planned to adjust the treatment protocol of Group 6.D using a longer treatment duration with daily REP 2055 (10 mg/kg) treatment for 28 days to sustain therapeutic intracellular drug levels. A follow-up period of 16 weeks was allowed to test the ability of REP treatment to clear DHBV and prevent the rebound of infection. A control Group of NS-treated ducks was monitored in a similar manner to that of the REP 2055-treated ducks to assess the efficacy of REP 2055 in clearing and preventing the rebound of persistent DHBV infection. Control ducks also helped to compare the effect of long-term REP 2055 treatment on clinicopathological, haematological and biochemical parameters. This experimental design mimics chronic HBV infection in humans and the outcomes of this study would be important to assess the potential clinical utility of REP 2055 to treat humans with chronic HBV infection. This extended study was performed and is discussed in Chapter 7 of this thesis.

Table 6.1: Experimental outline for prophylactic and therapeutic studies

Treatment Groups	Duck No	Treatment plan and follow-up
6.A	281 282 283 285 581	10 mg/kg REP 2055 IP daily starting from 1 day prior to DHBV infection for 15 days. Ducks were followed until 67 days p.i.
6.B	286 287 288 289 290	10 mg/kg REP 2055 IP daily starting from 4 days p.i. for 14 days. Ducks were followed until 67 days p.i.
6.C	291 292 294 593 594	2 mg/kg REP 2055 IP daily starting from 4 days p.i. for 14 days. Ducks were followed until 67 days p.i.
6.D	296 297 298 299 300	10 mg/kg REP 2055 IP daily starting from 12 days p.i. for 7 days followed by 7 weekly doses. Ducks were autopsied at last weekly treatment.

Table 6.2: Body weights of ducks during REP 2055 treatment and follow-up

Treatment Groups	Duck NO	Body weight (g)										
		Age (days)										
		14	**21**	**24**	**28**	**31**	**37**	**46**	**50**	**57**	**70**	**80**
6.A REP 2055 10 mg/kg IP[a] Daily	281	455	950	1295	1490	1890	2135	2730	3050	3390	3590	3800
	282	500	975	1290	1480	1740	2040	2580	2890	3135	3370	3595
	283	515	900	1285	1470	1690	2000	2550	2740	2990	3300	3650
	285	460	965	1295	1450	1620	2075	2600	2890	3210	3490	3765
	581	450	900	1240	1440	1850	2170	2600	2885	3100	3385	3700
Mean body weight		***476*** [b]	***938*** [c]	***1281*** [d]	***1466*** [e]	***1758*** [f]	***2084*** [g]	***2612*** [h]	***2891*** [i]	***3165*** [j]	***3427*** [k]	***3702*** [l]
6.B REP 2055 10 mg/kg IP[a] Daily	286	450	935	1225	1460	1690	1970	2450	2650	2985	3290	3550
	287	495	945	1310	1600	1850	2150	2650	2880	3155	3490	3800
	288	390	805	1000	1260	1470	1700	2200	2445	2815	3250	3650
	289	400	795	1070	Died							
	290	500	955	1300	1505	1870	2250	2900	3210	3505	3760	4000
Mean body weight		***447*** [b]	***887*** [c]	***1181*** [d]	***1456*** [e]	***1720*** [f]	***2018*** [g]	***2250*** [h]	***2796*** [i]	***3115*** [j]	***3448*** [k]	***3750*** [l]
6.C REP 2055 2 mg/kg IP[a] Daily	291	450	890	1120	1350	1680	1965	2500	2880	3100	3385	3750
	292	500	995	1155	1530	1800	2050	2465	2600	2880	3195	3600
	294	420	675	950	1155	Did not recover from anaesthesia after biopsy						
	593	475	905	1125	1430	1655	2000	2650	2850	3250	3490	3885
	594	525	1030	1295	1460	1865	2155	2670	2805	3370	3690	3950
Mean body weight		***474*** [b]	***889*** [c]	***1129*** [d]	***1385*** [e]	***1750*** [f]	***2043*** [g]	***2571*** [h]	***2784*** [i]	***3150*** [j]	***3440*** [k]	***3796*** [l]
6.D REP 2055 10 mg/kg IP[a] Daily	296	460	915	1145	1360	1735	2045	2540	2865	3180	3320	3650
	297	470	965	1125	1470	1805	2090	2495	2645	2925	3290	3770
	298	485	950	1190	1295	1485	1670	2095	2290	2590	2990	3445
	299	425	875	1105	1375	1665	1890	2200	2460	2760	3185	3660
	300	440	935	1195	1485	1690	1985	2450	2820	3000	3290	3695
Mean body weight		***456*** [b]	***928*** [c]	***1152*** [d]	***1397*** [e]	***1676*** [f]	***1936*** [g]	***2356*** [h]	***2616*** [i]	***2891*** [j]	***3215*** [k]	***3644*** [l]

[a]IP - Intraperitoneal injection; Mean body weight on 14[b], 21[c], 24[d], 28[e], 31[f], 37[g], 46[h], 50[i], 57[j], 70[k] and 80[l] days of age.

Table 6.3: Percentage of DHBsAg-positive hepatocytes in the liver at biopsy & autopsy

Treatment Groups	Duck No	Path No	%DHBsAg-positive hepatocytes	
			at biopsy	at autopsy
6.A REP 2055 10 mg/kg IP[a] Daily	281	5675/5769	<0.001	<0.001
	282	5677/5771	<0.001	<0.001
	283	5679/5773	0.001	<0.001
	285	5683/5775	0.002	>95
	581	5681/5777	<0.001	<0.001
Mean %DHBsAg-positive hepatocytes			***0.001***[b]	***<0.001***[c]
6.B REP 2055 10 mg/kg IP[a] Daily	286	5685/5779	>75	<0.001
	287	5687/5781	<0.001	<0.001
	288	5689/5783	>75	>65
	289	Died before biopsy		
	290	5691/5785	>75	<0.001
Mean %DHBsAg-positive hepatocytes			***45***[d]	***<0.001***[e]
6.C REP 2055 2 mg/kg IP[a] Daily	291	5695/5787	<0.001	<0.001
	292	5697/5789	>75	>55
	294	5699	>95	Died before autopsy
	593	5701/5791	>75	>65
	594	5703/5793	>95	>55
Mean %DHBsAg-positive hepatocytes			***>68***[f]	***>35***[g]
6.D REP 2055 10 mg/kg IP[a] Daily	296	5705/5795	>95	>95
	297	5711/5801	>95	<0.001
	298	5709/5799	>95	> 55
	299	5707/5797	>95	> 75
	300	5713/5803	>95	>75
Mean %DHBsAg-positive hepatocytes			***>95***[h]	***>60***[i]

[a] IP - intraperitoneal injection;

Mean DHBsAg-positive hepatocytes from livers of ducks in Group 6.A at biopsy[b] on day 12 p.i. and autopsy[c] on day 67 p.i.;

Mean DHBsAg-positive hepatocytes from livers of ducks in Group 6.B at biopsy[d] on day 16 p.i. and autopsy[e] on day 67 p.i.;

Mean DHBsAg-positive hepatocytes from livers of ducks in Group 6.C at biopsy[f] on day 16 p.i. and autopsy[g] on day 67 p.i.;

Mean DHBsAg-positive hepatocytes from livers of ducks in Group 6.D at biopsy[h] on day 12 p.i. and autopsy[i] on day 67 p.i.

Figure 6.1: DHBsAg levels in the sera of ducks treated with REP 2055 using 4 different treatment protocols.

14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with REP 2055 and monitored until 67 days p.i.

Panel A: Group 6.A. Treated with 10 mg/kg from 1 day prior to DHBV infection for 15 days and monitored for 53 days after stopping the treatment.

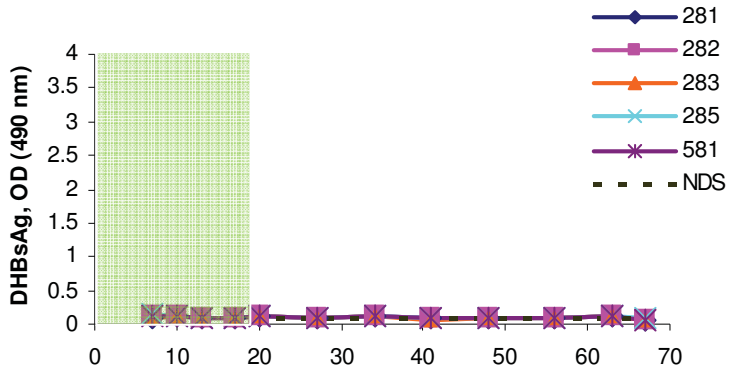
Panel B: Group 6.B. Treated with 10 mg/kg from 4 days p.i. for 14 days and monitored for 49 days after stopping the treatment.

Panel C: Group 6.C. Treated with 2 mg/kg from 4 days p.i. for 14 days and monitored for 49 days after stopping the treatment.

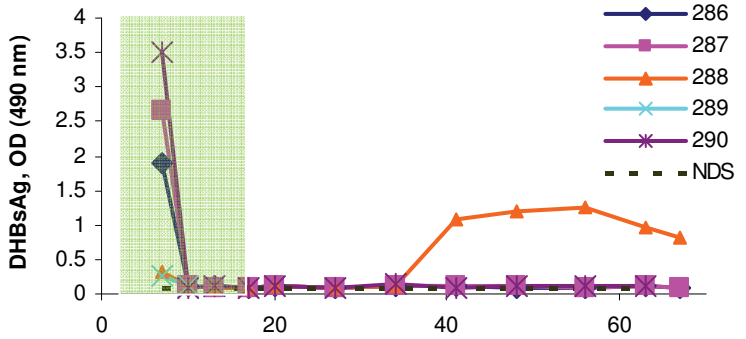
Panel D: Group 6.D. Treated with 10 mg/kg from 12 days p.i. for 7 days followed by 7 weekly doses

Serum samples were tested for DHBsAg levels using a qualitative ELISA described in Section 2.8.1.

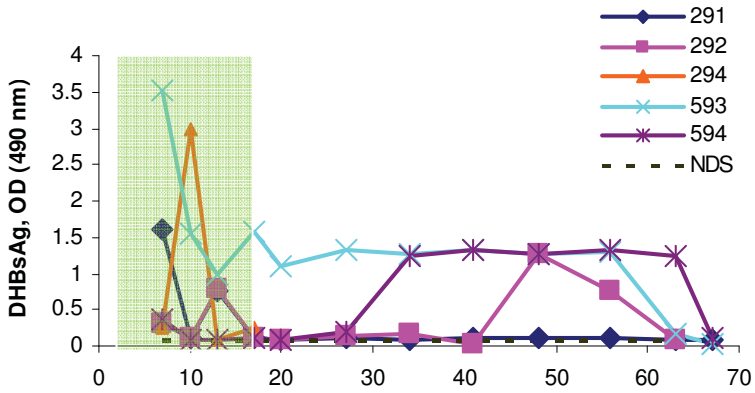
A


DHBsAg, OD (490 nm)
281
282
283
285
581
NDS

B


DHBsAg, OD (490 nm)
286
287
288
289
290
NDS

C


DHBsAg, OD (490 nm)
291
292
294
593
594
NDS

D

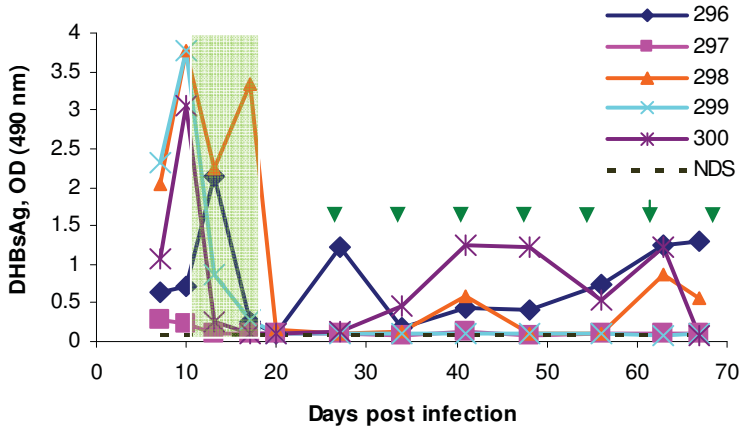

DHBsAg, OD (490 nm)
296
297
298
299
300
NDS
Days post infection

Figure 6.2: DHBV DNA levels in the sera of ducks treated with REP 2055 using 4 different treatment protocols.

14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with REP 2055 and monitored until 67 days p.i.

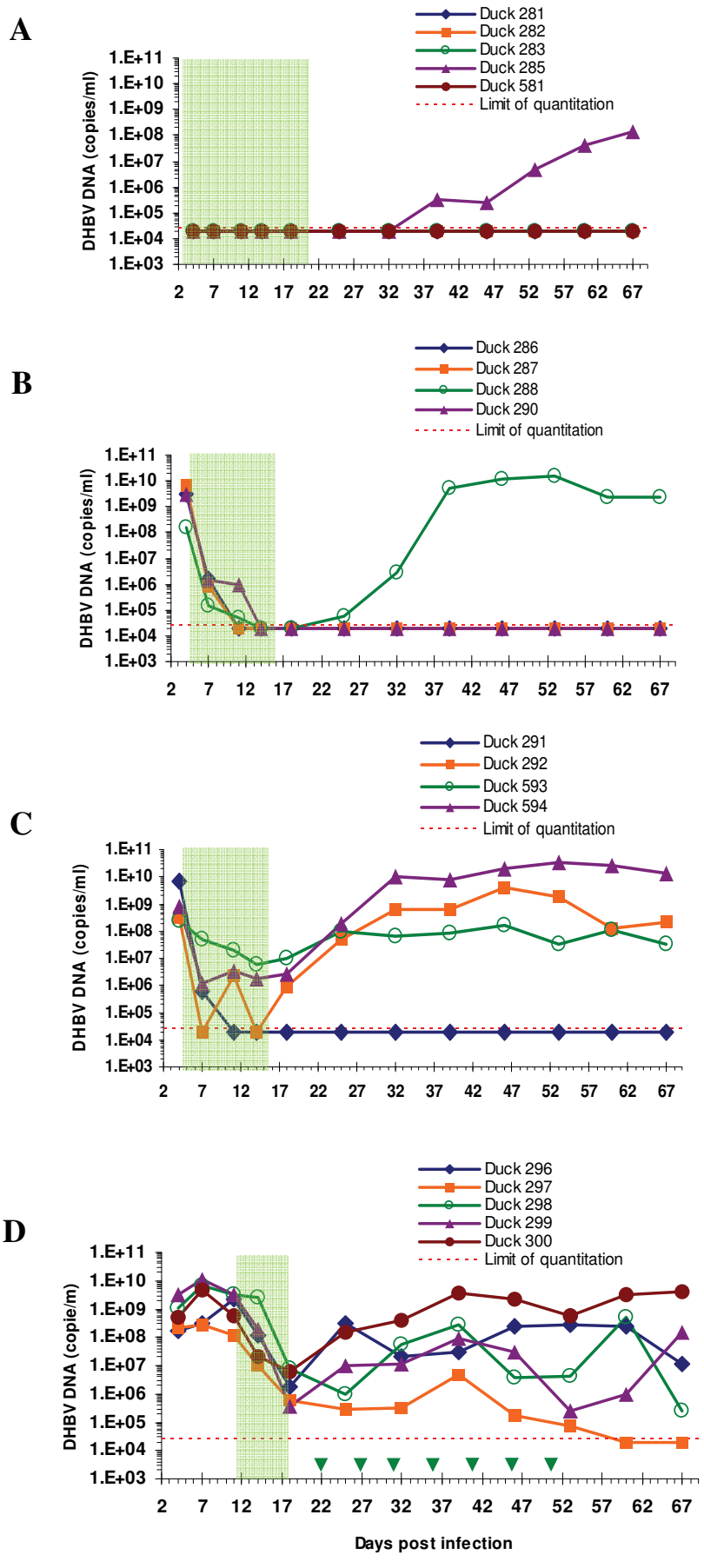
Panel A: Group 6.A. Treated with 10 mg/kg from 1 day prior to DHBV infection for 15 days and monitored for 53 days after stopping the treatment.

Panel B: Group 6.B. Treated with 10 mg/kg from 4 days p.i. for 14 days and monitored for 49 days after stopping the treatment.

Panel C: Group 6.C. Treated with 2 mg/kg from 4 days p.i. for 14 days and monitored for 49 days after stopping the treatment.

Panel D: Group 6.D. Treated with 10 mg/kg from 12 days p.i. for 7 days and followed by 7 weekly doses until 67 days p.i.

Serum viral DNA extracts were tested for DHBV DNA using a quantitative polymerase chain reaction (qPCR) assay as described in Section 2.10.4.


A
Duck 281
Duck 282
Duck 283
Duck 285
Duck 581
Limit of quantitation
DHBV DNA (copies/ml)
1.E+11
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
2 7 12 17 22 27 32 37 42 47 52 57 62 67
B
Duck 286
Duck 287
Duck 288
Duck 290
Limit of quantitation
DHBV DNA (copies/ml)
1.E+11
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
2 7 12 17 22 27 32 37 42 47 52 57 62 67
C
Duck 291
Duck 292
Duck 593
Duck 594
Limit of quantitation
DHBV DNA (copies/ml)
1.E+11
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
2 7 12 17 22 27 32 37 42 47 52 57 62 67
D
Duck 296
Duck 297
Duck 298
Duck 299
Duck 300
Limit of quantitation
DHBV DNA (copie/m)
1.E+11
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
2 7 12 17 22 27 32 37 42 47 52 57 62 67
Days post infection

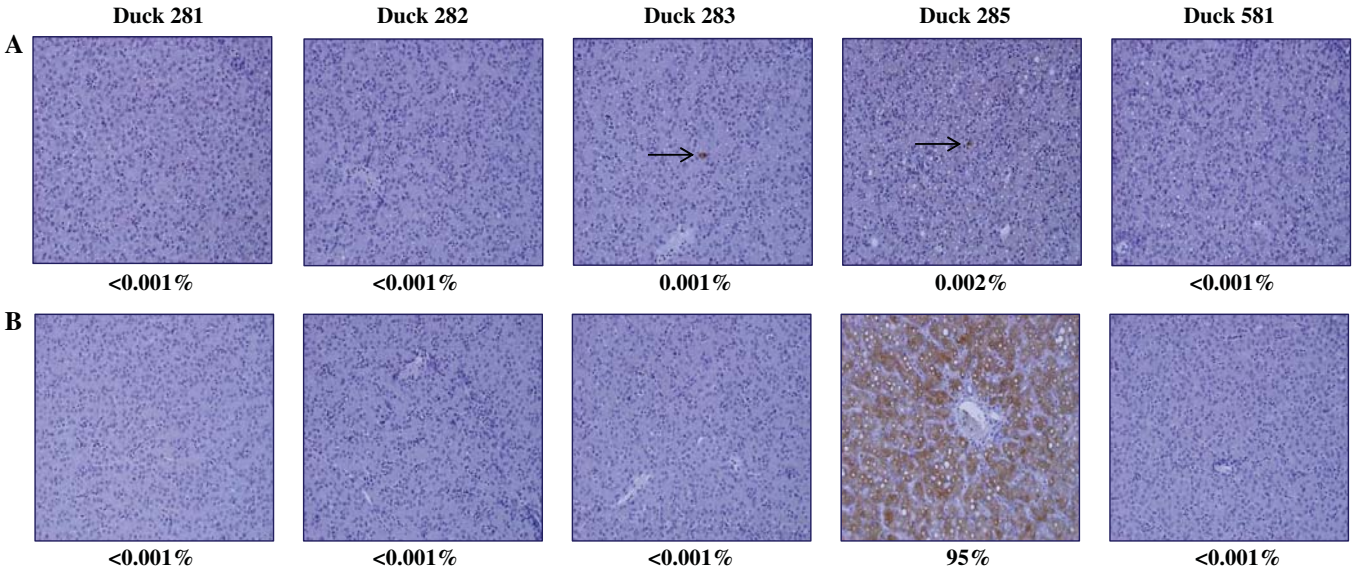


Figure 6.3a: The percentage of DHBsAg-positive hepatocytes in the liver of ducks treated with REP 2055 (10 mg/kg) from 1 day prior to DHBV infection for 15 days.

14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with REP 2055 and monitored until 67 days p.i. That is 53 days after stopping REP 2055 treatment.

Panel A: Liver sections of ducks in Group 6.A at biopsy on 12 days p.i.;

Panel B: Liver sections of ducks in Group 6.A at autopsy on 67 days p.i. Duck 285 experienced rebound of DHBV infection and shown in Panel B >95% DHBsAg-positive hepatocytes.

Liver samples were tested for the presence of 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.

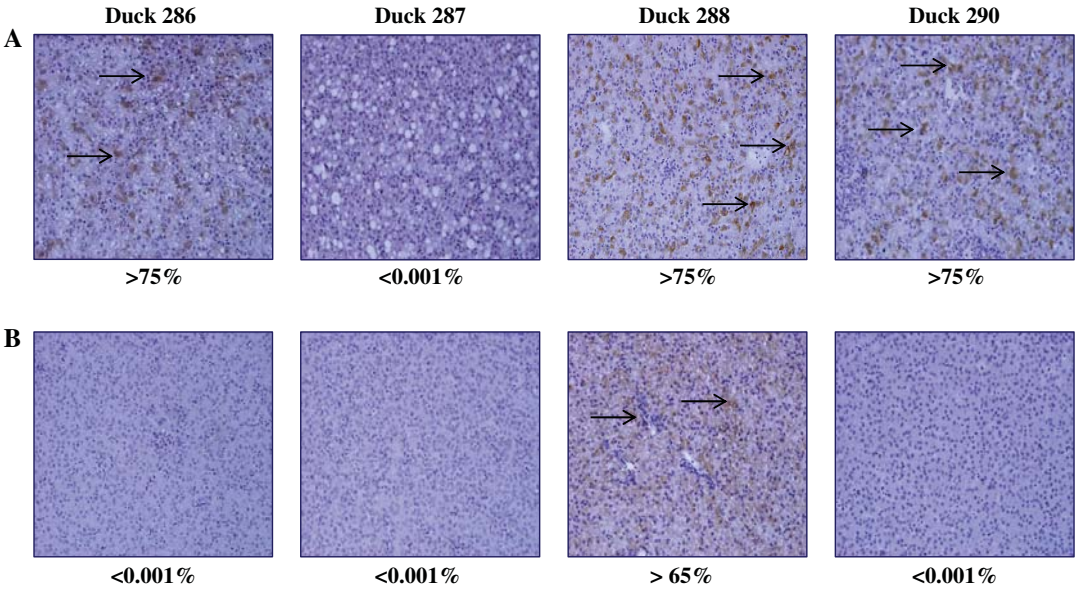


Figure 6.3b: The percentage of DHBsAg-positive hepatocytes in the liver of ducks treated with REP 2055. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with REP 2055 (10 mg/kg) starting from 4 days p.i. for 14 days.

Biopsy liver tissues were collected on 16 days p.i., two days before the treatment end point. Ducks were monitored until 67 days p.i. That is 49 days after ceasing REP 2055 treatment.

Panel A: Liver sections of ducks in Group 6.B at biopsy on 16 days p.i.;

Panel B: Liver sections of ducks in Group 6.B at autopsy on 67 days p.i. Duck 288 experienced a rebound and had widespread DHBV infection with >65% DHBsAg-positive hepatocytes in the liver.

Liver samples were tested for the presence of 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), which were widely distributed throughout the lobules of the liver.

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.

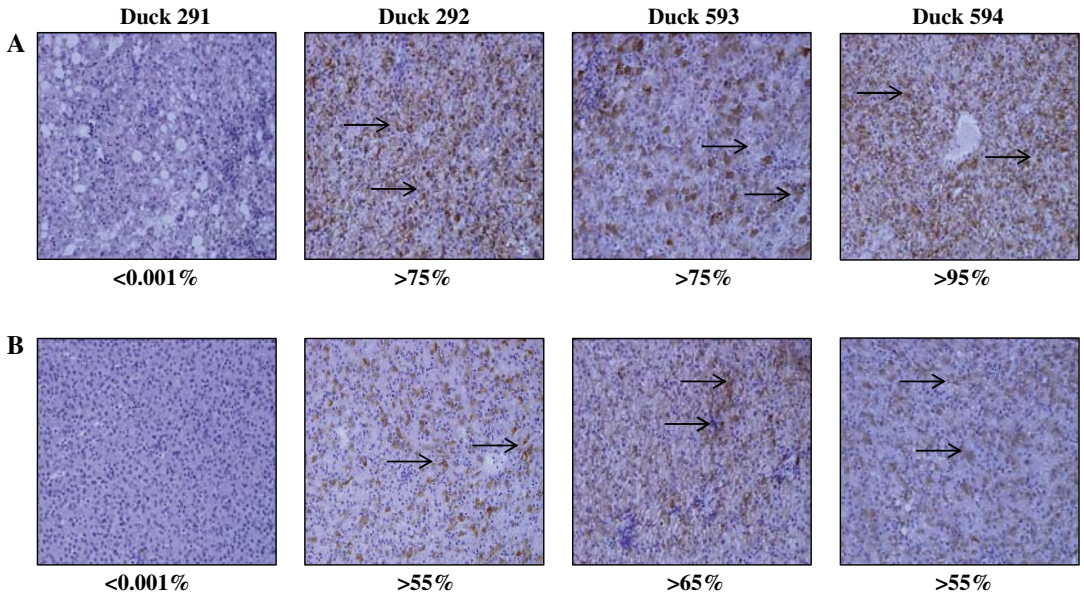


Figure 6.3c: The percentage of DHBsAg-positive hepatocytes in the liver of ducks treated with REP 2055. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with REP 2055 (2 mg/kg) starting from 4 days p.i. for 14 days.

Biopsy liver tissues were collected on 16 days p.i., two days before the treatment end point. Ducks were monitored until 67 days p.i. That is 49 days after ceasing REP 2055 treatment.

Panel A: Liver sections of ducks in Group 6.C at biopsy on 16 days p.i.;

Panel B: Liver sections of ducks in Group 6.C at autopsy on 67 days p.i. Duck 291 SVR and had <0.001% DHBsAg-positive hepatocytes on day 67 p.i. whereas Ducks 292, 593 and 594 had widespread DHBV infection with >55%-65% DHBsAg-positive hepatocytes.

Liver samples were tested for the presence of 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), which were widely distributed throughout the lobules of the liver.

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.

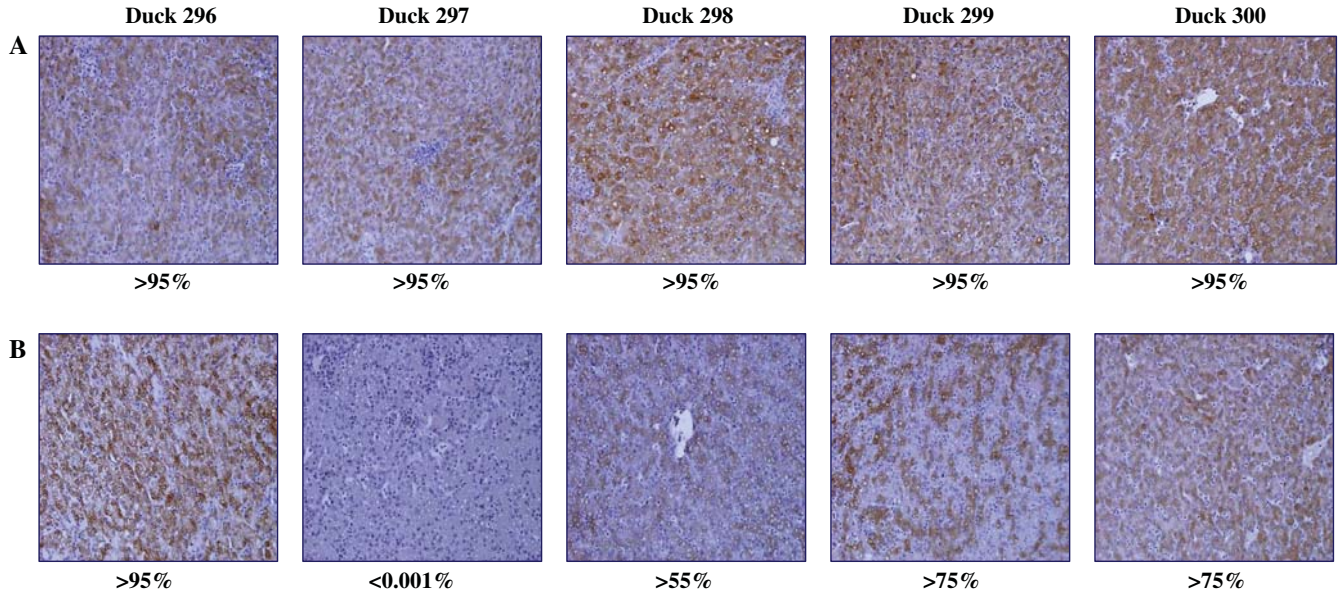


Figure 6.3d: The percentage of DHBsAg-positive hepatocytes in the liver of ducks treated with REP 2055. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with REP 2055 (10 mg/kg) starting from 12 days p.i. for 7 days, followed by 7 weekly doses until 67 days p.i.

Panel A: Liver sections of ducks in Group 6.D at biopsy on 12 days p.i., prior to REP 2055 treatment;

Panel B: Liver sections of ducks in Group 6.D at autopsy on day 67 p.i. Duck 297 achieved SVR in the liver with <0.001% DHBsAg-positive hepatocytes whereas Ducks 296, 298, 299 and 300 had widespread DHBV infection with >55%-95% DHBsAg-positive hepatocytes.

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. DHBsAg-positive hepatocytes (brown) were widely distributed throughout the lobules of the liver.

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.

Figure 6.4: DHBV DNA levels in the livers of ducks treated with REP 2055 using 4 different treatment protocols, Groups 6.A, 6.B, 6.C and 6.D at biopsy (Panel A) and autopsy (Panel B).

14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with REP 2055 and monitored until 67 days p.i.

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

6.A: Group 6.A. Treated with 10 mg/kg of REP 2055 from 1 day prior to DHBV infection for 15 days and monitored for 53 days after stopping the treatment.

6.B: Group 6.B. Treated with 10 mg/kg of REP 2055 from 4 days p.i.for 14 days and monitored for 49 days after stopping the treatment.

6.C: Group 6.C. Treated with 2 mg/kg of REP 2055 from 4 days p.i.for 14 days and monitored for 49 days after stopping the treatment.

6.D: Group 6.D. Treated with 10 mg/kg of REP 2055 from 12 days p.i. for 7 days and followed by 7 weekly doses until 67 days p.i.

Lane I: DHBV plasmid pBL4.8 X 2 (10 pg) and sizes of the bands are given in the figure.

Panel A: Group 6.A. Lanes 1-5 (days 12 p.i.);

Group 6.B. Lanes 6-9 (days 16 p.i.);

Group 6.C. Lanes 10-14 (days 16 p.i.);

Group 6.D. Lanes 15-19 (days 12 p.i.).

Panel B: Group 6.A. Lanes 1-5 (days 67 p.i.);

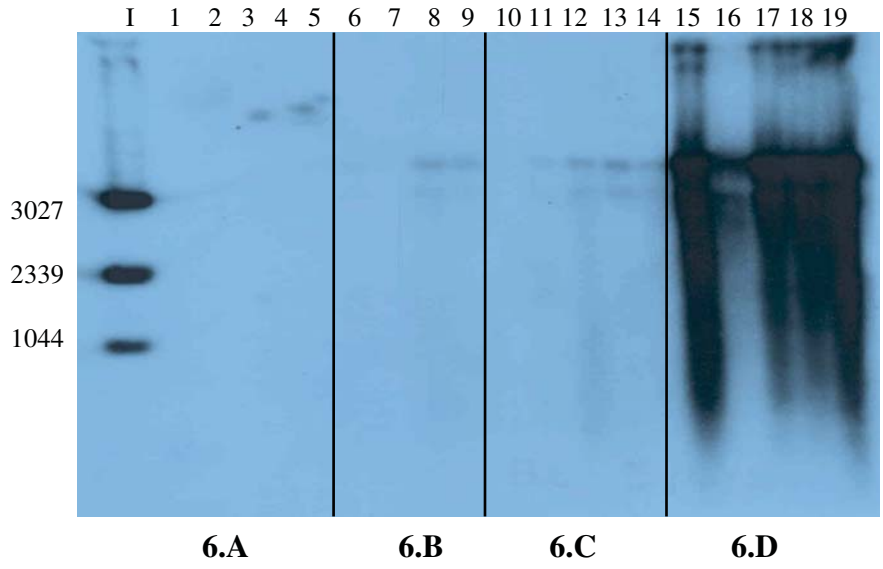
Group 6.B. Lanes 6-9 (days 67 p.i.);

Group 6.C. Lanes 10-13 (days 67 p.i.);

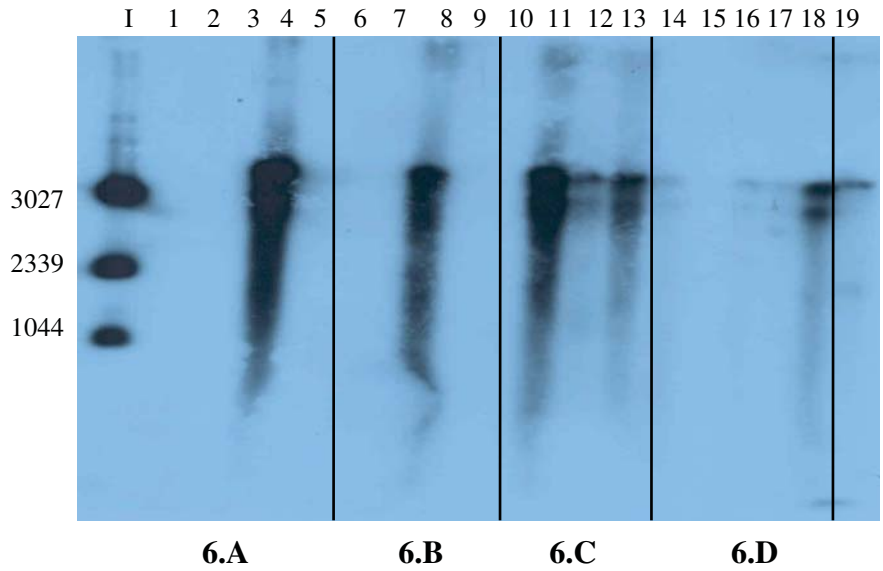
Group 6.D. Lanes 14-18 (days 67 p.i.).

Lane 19: Cellular and viral DNA extract of a duck from a different experiment.

A


I 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
3027
2339
1044
6.A
6.B
6.C
6.D

B


I 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
3027
2339
1044
6.A
6.B
6.C
6.D

Chapter 7: Testing the antiviral efficacy of REP 2055 against persistent DHBV infection

7.1 Introduction

To date six antiviral agents have been approved by the FDA for the treatment of chronic HBV infection including two parenteral immunomodulators (IFN-α and pegIFN-α) and four oral NAs (3TC, AFV, ETV, and TLB) (Zoulim and Perrillo 2008; Seetharam and Lisker-Melman 2009). IFN-α and pegIFN-α have significant side effects and low rates of HBeAg or HBsAg seroconversion while 3TC and AFV have been plagued by significant levels of antiviral resistance. Furthermore, recent data suggest that resistance rates to TLB are comparatively high in treatment-naïve patients and that TLB has decreased efficacy against 3TC-resistant HBV (Matthews 2007; Lui and Chan 2008; Nash 2009). ETV is relatively new in the field and therefore its ability to overcome problems associated with its predecessors has not been completely resolved; resistance to ETV in treatment naïve patients appears to be much lower than 3TC. With all these antivirals, the response to treatment with SVR upon treatment withdrawal occurs in <30% of patients (Zoulim and Perrillo 2008; Seetharam and Lisker-Melman 2009).

NAs do not have a direct effect on cccDNA molecules that act as the template for virus replication because the cccDNA in infected hepatocytes is highly stable (Foster *et al.* 2003; Seigneres *et al.* 2003). Consequently, the probability of a SVR is generally low unless there is a coincidental assistance from the host immune response to clear the infection. New pharmacological agents that target cccDNA or which can boost host immunity or prevent emergence of drug resistant virus are needed.

Studies in this chapter were designed to test the ability of REP 2055 to act as a therapeutic antiviral agent once persistent DHBV infection was established. Experiments in Chapter 6 tested the ability of REP 2055 to prevent the rebound of DHBV infection in a prophylactic and three therapeutic experiments using 10 or 2 mg/kg dose regimens in DHBV-infected ducks. Importantly, REP 2055 (10 mg/kg) prevented the development of persistent DHBV infection in 3/4 ducks when treatment was given from 4 days p.i. for 14 days, by which time 10-20% of hepatocytes are typically infected. This finding showed the

ability of REP 2055 to prevent the development and rebound of persistent DHBV infection when the drug treatment was started at an early stage of DHBV infection caused by a dose of DHBV that is capable of producing persistent DHBV infection in two week-old ducks (Jilbert *et al.* 1998; Foster *et al.* 2005).

In the next experiment, REP 2055 treatment was further delayed to 12 days p.i., to allow time for virus to spread to the entire liver. Daily REP 2055 treatment (10 mg/kg) was then carried out for 7 days, followed by 7 weekly doses, and an SVR of a 2-3 log reduction in serum DHBV DNA levels was obtained. One out of 5 ducks was protected against development and rebound of persistent DHBV infection and the other 4 ducks had fluctuating levels of DHBV DNA in the serum. Liver tissue analysis for DHBsAg and DHBV DNA were in agreement with serum based virological markers. At this point, it seemed possible that the short intrahepatic half life of REP 2055 together with the short term of daily therapy (7 days) minimised the success of this treatment regimen (Soni *et al.* 1998). We therefore carried out a longer term treatment with REP 2055 in hopes of obtaining an improved SVR.

To this end, REP 2055 (10 mg/kg) was administered daily for 28 days. Treatment was then stopped and the ducks were monitored for 16 weeks to test for SVR (Zoulim 2004; Hui *et al.* 2005; Marcellin *et al.* 2005; Zoulim and Perrillo 2008).

7.2 Experimental design

Approval to conduct the *in vivo* studies was obtained from the Animal Ethics Committees of the IMVS and University of Adelaide. The source and dose of DHBV, preparation and reconstitution of REP 2055 for IP injection, collection of blood samples, biopsy, autopsy and specimen collection procedures, analysis of serum and liver for DHBsAg and DHBV DNA, are given in Chapter 2. Moreover, anti-DHBs and anti-DHBc antibodies, haematological (CBE) and biochemical markers (liver enzymes) were also tested in blood or serum samples using methods described in Chapter 2

For this experiment, twenty-eight 14-day-old ducks were divided into 2 Groups of 14. The ducks were infected IV with 5 x 10^8 DHBV DNA genomes and examined for the next two weeks for serum and hepatic DHBV markers to confirm if persistent infection had been

established. The ducks were then treated daily with NS or REP 2055 from 14 days p.i. for 28 days.

Whole blood samples of ducks prior to, during, and at treatment endpoint, and after post-treatment follow-up, were tested for CBE and liver enzymes. Sera of NS-treated ducks were used as comparators for liver enzyme analysis. Furthermore, serum samples from 7 to 155 days p.i. were tested for DHBsAg using a quantitative ELISA and for DHBV DNA using qPCR. Sera were also tested for the presence of anti-DHBs and anti-DHBc antibodies. Liver tissues collected on days 14, 106 and 155 p.i. were examined for the percentage of DHBsAg-positive hepatocytes by immuno-staining and DHBV DNA (total and cccDNA) by qPCR. qPCR was performed in liver tissue extracts because PCR inhibition by REP 2055 was not a concern for the chosen time points. In particular, liver tissues were collected on 14 days p.i., prior to starting the REP 2055 treatment, or at 64 and 113 days after stopping REP 2055 treatment. Methods used for these analyses are discussed in detail in Chapter 2.

Ducks were assessed each day for any abnormalities in feed and water intake, weight loss and gait or changes in behaviour (Table 7.1; Figure 7.2). They were also clinically examined through a gentle palpation of the abdomen for signs of pain in the abdominal area. IP injection was given just below the sternum, which permits easy access to the peritoneal cavity. Ducks were also examined for signs of pain on IP injection.

Statistical analysis: Differences in mean body weights at weekly intervals, WBC counts and liver enzyme levels of Groups 7.A and 7.B were statistically analysed to evaluate the impact of REP 2055 treatment on the health of ducks when compared with NS treatment. Moreover, differences in mean serum and hepatic DHBsAg and DHBV DNA levels of ducks responding to REP 2055 treatment and those that did not respond to treatment were also analysed. All statistical analyses were performed using *Student's t-test* with the analytical software Graph Pad Prism Version 5. Differences were considered statistically significant when the p values were <0.05.

7.3 Results

The current study was undertaken to determine if treatment of an established DHBV infection with REP 2055 (10 mg/kg) for a duration of 28 days prevented rebound of infection when the treatment was withdrawn.

REP 2055 treatment for 28 days was well tolerated. IP injections in ducks can damage major abdominal blood vessels or lungs as birds do not have pleurae that cover the lungs. Careful injections minimised IP injection-related shock or mortality; however, Duck 200 died immediately after an IP injection. Moreover, repeated injections every day resulted in perihepatic abscess in 2 ducks possibly due to the introduction of bacteria from the skin to the peritoneal cavity. As this was an extended study, to accommodate the loss, the study was started with 14 ducks in each Group. Hence, 11 ducks were available for 28 days of REP 2055 treatment and follow-up while 13 ducks were used for NS treatment and follow-up (Figure 7.1).

7.3.1 Group 7.A: The effect of NS treatment on clinicopathological, haematological, biochemical and virological markers

Ducks in Group 7.A were treated with NS for 28 days via the IP route of administration. In general, ducks in this Group had neither abdominal tenderness nor abdominal pain on IP injection and no signs of pain were noted during the inspection and palpation of the abdomen. *In situ* examination of internal organs at autopsy (106 and 155 days p.i.) did not reveal any gross pathological changes. Duck 172 of this Group was found injured in its cage due to a physical insult that occurred when the duck inserted its neck and the wings through holes in the metal cage. This duck was autopsied and no abnormalities were observed in any internal organs at autopsy.

In contrast to the REP 2055-treated ducks, the majority of NS-treated ducks had elevated levels of serum DHBsAg and DHBV DNA throughout the course of the study (Figures 7.4a and 7.5a). Mean serum DHBsAg levels detected by qELISA remained between 10-100 µg/mL in NS-treated ducks throughout the course of the study except the levels were as low as 2 µg/mL at 14 and 21 days p.i. (Figure 7.4c). Fluctuating levels of serum DHBsAg levels have been reported in placebo-treated ducks in the past (Foster *et al.* 2005). This fluctuation in serum DHBsAg levels in placebo-treated ducks may be due to an early immune response and production of anti-DHBs antibodies at this time of the DHBV infection. Mean serum DHBV DNA levels detected by qPCR fluctuated between 10^5-10^8 copies/mL throughout the course of the study in NS-treated ducks (Figure 7.5c). Immuno-staining of liver sections on day 106 p.i from Group 7.A showed a widespread DHBV infection in the liver with >95%

DHBsAg-positive hepatocytes (data not shown). Consistent with immuno-staining results of the liver sections, ducks treated with NS had an individual and mean total DHBV DNA and cccDNA levels of 618 and 20 copies/cell, respectively on day 106 p.i. (Figure 7.9; Figure 7.11).

The hepatic dynamics of the 2nd follow-up period in 7 ducks in Group 7.A and 6 ducks in Group 7.B were analysed on 155 days p.i. Immuno-staining of liver sections from Group 7.A showed a widespread DHBV infection in the liver with >95% DHBsAg-positive hepatocytes in 7/7 ducks (data not shown). Agreeing with the percentage of DHBsAg-positive hepatocytes detected by immuno-staining, 7/7 ducks treated with NS had an individual and mean total DHBV DNA and cccDNA levels of 64 and 14 copies/cell, respectively on day 155 p.i. (Figure 7.9; Figure 7.11).

All ducks treated with NS had detectable levels of anti-DHBc antibodies that yielded an increased mean anti-DHBc antibody level (Figure 7.12a; Figure 7.12c). NS-treated ducks or "non responder" ducks did not produce levels of anti-DHBs antibodies that were comparable to "responder" ducks (Figure 7.13a; Figure 7.13c).

7.3.2 Group 7.B: The effect of REP 2055 (10 mg/kg) treatment of 28 days on clinicopathological, haematological, biochemical and virological markers

In general, Group 7.B ducks given daily IP administration of REP 2055 at a dose of 10 mg/kg for 28 days had neither abdominal tenderness nor abdominal pain on IP injection and no signs of pain during the inspection and palpation of the abdomen. *In situ* examination of internal organs at autopsy (106 or 155 days p.i.) did not reveal any gross pathological changes.

Moreover, no significant differences were noted in CBE and hepatic transaminases GGT, ALT and AST in ducks ($p>0.05$) treated with REP 2055 when compared to ducks treated with NS when tested prior to, during and at the end of treatment and follow up (Tables 7.2 and 7.3; Figure 7.3).

Duck 200 died after an IP injection. The cause of death was damage to a major abdominal blood vessel as post-mortem examination revealed the presence of fresh blood in the peritoneal cavity. Duck 190 of Group 7.B was found dead in its cage. Duck 195 of

Group 7.B became unwell, moribund and was euthanised. Ducks 190 and 195 had perihepatic abscess at autopsy and this could have resulted from introduction of pyogenic bacteria during repeated IP injections for several days. Pyogenic bacteria gain access to the circulation and cause septicaemia resulting in toxic shock that is often fatal.

Treatment of ducks with REP 2055 for 28 days reduced the serum DHBsAg levels within 1 week when the treatment was started at 14 days p.i. (Figure 7.4b; Figure 7.4c). By the third week of treatment, the DHBsAg levels in the majority of REP 2055-treated ducks were below the cut-off line except in Ducks 192 and 199 (Figure 7.4b; Figure 7.4c). DHBsAg levels continued to stay at low levels after completion of the 28 days of REP 2055 treatment in 6/11 ducks until 106 day p.i.; that is, 64 days after stopping REP 2055 treatment. However, rebound of infection with elevated DHBsAg levels in the serum occurred in Ducks 189, 191, 192, 194 and 199. DHBV infection rebounded in Duck 199 with elevated DHBsAg levels during the latter part of REP 2055 treatment whereas in Ducks 189, 191 and 192, the infection rebounded 3 weeks after stopping REP 2055 treatment (Figure 7.4b). In Duck 194, the infection rebounded during the latter part of 1st follow up (Figure 7.4b). Ducks 189, 191, 192, 194 and 199 were autopsied on day 106 p.i. and other virological markers were tested. Ducks 187, 188, 196, 197, 198 and 179 were monitored until 155 days p.i. to test for rebound. Ducks 187, 188, 196, 197, 198 and 179 continued to have low levels of serum DHBsAg mostly at the cut off levels or below until the end of follow-up on 155 days p.i., *i.e.* 113 days after stopping REP 2055 treatment (Figure 7.4b).

Treatment of ducks with REP 2055 reduced the serum DHBV DNA levels by ~1 log within a week when persistently DHBV-infected ducks were treated from 14 day p.i. At the end of REP 2055 treatment, 2 ducks had ~5 log reduction in serum DHBV DNA levels whereas as other 4 had ~3-4 log reduction in serum DHBV DNA levels (Figure 7.5b; Figure 7.5c). After stopping REP 2055 treatment, serum DHBV DNA levels continued to stay low in 6/11 ducks until 106 days p.i., *i.e.* 64 days after stopping REP 2055 treatment. However, Ducks 189, 191, 192, 194 and 199 experienced rebound of DHBV infection with elevated serum DHBV DNA levels. Among these 5 ducks that rebounded with DHBV viraemia, Duck 199 showed rebound of DHBV infection to pre-treatment levels immediately after stopping the REP 2055 treatment. Ducks 189, 191, 192, 194 and 199 were autopsied on 106 days p.i. and Ducks 187, 188, 196, 197, 198 and 179 were monitored until 155 days p.i. to

test for rebound. All 6 “responder” ducks (Ducks 187, 188, 196, 197, 198 and 179) continued to have undetectable levels of serum DHBV DNA until the end of follow-up on 155 day p.i., *i.e.* 113 days after stopping treatment (Figure 7.5b; Figure 7.5c).

Immuno-staining of liver sections from 14 days p.i from Group 7.B (Figure 7.6) showed widespread DHBV infection in the liver with >95% DHBsAg-positive hepatocytes prior to treatment (Figure 7.6). Total DHBV DNA and cccDNA copy numbers per liver cell were 615 and 10 copies/cell at first biopsy on 14 days p.i. prior to starting treatment (Figure 7.9).

Autopsies of ducks 189, 191, 192, 194 and 199 from Group 7.B and a second liver biopsy of the remaining 6 ducks were performed on day 106 p.i. to compare the DHBV dynamics 64 days after stopping REP 2055 treatment. Immuno-staining of liver sections on 106 days p.i from Group 7.B (Figure 7.7) showed no detectable DHBsAg-positive hepatocytes in 6/11 ducks while 5/11 ducks had a widespread DHBV infection in the liver with >95% DHBsAg-positive hepatocytes (Figure 7.7). On 106 days p.i., 6/11 REP 2055-treated ducks had a mean total DHBV DNA and cccDNA levels of 0.19 and 0.13 copies/cell, respectively (Figures 7.10 and 7.11) whereas 5/11 ducks from this Group had hepatic total DHBV DNA and cccDNA levels of 887 and 27 copies/cell, similar to the total DHBV DNA and cccDNA levels found in ducks treated with NS (Figures 7.10 and 7.11).

Ducks treated with REP 2055 on 155 days p.i. showed no detectable DHBsAg-positive hepatocytes in 6/6 ducks when tested by immuno-staining of liver sections (Figure 7.8). In REP 2055-treated Group 7.B, 6/6 ducks had total DHBV DNA and cccDNA levels of <0.07 copies/cell on 155 days p.i. (Figures 7.10 and 7.11).

Ducks that responded to the REP 2055 treatment had higher titres of anti-DHBs antibodies than “non-responder’’ and NS-treated ducks (Figures 7.13b and 7.13c). Two “responder” ducks developed a relatively high titre of anti-DHBs antibodies within 7 days of REP 2055 treatment and others had detectable anti-DHBs antibody levels during the treatment. Moreover, all 6 “responder” ducks that achieved SVR continued to have detectable levels of anti-DHBs antibodies throughout the study (Figure 7.13b). The “non responder” ducks did not produce levels of anti-DHBs antibodies that were comparable to “responder” ducks (Figures 7.13b and 7.13c). Similar to the “non responder” ducks, NS-treated ducks did not produce levels of anti-DHBs antibodies that are comparable to

"responder" ducks (Figure 7.13a). Both REP 2055- and NS-treated Groups developed anti-DHBc antibodies by 7 days p.i. and all these ducks had high levels of anti-DHBc antibodies throughout the study (Figures 7.12a, 7.12b and 7.12c).

In summary, 28 days of daily REP 2055 treatment (10 mg/kg) produced SVR in the serum and the liver of 6/11 ducks that was sustained at least 113 days after stopping treatment.

7.4 Discussion

Treating persistent HBV infection with a better therapeutic anti-HBV agent in order to achieve SVR in a higher number of patients is of paramount importance. In order to achieve this goal, persistently DHBV-infected ducks were treated with the novel therapeutic agent, REP 2055, for 28 days, with a dose of 10 mg/kg. The ducks were then monitored for 113 days after stopping treatment. It is important to monitor for sustained periods after stopping treatment in "responder ducks'' as rebound of infection is very common in CHB patients treated with NA therapy.

REP 2055 treatment for 28 days led to a significant decrease in DHBV viraemia and hepatic DHBV markers, although the infection rebounded in 5/11 ducks. DHBsAg levels decreased more rapidly than DHBV DNA in the current study. This finding is distinct for that seen during NA therapy. For instance, ducks treated with ETV for a period of 244 days against persistent DHBV infection exhibited a much more rapid decrease in serum DHBV DNA levels than serum DHBsAg levels (Foster *et al.* 2003). This difference is probably due to the differences in the mechanisms of action of REP 2055 and ETV. The latter has a direct effect on reducing the viral DNA replication and progeny virus production but does not directly alter DHBsAg production; hence, serum DHBV DNA levels are reduced more rapidly than serum DHBsAg levels. In contrast, APDPs appear to block the assembly and release of both DHBV and DHBsAg from infected hepatocytes. DHBsAg assembly is not blocked by ETV.

According to a recent clinical study, pegIFN-α-treated CHB patients that had an early reduction in serum HBsAg levels achieved SVR with a positive predictive value of 92% (Moucari *et al.* 2009). Based on this study, patients that had early reduction in serum HBsAg levels during and after ceasing the pegIFN-α therapy eventually lost HBsAg (Moucari *et al.*

2009). In this regard, reduction of serum DHBsAg levels in REP 2055-treated ducks that is sustained after treatment withdrawal is probably indicative of a strong anti-viral immune response that might be the ultimate cause of DHBV clearance. REP 2055 treatment led to reduction of liver infection to undetectable levels of DHBsAg-positive hepatocytes with total DHBV DNA and cccDNA levels of <0.2 copies/cell in 6/11 of ducks that responded to REP 2055 treatment. On 106 days p.i., the hepatic DHBV DNA levels (<0.2 copies/cell) of "responder'' ducks were 444 and 162 times less than the hepatic total DHBV DNA (>750 copies/cell) and cccDNA (>20 copies/cell) levels of "non responder'' and NS-treated ducks. On 155 days p.i. or at the end of follow-up, liver sections of "responder'' ducks had no detectable DHBsAg-positive hepatocytes with a further 914 and 233 fold decrease in the total DHBV DNA and cccDNA levels to <0.07 copies/cell when compared with 7/7 NS-treated ducks. This finding shows the effectiveness of antiviral activity with REP 2055 against DHBV infection and supports our postulate that APDPs including REP 2055 have the ability to target DHBV replication at multiple points including entry, post-entry events and release of progeny virus into the circulation, based on *in vitro* (Chapter 3, Noordeen *et al*. unpublished) and *in vivo* (Chapter 6, Noordeen *et al*. unpublished) data. The superior antiviral activity shown by REP 2055 on hepatic DHBV DNA has not been reported for any other antiviral agents (Seigneres *et al.* 2003; Foster *et al.* 2005) that have been investigated against persistent DHBV infection.

Other studies that tested long-term antiviral treatment with ETV (Foster *et al.* 2003), PEN (Lin *et al.* 1998) and AFV (Nicoll *et al.* 1998) against persistent DHBV infection showed a 96% reduction in total DHBV DNA and a three-fold reduction in cccDNA. The less dramatic changes in cccDNA levels reflect the inability of the NAs that act only on reverse transcription to markedly decrease the hepatic DHBV cccDNA levels.

The most promising finding of the present study was that 28 days of REP 2055 treatment had a dramatic impact on the levels of cccDNA (0.13 copies/cell), examined on 106 days p.i., and this level was 69 times less than the pre-treatment cccDNA levels (9 copies/cell). Moreover, cccDNA levels continued to decline to a level of 0.07 copies/cell when tested at 155 days p.i., indicating the effectiveness of REP 2055 to induce host mediated immune clearance of DHBV. This was demonstrated by the presence of anti-DHBs antibodies in "responder" ducks. With the decline of cccDNA levels, the levels of DHBsAg and DHBV

DNA released into the bloodstream and the numbers of hepatocytes expressing detectable levels of DHBsAg continued to remain undetectable between 106 and 155 days p.i. The levels of hepatic cccDNA levels may have started to decline during the 28 days of therapy, or from the time of stopping the REP 2055 treatment. However, no surgical biopsy was performed at the time that therapy was stopped.

This finding suggested that the hepatic cccDNA pool was sensitive to REP 2055 or immune clearance that was mediated by REP 2055 in "responder" ducks. On the other hand, the ability of REP 2055-related compounds to concentrate highly in the liver may have helped build a relatively higher intrahepatic concentration of REP 2055 during the 28 days of daily treatment. It elicited an antiviral activity, at least for a few weeks, and this would have helped to reduce the DHBV load. This protracted course of antiviral effect might be due to the PK of REP 2055 and its possible immunomodulatory effect, which would have had an effect on the levels of hepatic and serum DHBV markers including the stable cccDNA. In this respect, REP 2055 showed a superior antiviral efficacy over any of the NAs that have been studied against persistent DHBV infection in ducks (Lin *et al.* 1998; Nicoll *et al.* 1998; Foster *et al.* 2003; Seigneres *et al.* 2003). In "responder" ducks, the mechanism of action of REP 2055 on cccDNA is not known, however, it can be postulated that it is through immunomodulatory effects of REP 2055, as supported by the presence of anti-DHBs antibodies. This effect is similar to that of pegIFN-α and thus it may be through cytokines or through the death of DHBV infected hepatocytes or through both mechanisms that the infected liver cleared the relatively stable cccDNA molecules.

Following withdrawal of REP 2055 therapy, serum-based markers of viral replication continued to decline in all 6 "responder" ducks and this did not occur in "non responder" and NS-treated ducks. This was also the case in the liver when liver tissue was first examined for hepatic DHBV markers 64 days after withdrawing REP 2055 therapy, and hepatic DHBV markers further decreased when examined 113 days after stopping therapy. This phenomenon was very different to ETV or other NA-treated ducks in which the rebound of virological markers became detectable within 40 days of withdrawing the drugs, to levels comparable to those of untreated control ducks. Surprisingly, in the current study the rebound of virological markers did not occur even 113 days after stopping REP 2055, but these "responder" ducks eventually lost DHBsAg and seroconverted to anti-DHBs

antibodies that are true markers of DHBV resolution. In all 6 "responder" ducks that achieved SVR, serum and hepatic virological markers were not detectable, but these ducks developed anti-DHBs antibodies during REP 2055 treatment and continued to have detectable levels of anti-DHBs antibodies throughout the study.

All 6 "responder" ducks had undetectable levels or very low levels of serum DHBsAg and excess anti-DHBs antibodies (Figure 7.13b and 7.13c). As anti-DHBs antibodies are known markers of resolution of DHBV infections, it was evident that the 6/11 "responder" ducks had resolved the DHBV infection with no detectable level DHBsAg-positive hepatocytes and very low levels of DHBV DNA, which is probably similar to the residual DHBV infection that follows immune resolution of hepadnavirus infection (Le Mire *et al.* 2005; Reaiche 2008). In contrast to anti-DHBs antibodies, anti-DHBc antibodies do not neutralize the virus. Therefore the presence of anti-DHBc antibodies is only indicative of present or past DHBV infection. Furthermore, in the current study, the anti-DHBc antibodies were detected 7 days p.i. in the majority of ducks, as also reported by (Jilbert *et al.* 1998).

In summary, 28 days of daily REP 2055 treatment (10 mg/kg) produced a SVR in the serum and the liver in 6/11 ducks, 113 days after stopping the treatment. Resolution of DHBV infection was confirmed based on the virtual absence of hepatic DHBV DNA and the presence of anti-DHBs antibodies in these "responder" ducks, supporting the potential of REP 2055 to act as an antiviral agent against persistent DHBV infection. It can be postulated that REP 2055 has the potential to act as an effective antiviral agent through entry, post-entry and post-release inhibitory mechanisms, which prevented the development of serum and hepatic DHBV load. REP 2055's promising antiviral activity will have future applications for treating chronic HBV infection in humans. Since compounds that are pharmacologically similar to REP 2055 have been tested and used in human cancers and metabolic disorders, the clinical application of REP 2055 for chronic HBV infection in humans seems a practical option in the near future.

Table 7.1: The body weight of ducks treated with NS (Group 7.A) and REP 2055 (Group 7.B)

Treatment Groups	Duck NO	Body weight (g) Age (days)																							
		14	**21**	**24**	**31**	**37**	**46**	**50**	**57**	**64**	**71**	**78**	**85**	**92**	**97**	**106**	**113**	**120**	**127**	**134**	**141**	**148**	**155**	**162**	**165**	**169**
7.A NS IP[a] daily for 28 days	170	500	1100	1300	1650	2080	2365	2700	3010	3500	3635	3440	3450	3455	3495	3485	3550[b]	3565	3450	3445	3465	3380	3425	3430	3445	3450
	172	375	935	1225	1530	1840	2200	2450	2720	2950	2980	3135	3100	3095	3075	3110	3194[c]	3075	3095	-	-	-	-	-	-	-
	174	430	975	1250	1555	1975	2340	2690	3105	3275	3450	3785	3795	3870	3935	4005	3980[c]	4005	4015	-	-	-	-	-	-	-
	176	575	1190	1395	1840	2265	2675	2900	3235	3570	3660	3545	3555	3580	3355	3385	3295[b]	3345	3130	3145	3145	3130	3295	3330	3405	3510
	177	475	1025	1405	1740	2135	2445	2790	3065	3250	3460	3425	3495	3550	3445	3355	3360[c]	3450	3455	-	-	-	-	-	-	-
	178	470	1055	1325	1610	2015	2410	2695	2945	3155	3220	3250	3295	3315	3360	3220	3250[b]	3285	3000	3000	3010	2970	2970	3005	3270	3310
	180	495	1105	1345	1760	2145	2520	2995	3275	3450	3700	3735	3785	3810	3825	3675	3580[b]	3640	3505	3455	3370	3420	3475	3470	3485	3490
	181	305	775	1085	1395	1705	2210	2500	2680	2730	2900	2960	2905	2815	2830	2780	2800[b]	2760	2670	2605	2530	2495	2460	2550	2600	2660
	182	435	1045	1295	1675	1985	2385	2675	2840	3100	3200	3545	3500	3555	3530	3555	3550[b]	3605	3370	3410	3330	3445	3270	3495	3515	3600
	183	465	1165	1675	2050	2465	2975	3330	3620	3770	3900	4215	4255	4290	4340	4480	4380[c]	4370	4370	-	-	-	-	-	-	-
	185	505	1175	1465	1730	2150	2510	2880	3050	3120	3250	3515	3500	3515	3515	3570	3580[b]	3630	3425	3465	3425	3540	3535	3540	3530	3540
	186	475	1085	1375	1720	2120	2530	2990	3220	3390	3600	3640	3655	3675	3695	3465	3515[c]	3370	3385	-	-	-	-	-	-	-
	193	500	1100	1405	1670	1985	2425	2800	2990	3220	3450	3680	3600	3540	3460	3505	3400[c]	3340	3335	-	-	-	-	-	-	-
Mean body weight [d]		***470***	***1062***	***1355***	***1683***	***2062***	***2446***	***2776***	***3029***	***3268***	***3416***	***3528***	***3530***	***3543***	***3528***	***3507***	***3495***	***3500***	***3400***	***3218***	***3282***	***3197***	***3204***	***3260***	***3321***	***3366***
7.B REP 2055 IP[a] daily for 28 days	187	410	990	1325	1620	1890	2280	2400	2585	2800	3005	3095	3075	3100	3155	3045	3010[b]	3000	2965	2950	2905	2910	3235	2940	2965	2990
	188	460	1025	1375	1670	1960	2390	2750	2950	3025	3450	3740	3825	3900	3925	3865	3815[b]	3578	3635	3530	3545	3635	3715	3725	3775	3800
	189	405	995	1365	1690	2005	2345	2490	2580	**2035**	2550	3040	3100	3305	3325	3220	3190[c]	3180	3185	-	-	-	-	-	-	-
	191	420	895	1295	1510	1830	2025	2350	2560	2815	3250	3630	3695	3790	3975	3885	3985[c]	3965	3970	-	-	-	-	-	-	-
	192	505	1235	1575	1900	2270	2770	3200	3510	3695	4000	4095	4105	4120	4270	4300	4235[c]	4185	4195	-	-	-	-	-	-	-
	194	480	1045	1280	1580	2025	2300	2690	2820	2960	3400	3560	3590	3575	3625	3640	3600[b]	3965	3855	3520	3550	3530	3815	3855	3880	3910
	196	450	1055	1360	1740	1990	2365	2595	2720	**2615**	3250	3580	3695	3800	3880	3695	3665[b]	3625	3675	3600	3605	3650	3750	3795	3850	3975
	197	515	1105	1290	1670	1980	2275	2600	2870	**2375**	2500	2680	2865	3085	3600	3585	3550[b]	3530	3540	3505	3554	3550	3575	3700	3880	3915
	198	450	1025	1300	1560	1895	2240	2500	2665	2875	2950	3100	3100	3120	3080	2975	2895[b]	2860	2840	2785	2820	2765	2865	2890	2905	2955
	199	390	1045	1405	1720	2165	2485	2690	2920	3375	3500	3565	3570	3595	3660	3520	3590[c]	3460	3470	-	-	-	-	-	-	-
	179	415	1060	1375	1740	1890	2270	2390	2510	**2460**	3100	3405	3435	3495	3630	3475	3490[b]	3630	3590	3540	3505	3540	3540	3600	3760	3830
Mean body weight [e]		***443***	***1022***	***1338***	***1647***	***1953***	***2288***	***2532***	***2687***	***2769***	***3178***	***3408***	***3460***	***3535***	***3648***	***3564***	***3548***	***3543***	***3538***	***3347***	***3355***	***3369***	***3499***	***3501***	***3574***	***3625***

[a] IP - Intraperitoneal injection;

[b] Biopsy - 113 days of age = 106 days p.i.;

[c] Autopsy - 113 days of age = 106 days p.i.;

[d and e] Mean body weight of ducks treated with NS and REP 2055, respectively;

Differences in mean body weights of ducks in Groups 7.A and 7.B were analysed from 14 to 169 days of age and differences were not statistically significant ($p > 0.05$);

Mean body weight of ducks in Groups 7.A and 7.B is also depicted in Figure 7.1;

Ducks 189, 196, 197 and 179 had a weight reduction at 57 days of age (indicated in blue) and this did not affect the health of these ducks.

Table 7.2: Complete blood evaluation (CBE) of ducks treated with NS (Group 7.A) and REP 2055 (Group 7.B)

Treatment Groups	Duck NO	PCV [b] (L/L)				WBC [c] (x 10^9/L)				Platelets [d]			
		Pre Rx [e]	During Rx [f]	End of Rx [g]	End of follow up [h]	Pre Rx [e]	During Rx [f]	End of Rx [g]	End of follow up [h]	Pre Rx [e]	During Rx [f]	End of Rx [g]	End of follow up [h]
7.A NS IP [a] daily for 28 days	170	0.36	0.36	0.40	0.42	5.2	7.8	14.8	23	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	172	0.36	0.37	0.42	0.59	7.2	16.3	16.7	42	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	174	0.40	0.39	0.43	0.57	5.8	12.6	16.0	43	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	176	0.36	0.40	0.43	0.46	7.7	4.8	14.4	31	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	177	0.36	0.38	0.43	0.44	11.8	13.3	18.5	43	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	178	0.37	0.41	0.43	0.50	6.3	18.8	16.0	32	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	180	0.39	0.40	0.44	0.49	14.5	24.4	24.0	35	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	181	0.37	0.42	0.46	0.50	10.1	16.7	17.2	39	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	182	0.35	0.38	0.43	0.43	11.4	22.8	14.0	37	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	183	0.35	0.35	0.41	0.52	13.3	14.7	18.5	39	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	185	0.38	0.36	0.41	0.45	13.0	19.9	17.2	39	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	186	0.36	0.38	0.45	0.46	7.5	10.4	14.4	30	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	193	0.33	0.35	0.39	0.46	6.6	12.5	9.5	26	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
Mean [i]		***0.37***	***0.38***	***0.43***	***0.48***	***9.02***	***14.20***	***16.25***	***35.31***	***Overall, clumped and adequate levels of platelets were observed on microscopy***			
7.B REP 2055 IP [a] daily for 28 days	187	0.36	0.32	0.29	0.46	9.2	20	17.2	18	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	188	0.36	0.31	0.35	0.46	8.2	16.6	25.8	4	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	189	0.36	0.29	0.31	0.43	11.0	16.0	36.8	8	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	191	0.30	0.31	0.27	0.46	4.7	18.6	31.0	39	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	192	0.37	0.34	0.37	0.48	11.1	22.0	35.5	19	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	194	0.35	0.34	0.33	0.47	7.6	19.4	29.2	16	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	196	0.36	0.35	0.26	0.48	11.2	15.8	31.6	15	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	197	0.34	0.36	0.33	0.45	10.4	22.4	39.4	17	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	198	0.34	0.29	0.34	0.48	8.2	20.0	26.8	13	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	199	0.36	0.32	0.37	0.52	9.0	17.2	19.6	22	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
	179	0.37	0.29	0.35	0.45	9.3	18..0	20.0	31	Clumped and adequate	Clumped and adequate	Clumped and adequate	Clumped and adequate
Mean [j]		***0.36***	***0.33***	***0.32***	***0.47***	***9***	***19***	***30***	***18***	***Overall, clumped and adequate levels of platelets were observed on microscopy***			

[a] IP - Intraperitoneal injection;

[b] PCV - Packed cell volume expressed as L/L;

[c] WBC - White blood cells expressed per litre (WBC/L);

[d] Platelet quality and approximate quantity is expressed as an estimate based on microscopic examination;

[e, f, g and h] Prior to, during, end of treatment and follow up, respectively;

[i and j] Mean levels of PCV and WBC in ducks treated with NS and REP 2055, respectively;

Differences of mean PCV and WBC counts of ducks in Groups 7.A and 7.B were analysed but the differences were not statistically significant ($p > 0.05$).

Table 7.3: Liver function of ducks as tested by liver enzymes GGT, ALT and AST in ducks treated with NS (Group 7.A) and REP 2055 (Group 7.B)

Treatment Groups	Duck NO	Nature of plasma [b]	GGT [c] (IU/L)				ALT [d] (IU/L)				AST [e] (IU/L)			
			Pre Rx [f]	During Rx [g]	End of Rx [h]	End of follow up [i]	Pre Rx [f]	During Rx [g]	End of Rx [h]	End of follow up [i]	Pre Rx [f]	During Rx [g]	End of Rx [h]	End of follow up [i]
7.A NS IP [a] daily for 28 days	170	Normal	3	13	9	7.1	22	26	22	21	12	14	18	15
	172	Normal	7	7	4	8	27	27	18	27	20	22	23	20
	174	Normal	3	8	3	8.4	77	36	28	12	29	22	33	23
	176	Normal	13	3	3	5.8	41	22	15	12	44	36	113	18
	177	Normal	3	6	3	8.2	64	38	28	19	51	25	35	14
	178	Normal	3	7	3	6	82	25	22	15	74	19	26	22
	180	Normal	15	12	3	8.9	72	38	48	25	34	21	45	19
	181	Normal	12	20	16	10.6	67	26	26	30	32	15	23	46
	182	Normal	5	4	3	8.6	39	21	18	15	16	12	20	21
	183	Normal	4	6	3	5.3	57	23	52	15	19	10	38	23
	185	Normal	5	13	3	11	47	24	22	27	26	15	25	26
	186	Normal	6	11	6	9.4	53	29	23	16	25	15	36	17
	193	Normal	7	14	6	11.5	46	32	27	23	29	15	21	30
Mean [j]			***6.36***	***9.07***	***5***	***8.37***	***52.43***	***29.36***	***26.85***	***19.77***	***31.36***	***19.21***	***35.08***	***22.62***
7.B REP 2055 IP [a] daily for 28 days	187	Normal	11	4	5	9.9	42	26	23	16	21	32	25	16
	188	Normal	8	11	6	11	51	23	19	26	26	15	29	19
	189	Normal	9	31	18	8	58	27	27	16	23	13	28	14
	191	Normal	4	9	10	11.2	39	18	25	16	15	12	14	16
	192	Normal	12	10	5	9.7	55	20	19	20	22	16	18	17
	194	Normal	15	14	12	9.2	46	33	28	19	17	14	20	18
	196	Normal	12	23	6	6.9	41	26	40	18	23	15	25	14
	197	Normal	16	23	35	13.2	45	17	8	12	24	20	30	18
	198	Normal	16	23	9	3.4	49	15	25	27	20	17	19	23
	199	Normal	17	18	6	5	50	37	24	28	17	22	20	25
	179	Normal	3	4	16	9.9	88	27	32	24	51	14	32	20
Mean [k]			***11.36***	***16.64***	***11.83***	***8.85***	***49.79***	***23.50***	***25***	***20.18***	***23.50***	***17***	***23.64***	***18.18***

[a] IP - Intraperitoneal injection;

[b] Nature of plasma;

[c] Gamma glutamyl transferase (GGT),

[d] Alanine amino transferase (ALT);

[e] Aspartate transferase (AST);

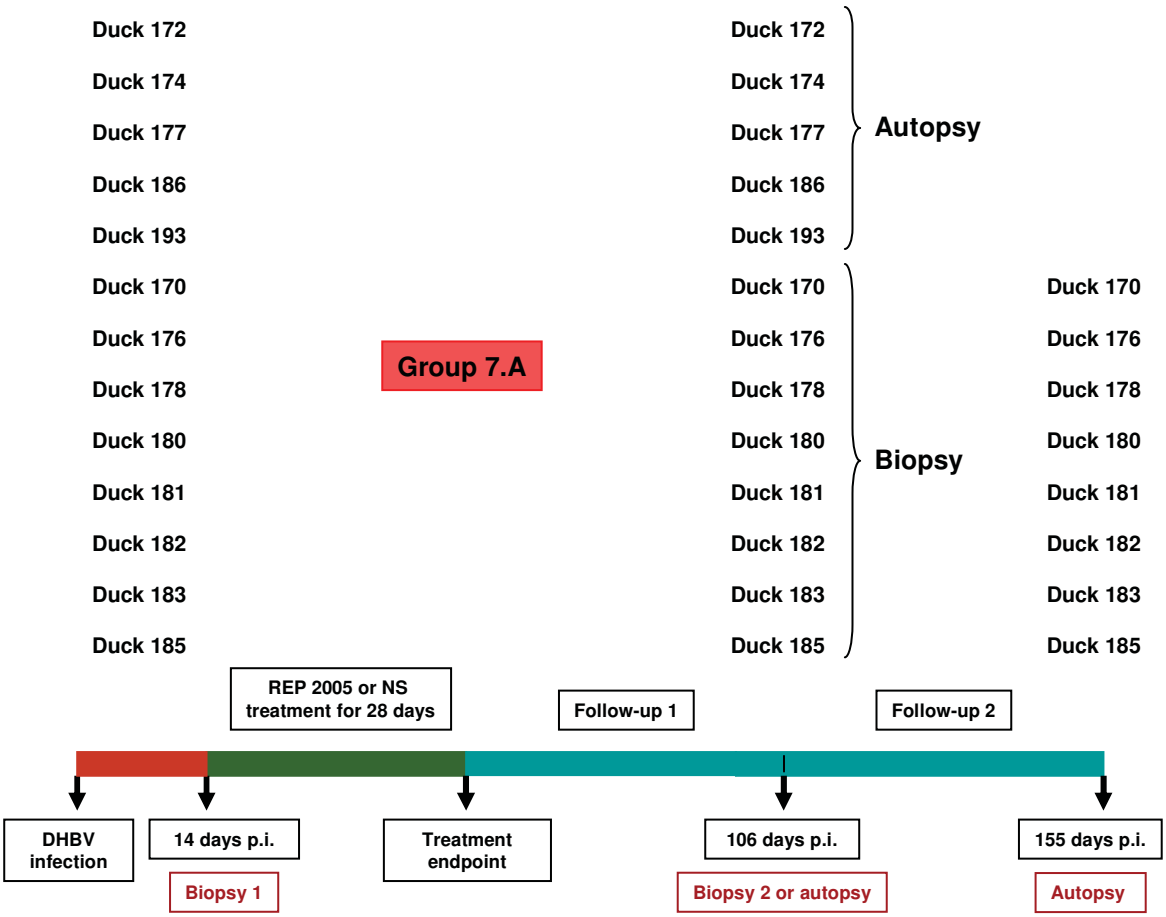
[f, g, h and i] Prior to, during, end of treatment and follow up, respectively;

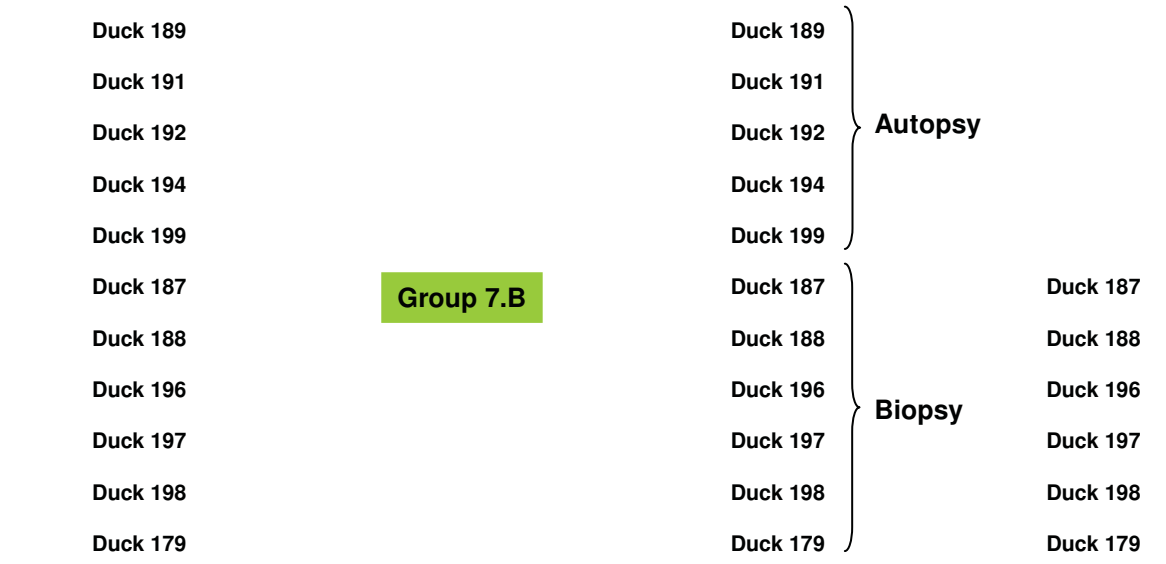
[j and k] Treatment with NS and REP 2055, respectively; and the graphical representation is given in Figure 7.3;

Levels of GGT, ALT and AST in ducks from Groups 7.A and 7.B were analysed and differences in means were not statistically significant ($p > 0.05$).

Figure 7.1: Experimental outline of studies discussed in Chapter 7. 14-day-old ducks infected with 5 x 10^8 DHBV DNA genomes, were treated from 14 days p.i. with NS **(Group 7.A)** and REP 2055 **(Group 7.B)** for 28 days.

Ducks were then followed up until 106 days p.i. at which point 6 ducks from Group 7.A and 5 ducks from Group 7.B were autopsied. Seven ducks from Group 7.A and 5 ducks from Group 7.B were biopsied on 106 days p.i. and these ducks were followed up until 155 days p.i.


Duck 172
Duck 174
Duck 177
Duck 186
Duck 193
Duck 172
Duck 174
Duck 177
Duck 186
Duck 193
Autopsy
Duck 170
Duck 176
Duck 178
Duck 180
Duck 181
Duck 182
Duck 183
Duck 185
Group 7.A
Duck 170
Duck 176
Duck 178
Duck 180
Duck 181
Duck 182
Duck 183
Duck 185
Biopsy
Duck 170
Duck 176
Duck 178
Duck 180
Duck 181
Duck 182
Duck 183
Duck 185
REP 2005 or NS treatment for 28 days
Follow-up 1
Follow-up 2
DHBV infection
14 days p.i.
Biopsy 1
Treatment endpoint
106 days p.i.
Biopsy 2 or autopsy
155 days p.i.
Autopsy


Duck 189
Duck 191
Duck 192
Duck 194
Duck 199
Duck 189
Duck 191
Duck 192
Duck 194
Duck 199
Autopsy
Duck 187
Duck 188
Duck 196
Duck 197
Duck 198
Duck 179
Group 7.B
Duck 187
Duck 188
Duck 196
Duck 197
Duck 198
Duck 179
Biopsy
Duck 187
Duck 188
Duck 196
Duck 197
Duck 198
Duck 179

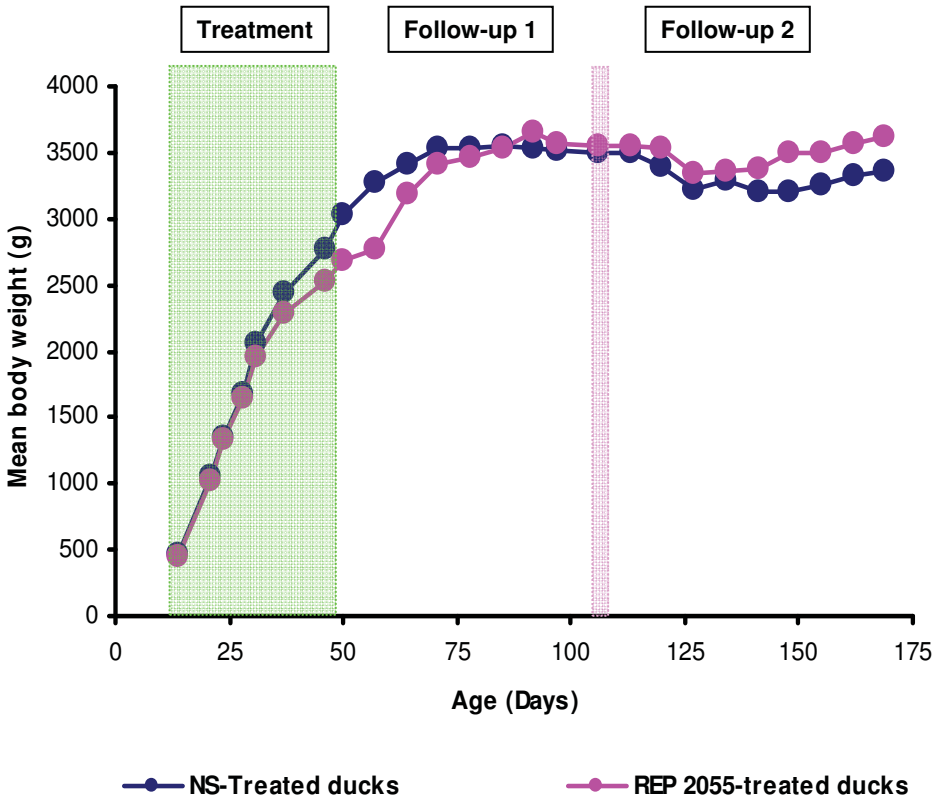


Figure 7.2: Mean body weight of ducks treated with NS (Group 7.A) and REP 2055 (Group 7.B). A complete analysis of individual body weights of ducks is given in Table 7.1.

Differences of the mean body weights of ducks in Groups 7.A and 7.B were analysed at time points from 14 to 169 days and the differences were not statistically significant ($p > 0.05$).

Figure 7.3: Mean levels of GGT (Panel A), ALT (Panel B) and AST (Panel C) in ducks treated with NS (Group 7.A) and REP 2055 (Group 7.B) prior to, during, at the end of treatment and follow up.

The original data with mean enzyme levels prior to, during, at the end of treatment and at the end of follow up are given in Table 7.3.

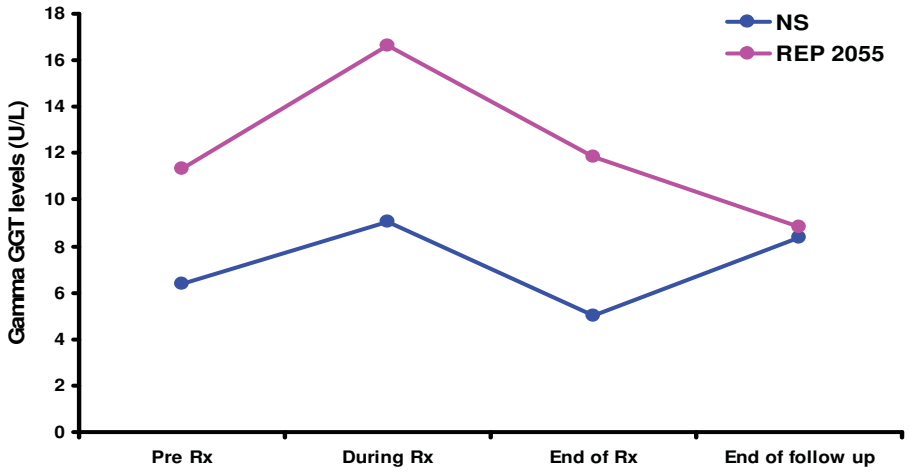
Panel A: Mean levels of GGT;

Panel B: Mean levels of ALT;

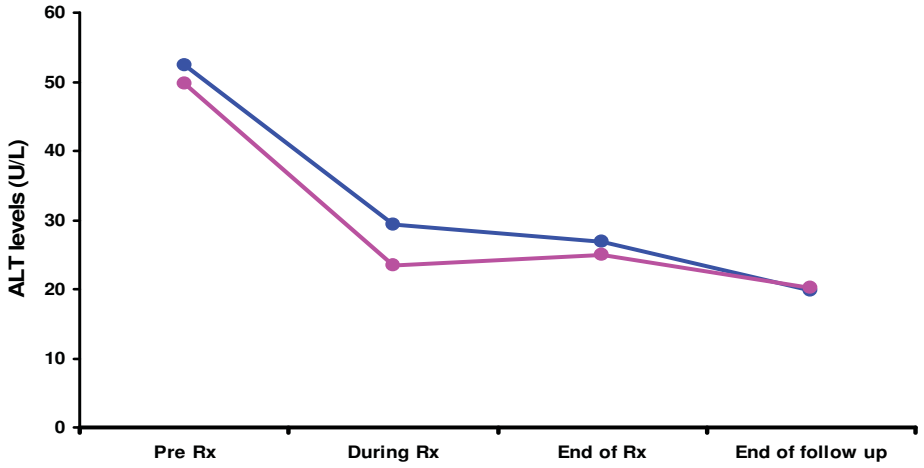
Panel C: Mean levels of AST.

Differences of mean liver enzyme levels between ducks treated with NS and REP 2055 were analysed and differences were not statistically significant ($p > 0.05$).

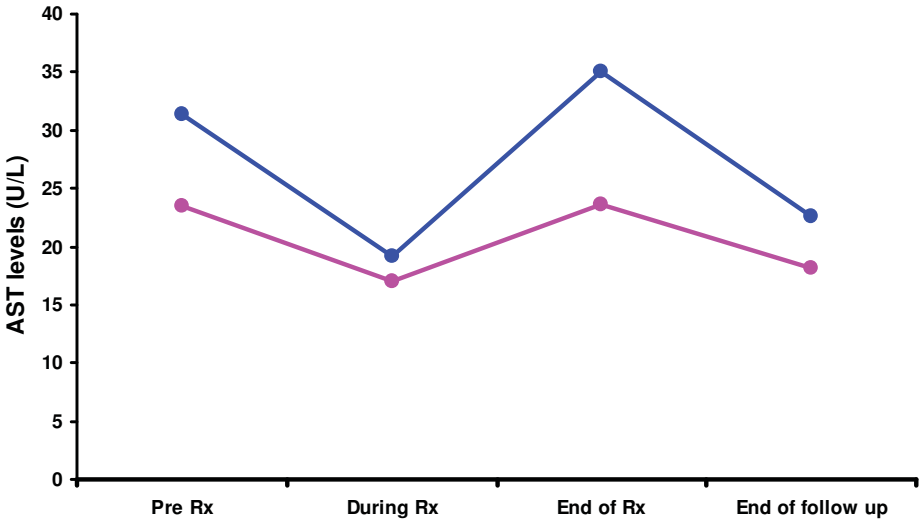
A


NS
REP 2055
Gamma GGT levels (U/L)
18
16
14
12
10
8
6
4
2
0
Pre Rx
During Rx
End of Rx
End of follow up

B


ALT levels (U/L)
60
50
40
30
20
10
0
Pre Rx
During Rx
End of Rx
End of follow up

C


AST levels (U/L)
40
35
30
25
20
15
10
5
0
Pre Rx
During Rx
End of Rx
End of follow up

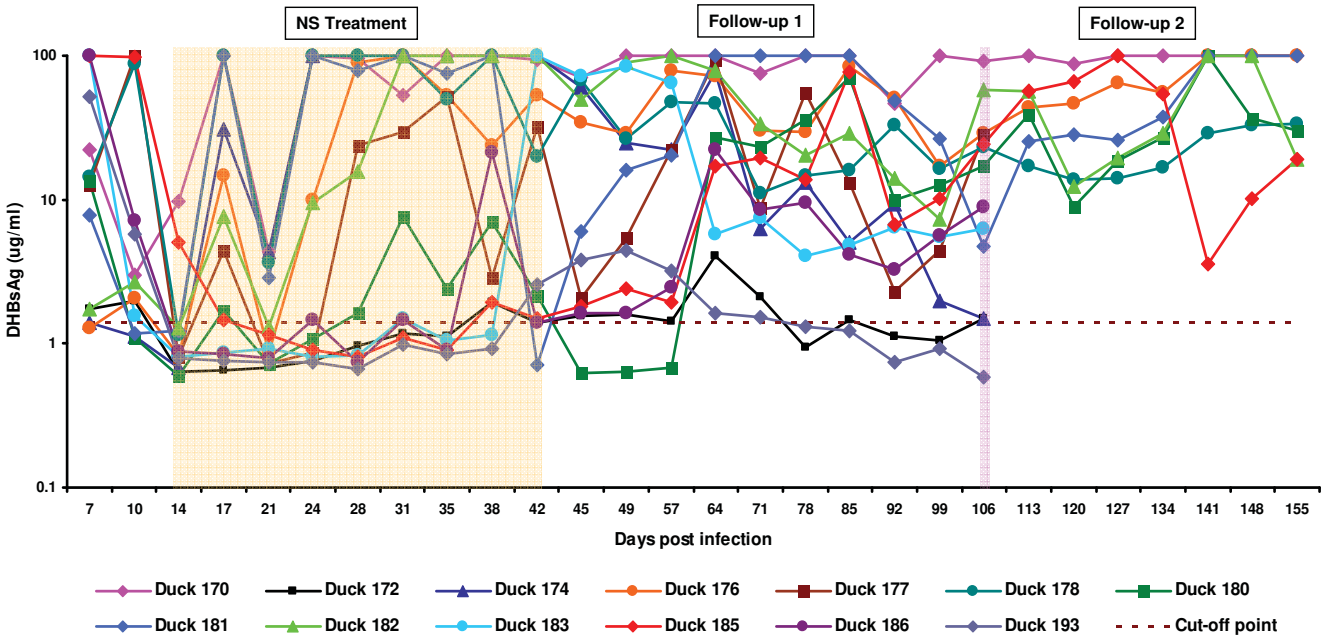


Figure 7.4a: Group 7.A - Serum DHBsAg levels of ducks treated with NS were detected by a qualitative ELISA using methods described in Section 2.8.1. 14-day-old ducks were infected with 5 x 10^8 DHBV DNA genomes and treated with NS from 14 days p.i. for 28 days. Ducks were monitored for 16 weeks after stopping the treatment.

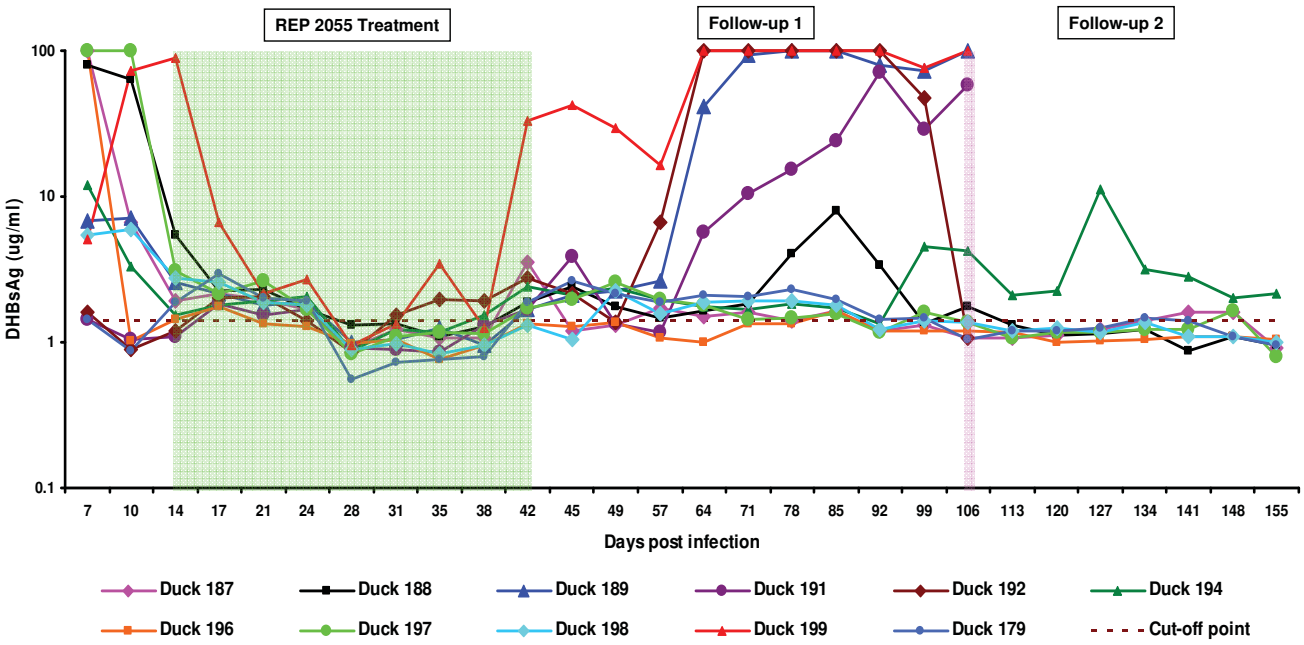


Figure 7.4b: Group 7.B - Serum DHBsAg levels of ducks treated with REP 2055 were detected using a qualitative ELISA described in Section 2.8.1. 14-day-old ducks were infected with 5 x 10^8 DHBV DNA genomes and treated with REP 2055 (10 mg/kg) from 14 days p.i. for 28 days. Ducks were monitored for 16 weeks after stopping the treatment.

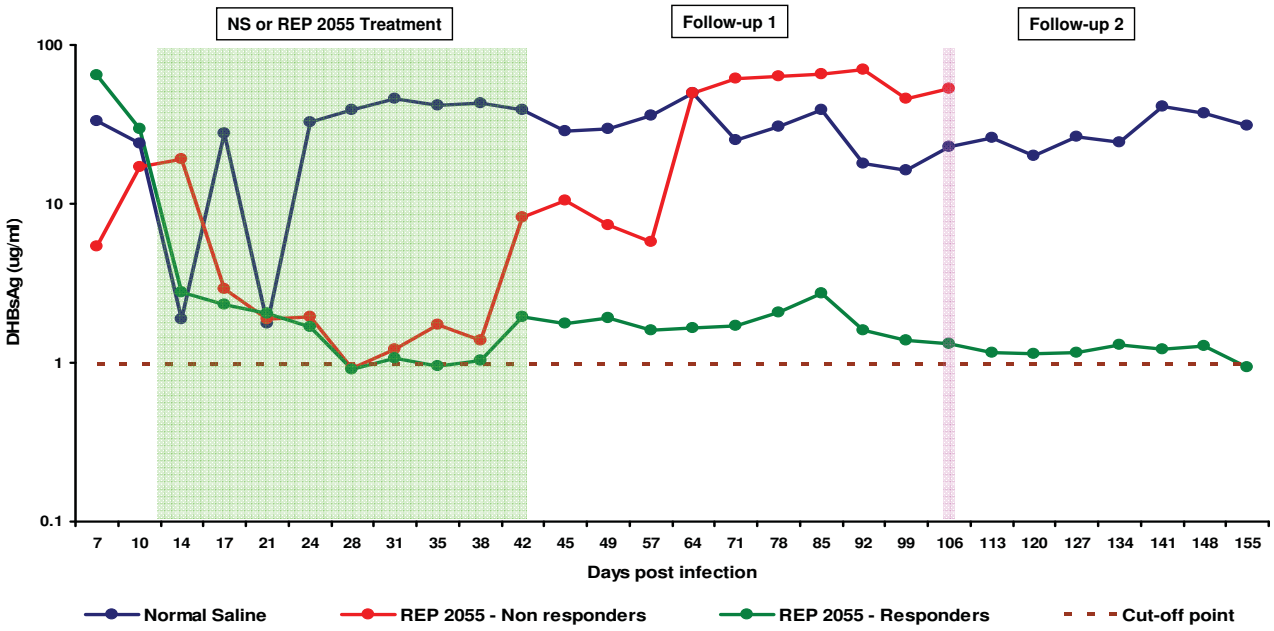


Figure 7.4c: Mean serum DHBsAg levels in ducks treated with NS (n=13), ducks that did not respond to REP 2055 (n=5) treatment and ducks that responded to REP 2055 treatment (n=6). 14-day-old ducks were infected with 5 x 10^8 DHBV DNA genomes and treated with NS or REP 2055 from 14 days p.i. for 28 days. Ducks were monitored for 16 weeks after stopping the treatment.

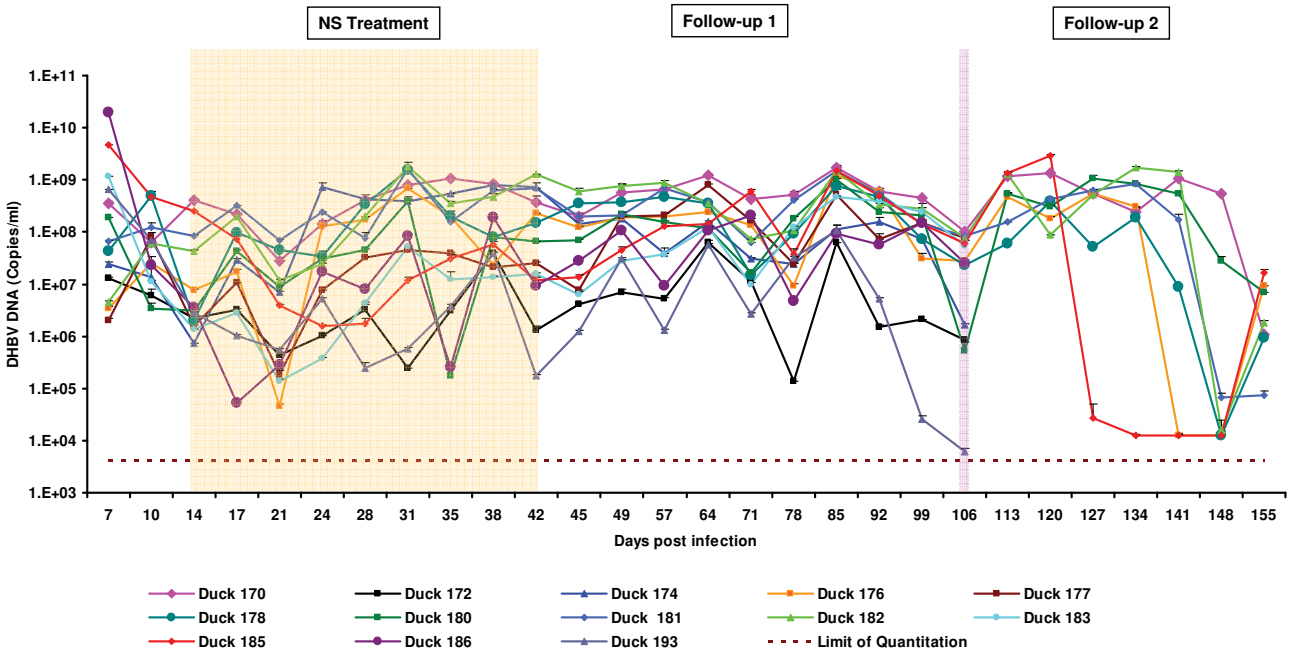


Figure 7.5a: Group 7.A - Serum DHBV DNA levels of ducks treated with NS using a qPCR assay as described in Section 2.10.4. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with NS from 14 days p.i. for 28 days. Ducks were monitored for 16 weeks after stopping the treatment.

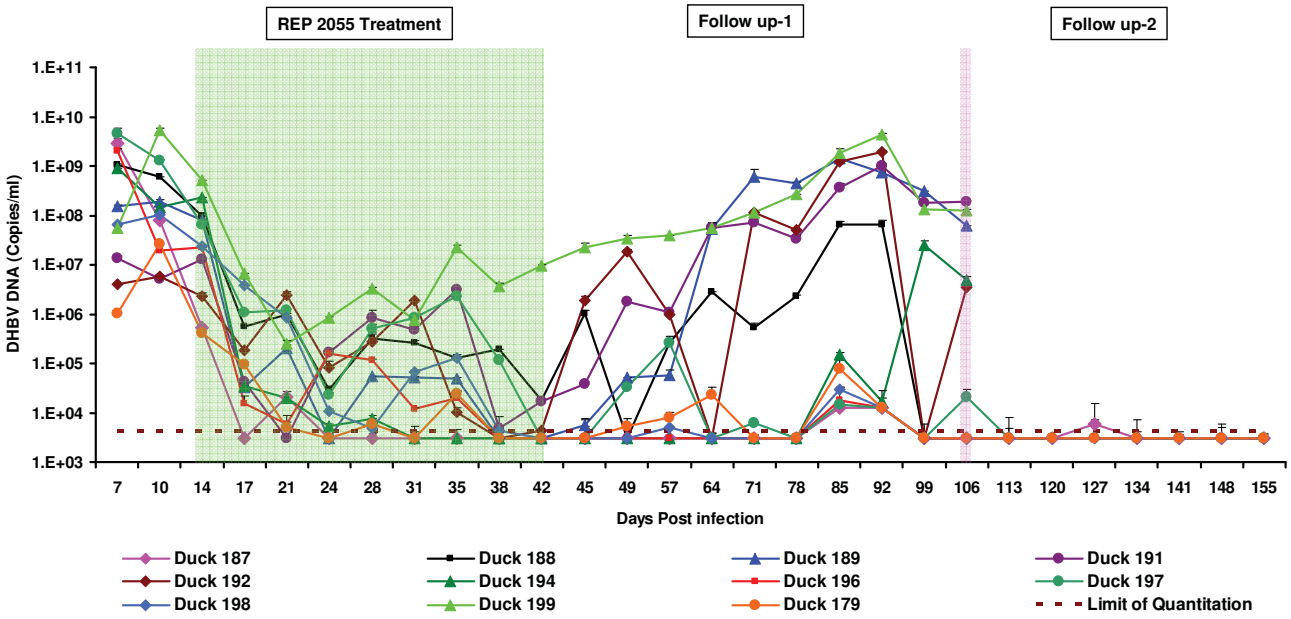


Figure 7.5b: Group 7.B - Serum DHBV DNA levels of ducks treated with REP 2055 using a qPCR assay as described in Section 2.10.4. 14-day-old ducks were infected with 5 x 10^8 DHBV DNA genomes and treated with REP 2055 (10 mg/kg) from 14 days p.i. for 28 days. Ducks were monitored for 16 weeks after stopping the treatment.

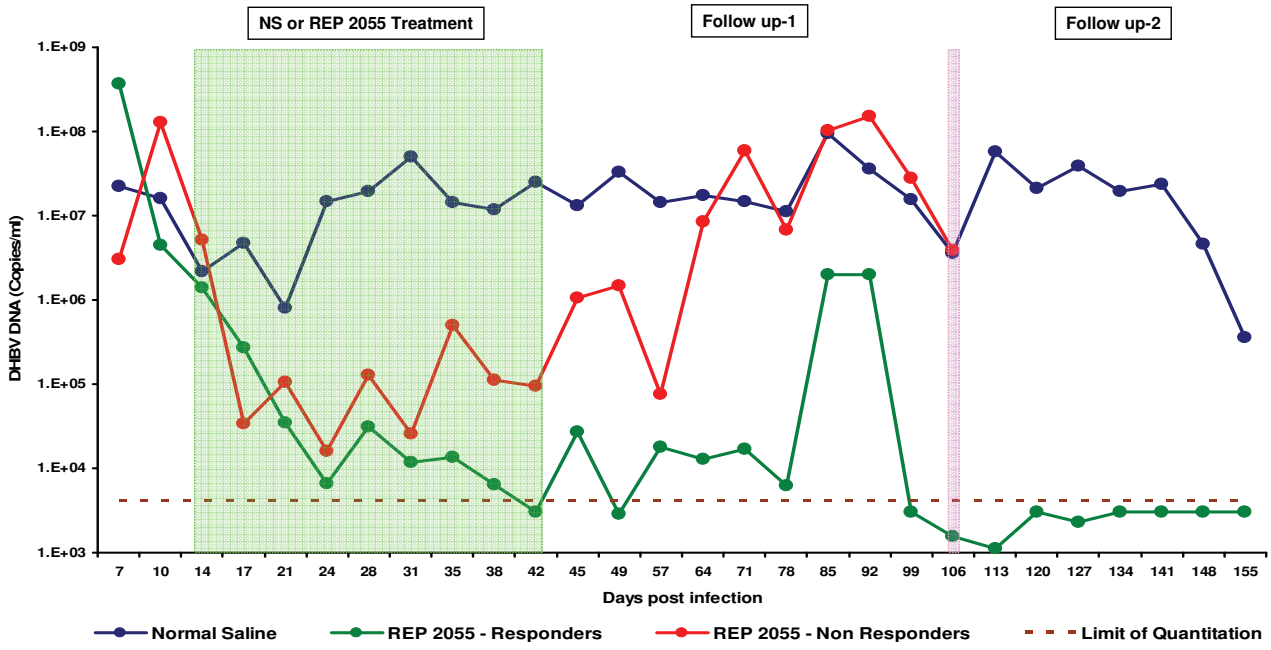


Figure 7.5c: Mean serum DHBV DNA levels in ducks treated with NS (n=13), ducks that did not respond to REP 2055 treatment (n=5) and ducks that responded to REP 2055 treatment (n=6). 14-day-old ducks were infected with 5 x 10^8 DHBV DNA genomes and treated with NS or REP 2055 from 14 days p.i. for 28 days. Ducks were monitored for 16 weeks after stopping the treatment.

Figure 7.6: The percentage of DHBsAg-positive hepatocytes in liver sections of ducks with persistent DHBV infection. These liver sections were from ducks on 14 days p.i. *i.e.* prior to starting the REP 2055 therapy.

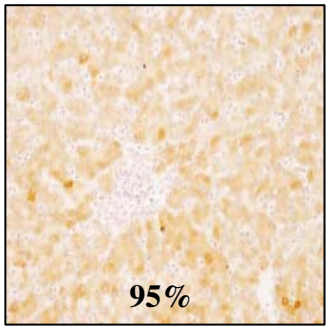
All ducks (Groups 7.A and 7.B) had >95% DHBV-positive hepatocytes prior to treatment.

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

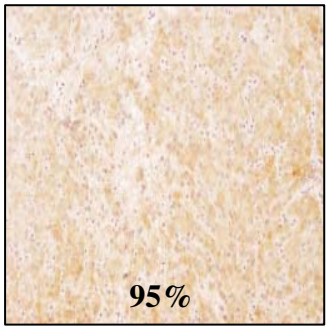
All the sections were counter stained with haematoxylin and the sections were photographed using 400x magnification.

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.

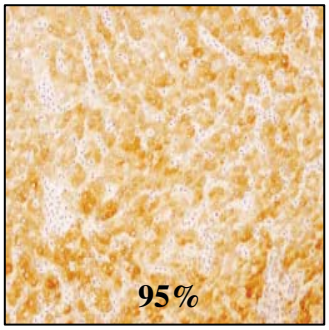
Duck 187



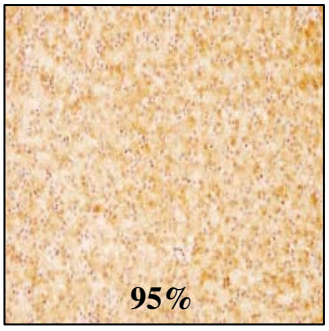
Duck 188



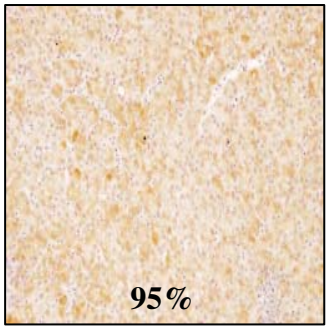
Duck 189



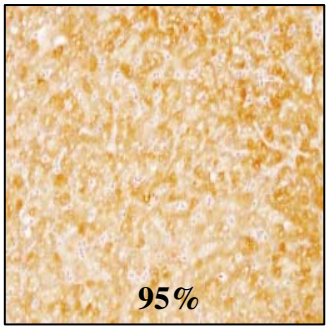
Duck 191



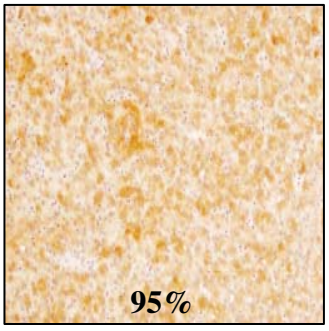
Duck 192



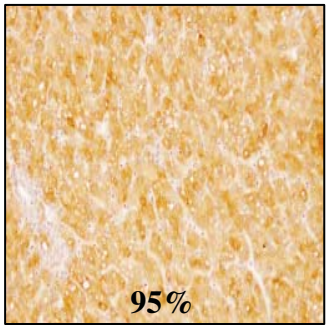
Duck 194



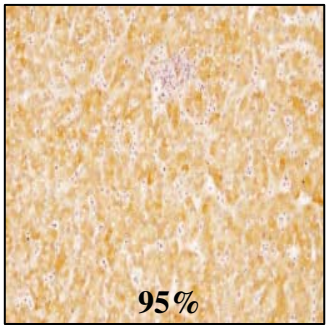
Duck 196



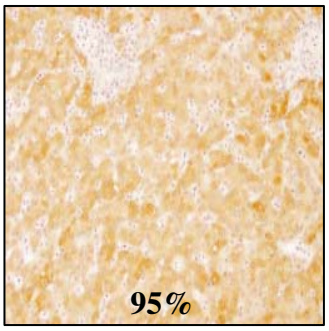
Duck 197



Duck 198



Duck 199



Duck 179

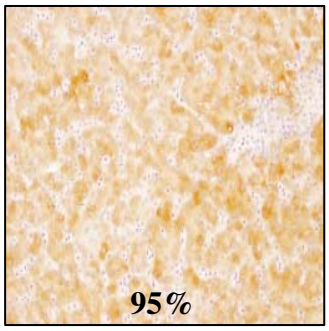


Figure 7.7: The percentage of DHBsAg-positive hepatocytes in the liver sections on 106 days p.i. *i.e.* 64 days after ceasing REP 2055 treatment. Ducks were treated with NS (Group 7.A) or REP 2055 (Group 7.B) starting from 14 days p.i. for 28 days.

Ducks 187, 188, 196, 197, 198 and 179 had no detectable DHBs-Ag positive hepatocytes (<0.001) and ducks 189, 191, 192, 194 had >95% DHBV-positive hepatocytes.

All 13 Group 7.A ducks had 95% DHBV-positive hepatocytes in the liver on day 106 p.i. *i.e* 64 days after ceasing the NS therapy (data not shown).

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

All the sections were counter stained with haematoxylin and the sections were photographed using 400x magnification.

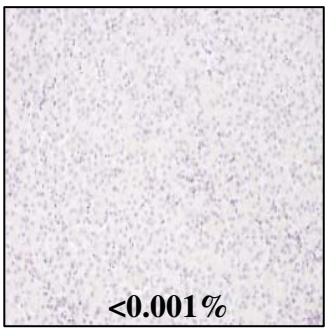
The sensitivity of detection of cytoplasmic DHBsAg by immuno-staining is <0.001. This level of sensitivity was calculated by counting a total of 100,000 hepatocytes in the liver tissue sections as explained in Section 2.9.2.

Duck 187

Duck 188

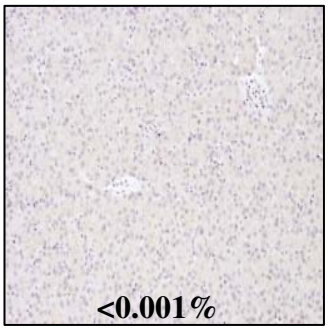
Duck 196

Duck 197

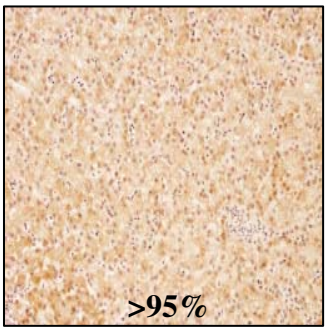


Duck 198

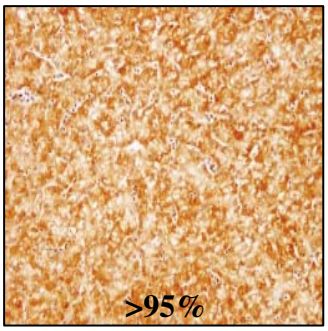
Duck 179



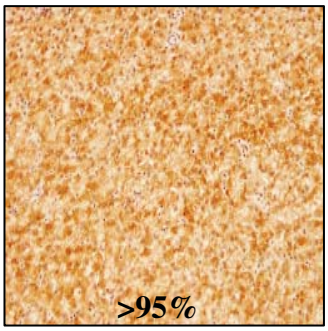
Duck 189



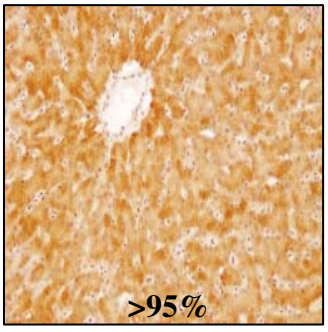
Duck 191



Duck 192



Duck 194



Duck 199

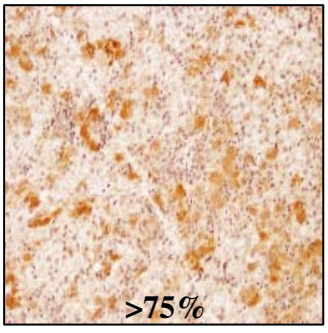
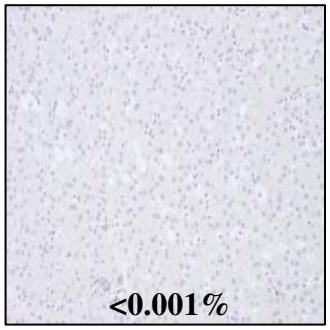


Figure 7.8: Detection of DHBsAg-positive hepatocytes by immuno-staining of liver sections. Ducks with persistent DHBV infection were treated with REP 2055 starting from 14 days p.i. for 28 days. These liver sections were from ducks at autopsy on 155 days p.i.

All 7 Group 7.A ducks had 95% DHBV-positive hepatocytes in the liver on day 155 p.i. *i.e* 113 days after ceasing the NS therapy (data not shown).

Group 7.B Ducks 187, 188, 196, 197, 198 and 179 had no detectable DHBV-positive hepatocytes in the liver on day 155 p.i. *i.e* 113 days after ceasing the REP 2055 therapy.

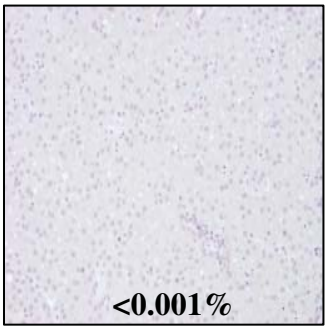
Duck 187



Duck 188

Duck 196

Duck 197



Duck 198

Duck 179

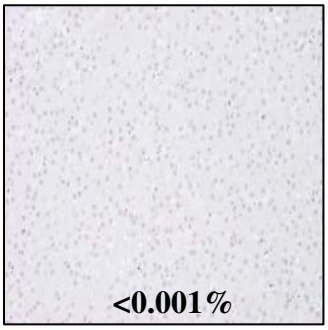


Figure 7.9: The levels of hepatic total and cccDNA of ducks treated with NS on 14 (prior to starting the NS treatment), 106 (64 days after ceasing the REP 2055 therapy) and 155 (113 days after ceasing the REP 2055 therapy) days p.i.

There were no dramatic differences in the levels of hepatic total and cccDNA between the three time points (14, 106 and 155 days p.i.) as NS treatment was expected to produce any antiviral activity against DHBV infection and its spread.

Total DHBV DNA and cccDNA were detected by qPCR of cellular and viral DNA extracts using primers and PCR conditions as described in Sections 2.10.5 and 2.10.6.

DHBV DNA and cccDNA levels are expressed as copies of DNA/cell. 150 ng of DNA was used for each qPCR reaction is equivalent to ~53000 cells. The analysis was performed using methods described in Section 2.10.5.

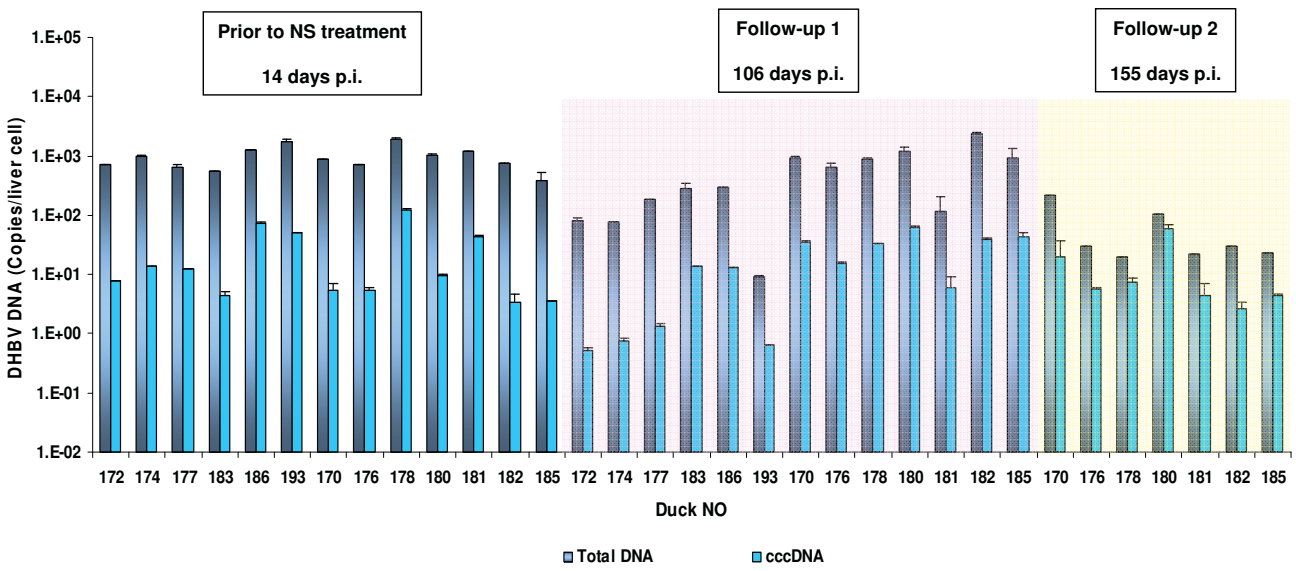

Prior to NS treatment
14 days p.i.
Follow-up 1
106 days p.i.
Follow-up 2
155 days p.i.
DHBV DNA (Copies/liver cell)
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
1.E-01
1.E-02
172 174 177 183 186 193 170 176 178 180 181 182 185 172 174 177 183 186 193 170 176 178 180 181 182 185 170 176 178 180 181 182 185
Duck NO
Total DNA
cccDNA

Figure 7.10: The levels of hepatic total and cccDNA of ducks treated with REP 2055 on 14 (prior to starting the REP 2055 treatment), 106 (64 days after ceasing the REP 2055 therapy) and 155 (113 days after ceasing the REP 2055 therapy) days p.i.

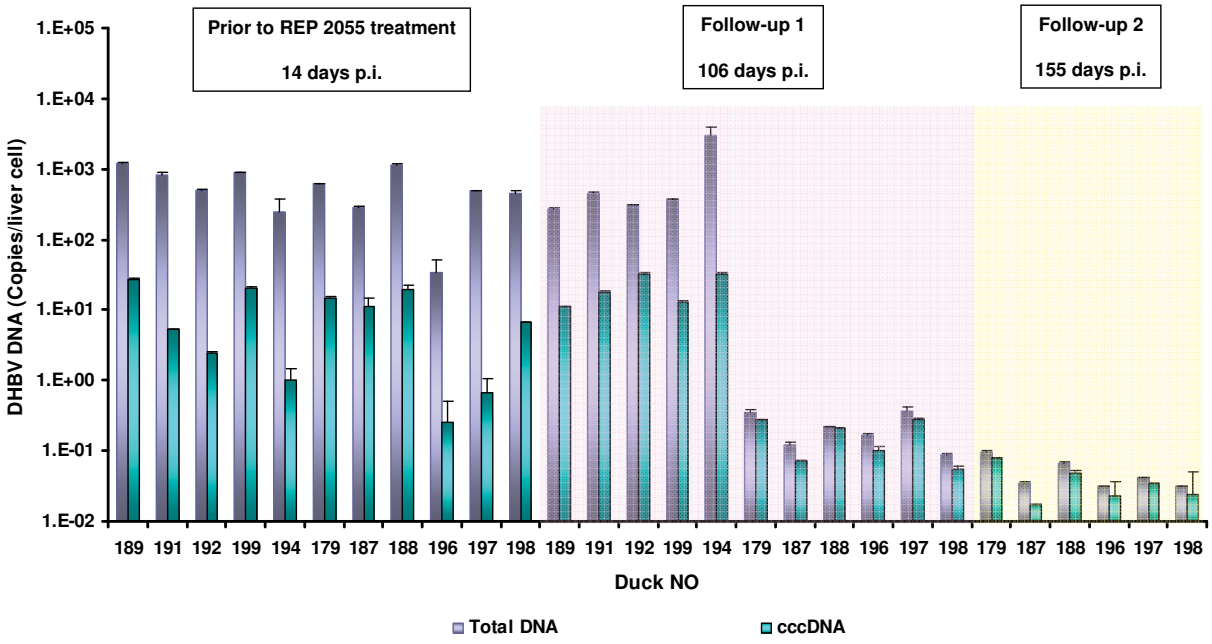
There was a significant drop in the levels of hepatic total and cccDNA of ducks that responded to the REP 2055 treatment (n=6) when compared with the ducks that did not respond to REP 2055 treatment (n=5). Hepatic total and cccDNA levels of "responder ducks" on 106 and 155 days p.i. were significantly lower than the levels detected prior to treatment in these ducks ($p<0.05$).

Furthermore, there was a significant drop in the levels of hepatic total and cccDNA of ducks that responded to the REP 2055 treatment (n=6) when compared with the ducks treated with NS (n=13) ($p<0.05$) (Figure 7.9).

Total DHBV DNA and cccDNA were detected by qPCR of cellular and viral DNA extracts using primers and PCR conditions as described in Sections 2.10.5 and 2.10.6.

DHBV DNA and cccDNA levels are expressed as copies of DNA/cell. 150 ng of DNA was used for each qPCR reaction is equivalent to ~53000 cells. The analysis was performed using methods described in Section 2.10.5.

The error bars indicate the standard deviation (SD).


Prior to REP 2055 treatment
14 days p.i.
Follow-up 1
106 days p.i.
Follow-up 2
155 days p.i.
DHBV DNA (Copies/liver cell)
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
1.E-01
1.E-02
189 191 192 199 194 179 187 188 196 197 198 189 191 192 199 194 179 187 188 196 197 198 179 187 188 196 197 198
Duck NO
Total DNA
cccDNA

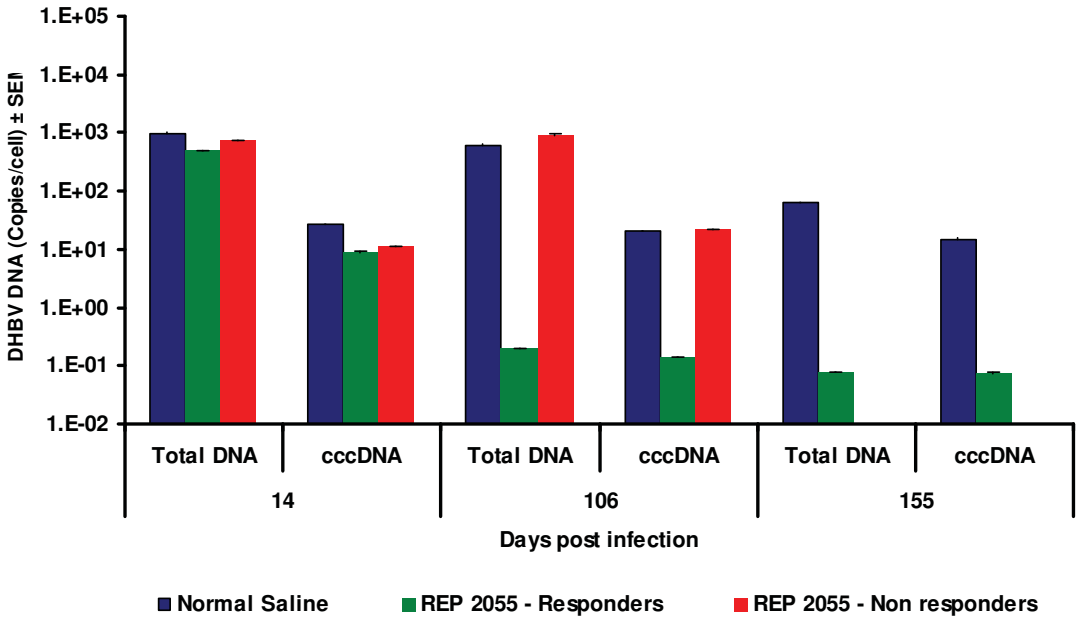


Figure 7.11: The mean levels of total DHBV DNA and cccDNA as detected by qPCR of cellular and viral DNA extracts using primers and conditions as described in Sections 2.10.5 and 2.10.6.

Ducks with persistent DHBV infection were treated with either NS or REP 2055 starting from 14 day p.i. for 28 days and monitored for 16 weeks after ceasing REP 2055 treatment.

These total DHBV DNA and cccDNA were from livers of ducks on 14 (prior to treatment), 106 (46 days after ceasing the therapy) and 155 (113 days after ceasing the therapy) days p.i.

The mean levels of hepatic total and cccDNA were significantly less in ducks that responded to REP 2055 treatment (n=6) than ducks treated with NS (n=13) *($p<0.05$)* or ducks that did not respond to REP 2055 treatment (n=5) *($p<0.05$)*.

The error bars indicate the standard error of mean (SEM).

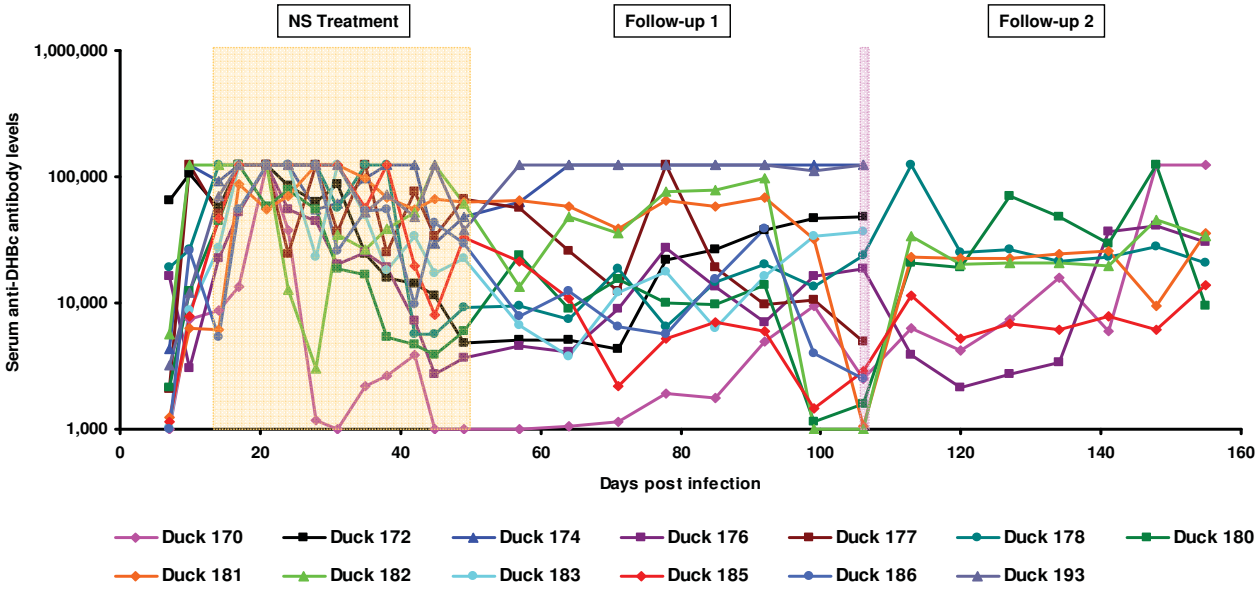


Figure 7.12a: Group 7.A - Anti-DHBc antibody titre in the sera of ducks treated with NS using an ELISA. Anti-DHBc antibody titres were calculated using methods described in Section 2.8.4. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes, treated with NS from 14 days p.i. for 28 days and monitored for 16 weeks after ceasing the treatment.

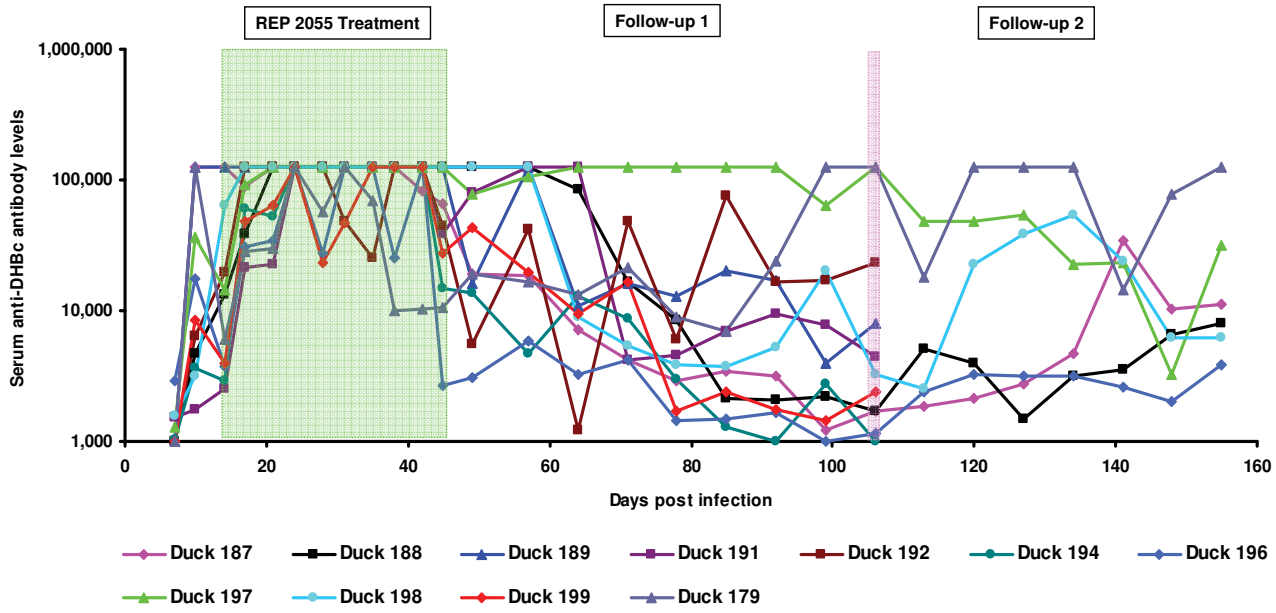


Figure 7.12b: Group 7.B - Anti-DHBc antibody titre in the sera of ducks treated with REP 2055 using ELISA. The anti-DHBc antibody titres were calculated using methods described in Section 2.8.4. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes, treated with REP 2055 from 14 days p.i. for 28 days and monitored for 16 weeks after ceasing the treatment.

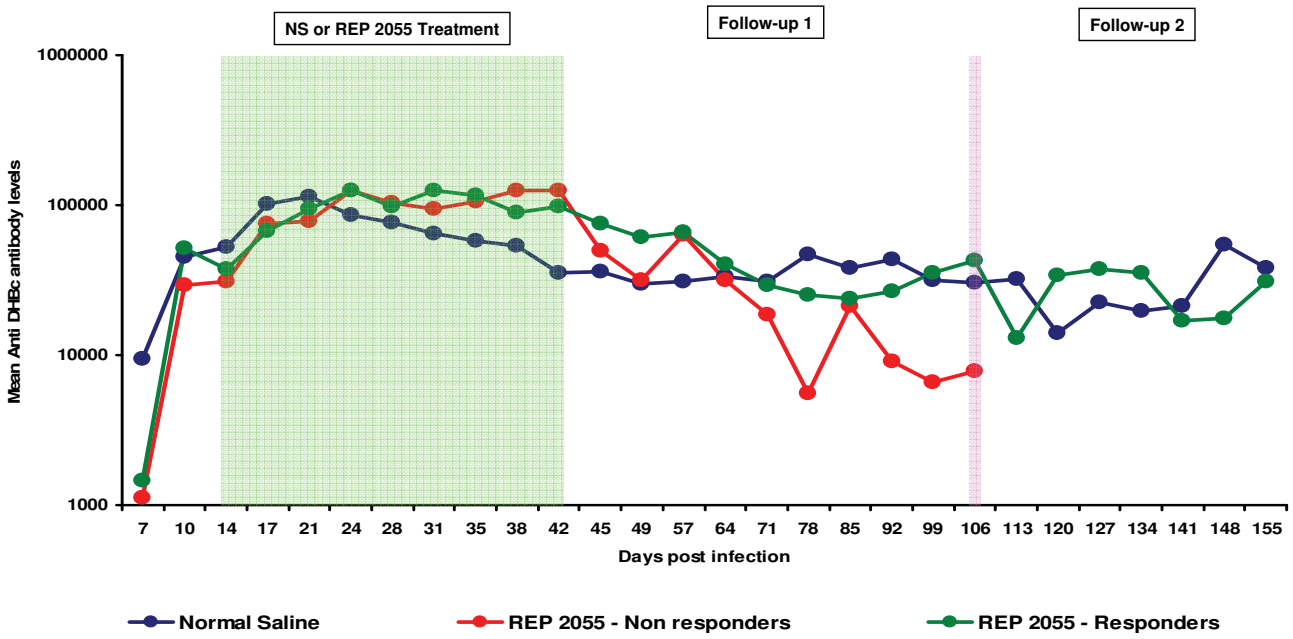


Figure 7.12c: Mean anti-DHBc antibody levels in ducks treated with NS (n=13), ducks that did not respond to REP 2055 treatment (n=5) and ducks that responded to REP 2055 treatment (n=6). 14-day-old ducks were infected with 5 x 10^8 DHBV DNA genomes and treated with NS or REP 2055 from 14 days p.i. for 28 days. Ducks were monitored for 16 weeks after stopping the treatment.

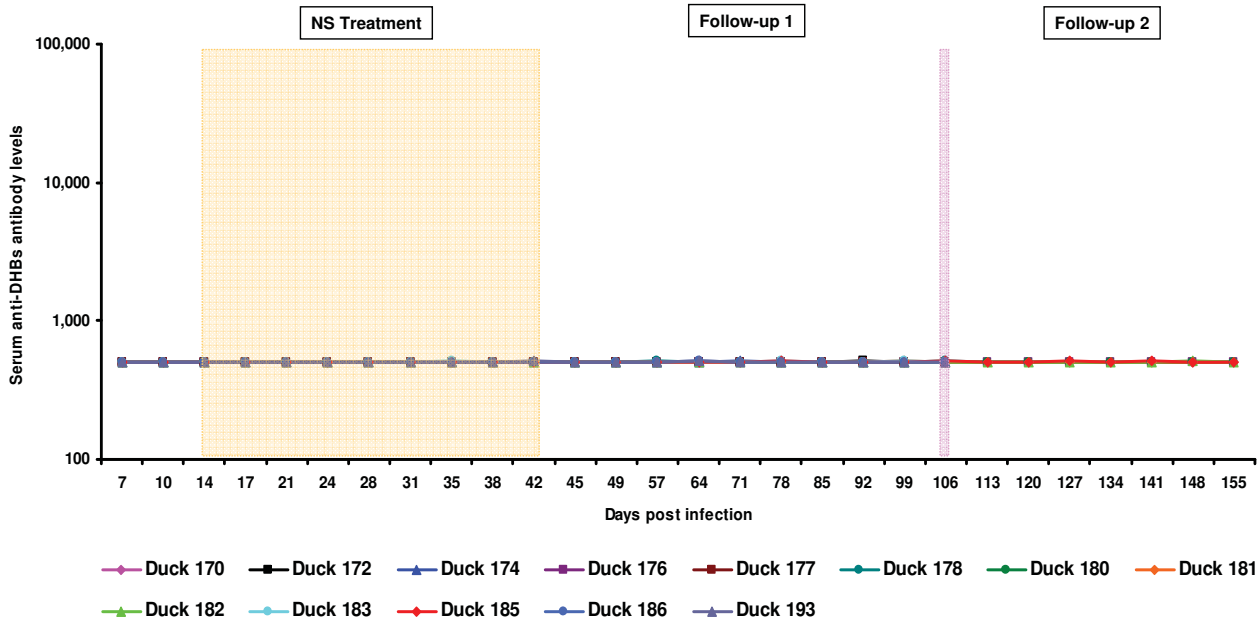


Figure 7.13a: Group 7.A - Anti-DHBs antibody titre in the sera of ducks treated with NS using ELISA and the titres were calculated using methods described in Section 2.8.3. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes, treated with NS from 14 days p.i. for 28 days and monitored for 16 weeks after ceasing the treatment.

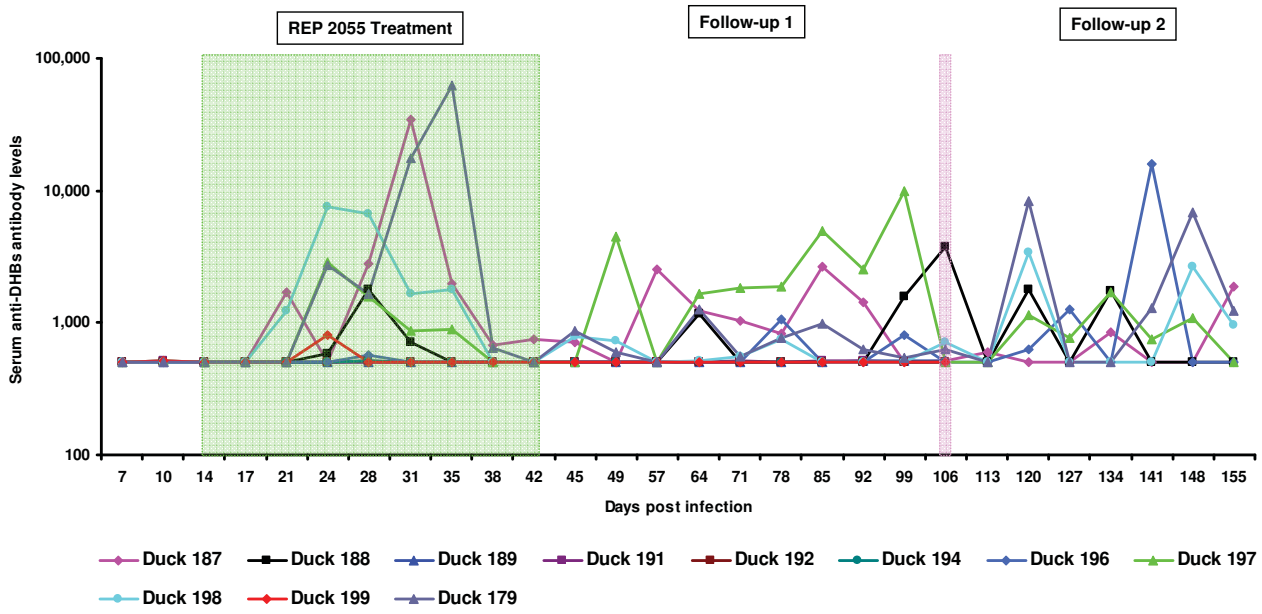


Figure 7.13b: Group 7.B - Anti-DHBs antibody titre in the sera of ducks treated with REP 2055 using ELISA and the titres were calculated using methods described in Section 2.8.3. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes, treated with REP 2055 from 14 days p.i. for 28 days and monitored for 16 weeks after ceasing the treatment.

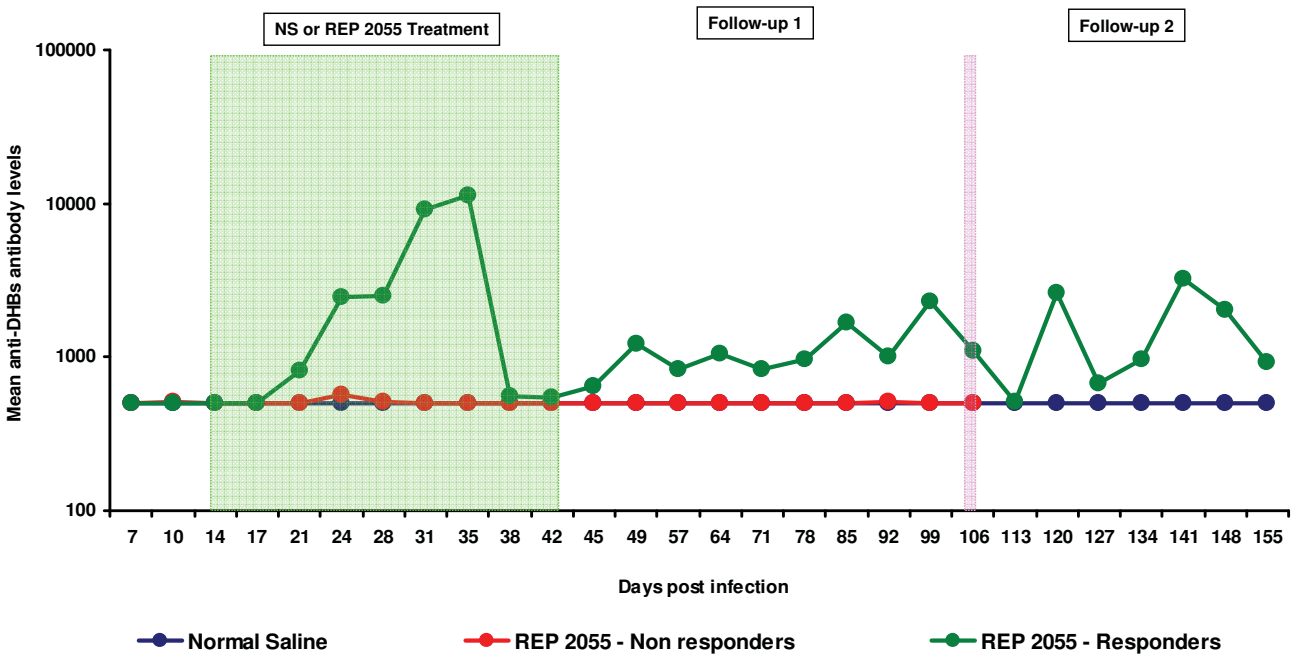


Figure 7.13c: Mean anti-DHBs antibody levels in ducks treated with NS (n=13), ducks that did not respond to REP 2055 treatment (n=5) and ducks that responded to REP 2055 treatment (n=6). 14-day-old ducks were infected with 5 x 10^8 DHBV DNA genomes and treated with NS or REP 2055 from 14 days p.i. for 28 days. Ducks were monitored for 16 weeks after stopping the treatment.

Chapter 8: Antiviral efficacy of NAs, TFV and FTC against persistent DHBV infection

8.1 Introduction

NAs were first identified as antiviral agents two decades ago and have been used in the treatment of several persistent viral infections, including HIV, members of the herpesviridae family and HBV (Karayiannis 2003; Zoulim 2004; Mailliard and Gollan 2006). The NAs competitively inhibit the reverse transcriptase activity of the HBV Pol to prevent: firstly, reverse transcription of the HBV pregenomic mRNA into the minus DNA; and secondly, formation of positive strand DNA from the negative strand DNA (Karayiannis 2003; Younger *et al.* 2004). The ability of NAs to block conversion of rcDNA to cccDNA and prevent supply of the cccDNA template for viral DNA synthesis determines their activity against HBV (Marcellin *et al.* 2005; Zoulim 2005).

The need for combination chemotherapy for chronic HBV infection using NAs has been suggested by many as a means to combat antiviral resistance, whicht often occurs during NA monotherapy (Sasadeusz *et al.* 2007; Balsano and Alisi 2008; Zoulim and Perrillo 2008). However, very little has been done to investigate the effectiveness of combination chemotherapy. In a preclinical study (Delaney *et al.* 2004), using HepG2 cells a wide range of NAs were tested to identify any additive or synergistic effects of combination chemotherapy. The additive effect of AFV with ETV, FTC, 3TC and TLB in dual combinations has been identified as being effective against HBV. The combination of AFV and 3TC provided a better additive effect than other combinations against HBV infection in HepG2 cells. Furthermore, no cytotoxic effects were seen in the cells treated with dual combinations (Delaney *et al.* 2004). It has been widely postulated that combination of NAs has the potential to enhance the therapeutic efficacy of antiviral treatment against chronic HBV infection. It has also been shown that combining PEN and 3TC can potentially reduce the levels of cccDNA (Lau *et al.* 2000), which often allows rebound viraemia and hepatitis following the withdrawal of NA monotherapy. Combination chemotherapy with NAs is likely to block the formation of cccDNA more efficiently than a single drug when the right combination is selected. The spread of HBV from one infected hepatocyte to other non-

infected hepatocytes is also expected to be lower. Furthermore, combination therapy is expected to reduce levels of cccDNA and replicative intermediates (RI DNA) in an established HBV infection (Zoulim 2005; Rapti *et al.* 2007; Zoulim and Perrillo 2008; Seetharam and Lisker-Melman 2009).

HBV and HIV follow a reverse transcription step in their replication strategy and combination chemotherapy has been widely used against HIV since the late 1990s. More new NAs have been introduced for anti-HIV combination regimens ever since. For example, the combination of TDF and FTC is licensed to treat HIV infection in humans and has been used in many countries for the last 3 years (Gazzard 2006; Munoz de Benito and Arribas Lopez 2006).

TDF is an oral prodrug of TFV, an acyclic nucleoside phosphonate analogue of adenosine monophosphate and it requires the initial diester hydrolysis to become its active drug TFV. The latter then undergoes phosphorylation by cellular enzymes to become TFV diphosphate. This inhibits the HBV replication by competing with the natural substrate deoxyadenosine 5'-triphosphate, allowing its incorporation into the newly formed viral DNA and causes DNA chain termination (De Clercq and Field 2008; Delaney and Borroto-Esoda 2008; Marcellin *et al.* 2008).

FTC is a synthetic NA of cytosine and is phosphorylated by cellular enzymes to form FTC 5'-triphosphate. The latter inhibits the HBV reverse transcriptase activity of Pol by competing with its natural substrate deoxycytosine 5'-triphosphate by being incorporated into the newly formed viral DNA resulting in chain termination (De Clercq and Field 2008).

As TDF and FTC are potent inhibitors of the HIV-1 reverse transcriptase and the HBV Pol, it can therefore be hypothesised that this combination has the potential to produce a more effective treatment outcome against chronic HBV infection than either drug alone (De Clercq and Field 2008). To investigate this hypothesis, treating persistently DHBV infected ducks with TFV or FTC as monotherapies and TFV and FTC as combination therapy was used as a model system for HBV infection. As a first step, PK properties of TFV and TDF were investigated in ducks because no data was available. PK data for FTC was already available for ducks as previously published by Seigneres *et al.* (2003) and these data were modified and used in a rational manner in the current study. The ability of TFV and FTC to

suppress DHBV replication when used as monotherapies and then in combination therapies were investigated *in vivo*.

8.2 Experimental design

Approval to conduct the *in vivo* studies was obtained from the Animal Ethics Committees of the IMVS and University of Adelaide. Chapter 2 outlines the following experimental requirements: source and dose of DHBV; preparation and reconstitution of TFV and FTC; collection of blood samples; autopsy and specimen collection procedures; analysis of blood and serum for virological (DHBsAg and DHBV DNA); and biochemical markers (liver enzymes) and liver tissues to assess the level of DHBV infection.

For Experiment I, 8-month-old ducks were divided into 4 Groups of 2 (Table 8.1). For Experiment II, 14-day-old ducks were divided into 6 Groups of 3 (Table 8.2) and for Experiment III, 14-day-old ducks were divided into 3 Groups of 5 (Figure 8.1; Table 8.3).

The ducks in Experiment I received single treatment of 5 or 15 mg/kg of TDF orally or TFV IP and then the blood samples were collected in EDTA impregnated tubes (VACUETTE®$_{CE,}$ GrenierBio-One Gmbh, Austria) from 30 min post-administration of the drug for a 24 h period. Plasma was separated and used for PK analysis, *i.e.* 30 min, 1, 2, 4, 6, 8, 12 and 24 h after administering 5 or 15 mg/kg of TDF orally or TFV IP. In all cases ducks were weighed and bled prior to drug administration (Figure 8.1). Plasma samples were separated within 30 min of blood collection by centrifuging the tubes at 4^{o}C for 10 min at a relative centrifugal force of 100g. Plasma samples were stored at -20^{o}C until they were transported to Gilead Sciences Bio-Analytical Group Laboratory, USA for PK analysis.

Plasma TFV concentrations were measured by liquid chromatography and mass spectrometry (LC/MS). The LC/MS method used in this study is specific and sensitive for TFV with a lower limit of quantitation of 10 ng/mL. PK parameters of TFV in plasma were estimated by application of a non-linear model using standard non-compartmental methods (WinNonlin® Professional software version 5.0.1 California, USA). The linear/log trapezoidal rule was applied in conjunction with an extra-vascular input model. Input values for dose, plasma concentration, corresponding time points based on drug dosing times and all pre-dose sample times set to zero were used for the analysis of PK for TDF and TFV.

For Experiments II and III, 14-day-old ducks were infected with 5 x 10^8 DHBV DNA genomes by inoculating the jugular vein. The ducks in Experiment II then received daily treatment of 5 (Group 8.A) or 25 (Group 8.B) or 50 (Group 8.C) mg/kg of TFV or 100 (Group 8.D) or 200 mg/kg of FTC (Group 8.E) or NS (Group 8.F) from 14 days p.i. for 28 days. The ducks in Experiment III received daily treatment of 5 mg/kg TFV + 100 mg/kg of FTC (Group 8.G) or 5 mg/kg TFV + 200 mg/kg of FTC (Group 8.H) or NS (Group 8.I) from 14 day p.i. for 28 days (Figure 8.1).

Ducks were assessed each day for any abnormalities in feed and water intake, weight loss and gait or changes in behaviour. Ducks were clinically examined through a gentle palpation of the abdomen and on IP injection for signs of pain on the abdominal area. IP injection was administered just below the sternum that permits an easy access to the peritoneal cavity.

CBE was performed using whole blood samples of ducks in Experiments II and III. Sera were also analysed for liver enzymes to elucidate the liver function of ducks after TFV and FTC treatment at various dose regimens in the mono and combination therapy studies. NS-treated ducks were used as comparators for CBE and liver enzyme analysis. Blood samples collected on days 0, 7, 14 p.i. and 7, 10, 14, 17, 21, 24 and 28 days post-treatment (Dp Rx) were tested for DHBsAg using a quantitative ELISA. Liver tissues collected at autopsy on day 42 p.i. (28Dp Rx) were tested for histology, percentage of DHBsAg-positive hepatocytes by immuno-staining and DHBV DNA by qPCR. The detailed methods used for these analyses are discussed in Sections 2.8.2 and 2.10 of Chapter 2.

***Statistical analysis*:** Differences in mean body weights, haematological parameters, liver enzyme levels and percentage of DHBsAg-positive hepatocytes and DHBV DNA levels in liver tissues of ducks in Groups 8.A, 8.B, 8.C, 8.D, 8.E and 8.F (Experiment II) and Groups 8.G, 8.H and 8.I (Experiment III) 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.

8.3 Results

8.3.1 Experiment I: PK study on TFV & TDF in healthy DHBV-negative ducks

Plasma drug distribution of TFV was investigated after oral administration of TDF (Table 8.1) or IP administration of TFV (Table 8.2) to healthy adult ducks using either 5 or 15 mg/kg dose rates for a 24 h period. PK parameters included the time taken to achieve the maximum drug concentration (T_{max}), maximum drug concentration (C_{max}), area under the curve (AUC_{inf}), drug elimination kinetics (λz). Furhermore, plasma half life ($T^{½}$) of TFV was investigated after administering oral TDF (Table 8.3) or IP TFV (Table 8.4) to healthy adult ducks either at 5 or 15 mg/kg dose rates.

After an oral administration of TDF, reasonable dose-related increase in AUC_{inf} was observed and such dose-related effect was not observed following IP administration of TFV (Tables 8.3 and 8.4). IP administration of TFV produced a significantly higher AUC_{inf} in both doses compared to those involving oral administration of TDF (Tables 8.3 and 8.4). An IP dose of 5 mg/kg TFV once daily would be expected to achieve C_{max} (12X), C_{min} (0.5X) and AUC_{inf} (4.5X) compared to values achieved in humans at therapeutic doses (Yadav *et al.* 2009). On the other hand, an oral dose of 5 mg/kg TDF twice daily would be expected to achieve C_{max} (2X), C_{min} (3X) and AUC_{inf} (0.9X) compared to values achieved in humans at therapeutic doses.

8.3.2 Experiment II: Monotherapy studies on TFV and FTC against persistent DHBV infection

8.3.2.1 Groups 8.A, 8.B and 8.C: The effect of TFV treatment on clinicopathological, haematological, biochemical and virological markers

Ducks treated with TFV alone as a monotherapy in Group 8.A (5 mg/kg TFV), 8.B (25 mg/kg TFV) and 8.C (50 mg/kg TFV) had neither abdominal tenderness nor abdominal pain on IP injection and no abnormal findings were noted during the clinical examination of the abdomen. Furthermore, *in situ* examination of internal organs at autopsy did not reveal any gross pathological changes (data not shown).

Ducks in Groups 8.A-8.C did not show any significant changes ($p>0.05$) in their mean body weight compared to the body weight of ducks treated with NS (Table 8.5). A normal body weight gain throughout the studies indicated that TFV did not affect the general health and behavior of the ducks in all three dose regimens.

Blood samples collected at treatment endpoint or day 28Dp Rx from all ducks in Groups 8.A–8.C were tested and no significant changes were observed in either total RBC ($p>0.05$), WBC ($p>0.05$) or platelet ($p>0.05$) counts of ducks treated with any of the monotherapy regimens of TFV when compared with NS-treated ducks (Table 8.7). There were no significant differences ($p>0.05$) in liver enzymes in the Groups treated with mono therapy regimens of TFV when compared with NS-treated ducks (Table 8.9). This result suggests that the liver function was not affected by treatment with TFV.

Treatment of ducks with TFV using 3 monotherapy regimens was able to reduce the serum DHBsAg to a significantly lower level than DHBsAg levels of NS-treated ducks 2 weeks after treatment ($p<0.05$) and the serum DHBsAg levels continued to decline during the remainder of treatment (Figure 8.2). Moreover, treatment of ducks with TFV using 3 different dose regimens (5, 25 and 50 mg/kg) was able to reduce the individual Group mean levels of serum DHBV DNA by 3 logs when compared with that of NS-treated ducks (Figure 8.4). This finding shows that TFV was equally active in suppressing the serum DHBV DNA levels in all 3 dose regimens.

Group means of DHBsAg-positive hepatocytes fell to 10-15% in ducks treated with TFV in all 3 dose regimens (Figure 8.5). DHBV DNA detected from the liver agreed with the percentage of DHBsAg-positive hepatocytes detected in all 3 Groups of ducks in Experiment II (Figures 8.5 and 8.6). Only <4 copies of DHBV DNA detected per hepatocyte in all TFV-treated ducks in the 25 mg/kg dose regimen (1.3 copies/cell) were superior to 5 and 50 mg/kg dose regimens (3.8 copies/cell) (Figure 8.6). Ducks treated with NS had >95% of DHBsAg-positive hepatocytes in the liver and >144 copies of DHBV DNA per hepatocyte clearly showed the ongoing high levels of DHBV replication and spread in the liver. This was in the absence of antiviral treatment when compared with TFV-treated ducks (Figures 8.5 and 8.6).

Overall, TFV treatment in all 3 doses showed a better SVR in the serum and in the liver when compared to NS-treated control ducks. It did not produce any observable changes in duck health, body weight, haematological and biochemical markers.

8.3.2.2 Groups 8.D and 8.E: The effect of FTC treatment on clinicopathological, haematological, biochemical and virological markers

Ducks in Group 8.D (100 mg/kg FTC) and 8.E (200 mg/kg FTC) treated with FTC alone as a monotherapy had neither abdominal tenderness nor abdominal pain on IP injection and no abnormal findings were detected on clinical examination of the abdomen. Furthermore, *in situ* examination of internal organs at autopsy did not reveal any gross pathological changes. Moreover, ducks in Groups 8.D and 8.E did not show any significant changes in their mean body weight ($p>0.05$) when compared with the body weight of ducks treated with NS (Table 8.5). A normal body weight gain indicated that FTC treatment with 2 dose regimens did not affect the general health and behavior of ducks.

No significant changes were observed in mean total RBC, WBC and platelet counts ($p>0.05$) of ducks treated with any of the monotherapy regimens of FTC compared with NS-treated ducks. There were no significant differences in the mean levels of liver enzymes GGT, ALT and AST ($p>0.05$) among the Groups treated with two mono therapy regimens of FTC when compared with NS-treated ducks. This result suggests that the liver function was not affected by treatment with FTC in both doses (Table 8.9).

FTC treatment was also able to reduce the serum DHBsAg to a significantly low level 2 weeks into treatment (Figure 8.2) for the dose regimen of 200 mg/kg does. The 100 mg/kg dose regimen, however, was inferior to the 200 mg/kg dose regimen of FTC in terms of reducing the serum DHBsAg levels (Figure 8.2). All NS-treated ducks had high levels of serum DHBsAg throughout the course of study, indicating the presence of persistent viraemia in the absence of antiviral treatment (Figure 8.2). FTC treatment produced a dose-related response in suppressing the Group mean levels of serum DHBV DNA. A dose regimen of 200 mg/kg of FTC suppressed the Group mean levels of serum DHBV DNA by 2 logs while the 100 mg/kg of FTC suppressed the Group mean levels of serum DHBV DNA by 1 log when compared to NS-treated ducks (Figure 8.4).

FTC treatment had reduced the mean percentage of DHBsAg-positive hepatocytes to 35.54% and 30.8% with 100 and 200 mg/kg dose regimens, respectively (Table 8.11; Figure 8.5). DHBV DNA detected in the liver agreed with the percentage of DHBsAg-positive hepatocytes detected in all ducks (Figures 8.5 and 8.6). DHBV DNA levels of 104 copies/cell were detected in FTC-treated ducks in 100 mg/kg dose regimen and that decreased to 56 copies/cell with a 200 mg/kg FTC regimen. This clearly demonstrated a dose-related response in the hepatic DHBV suppression. Ducks treated with NS had >95% of DHBsAg-positive hepatocytes in the liver and >145 copies/cell clearly showing high levels of DHBV replication and spread in the liver in the absence of antiviral treatment (Figures 8.5 and 8.6).

In summary, FTC treatment of ducks with 100 and 200 mg/kg/day for 28 days produced a dose-related response in the serum and hepatic virological markers when compared with serum and hepatic virological markers of NS-treated ducks.

8.3.3 Experiment III: Combination therapy studies on TFV and FTC against persistent DHBV infection

8.3.3.1 Groups 8.G and 8.H: The effect of TFV and FTC treatment in combination on clinicopathological, haematological, biochemical and virological markers

Ducks in Groups 8.G (5 mg/kg TFV + 100 mg/kg FTC) and 8.H (5 mg/kg TFV + 200 mg/kg FTC) treated with TFV and FTC in combination showed neither abdominal tenderness nor abdominal pain on IP injection. No abnormal findings were noted during the clinical examination of the abdomen. Furthermore, *in situ* examination of internal organs at autopsy did not reveal any gross pathological changes. Moreover, ducks in Groups 8.G and 8.H treated with TFV and FTC in combination did not show any significant changes in their mean body weight ($p>0.05$) in comparison to the body weight of ducks treated with NS (Table 8.6). A normal body weight gain indicates that TFV and FTC in combination did not affect the general health and behavior of ducks in regard to the dose regimens.

The individual Group means of RBC, WBC and platelet counts (or CBE) of ducks treated with the combination therapy regimens of TFV and FTC showed no significant difference when compared with Group mean of RBC, WBC and platelet counts of NS-

treated ducks ($p>0.05$) (Table 8.8). Ducks treated with combination therapy regimens of TFV and FTC had no observable changes in CBE, suggesting that TFV and FTC in combination had not influenced the synthesis of RBC, WBC and platelets in the bone marrow or their release to the peripheral circulation with 28 days of treatment. There were no significant differences ($p>0.05$) in liver enzymes, GGT, ALT and AST among the Groups treated with combination therapy regimens when compared with NS-treated ducks (Table 8.10). These results suggest that the liver function was not affected by treatment with TFV and FTC in combination.

Combination therapy with 5 mg/kg TFV and 100 mg/kg FTC (Group 8.G) brought serum DHBsAg levels to a low level from 21Dp Rx onwards in ducks with persistent DHBV infection. Compared to the levels in NS-treated ducks, serum DHBsAg declined much earlier in ducks receiving combination therapy. In addition, serum DHBsAg levels in these ducks declined further with continued treatment from 21Dp Rx onwards and reached low or undectable levels at the end of treatment (Figure 8.3). The same can be said for the group of ducks (Group 8.H) treated with a combination of 5 mg/kg TFV and 200 mg/kg FTC in which a pronounced decline in serum DHBsAg levels was observed much earlier than at 17Dp Rx (Figure 8.3). In contrast, ducks treated with NS had fluctuating high levels of serum DHBsAg throughout the course of treatment (Figure 8.3).

In the combination therapy protocol, treatment of ducks with either 5 mg/kg TFV and 100 mg/kg of FTC or 5 mg/kg TFV and 200 mg/kg of FTC reduced the individual Group mean levels of serum DHBV DNA by 4 logs when compared with NS-treated ducks (Figure 8.4). Serum DHBV DNA suppression by combination regimens proved to be more effective than the levels of DHBV DNA suppression achieved by TFV or FTC in the monotherapy regimens ($p<0.05$) (Figure 8.4).

Combination therapy with 5 mg/kg TFV and 100 mg/kg FTC reduced the Group mean of DHBsAg-positive hepatocytes to 10% (Table 8.12; Figure 8.5). In contrast, 5 mg/kg TFV and 200 mg/kg FTC in combination reduced the Group mean of DHBsAg-positive hepatocytes to 5% (Table 8.12; Figure 8.5) in ducks with persistent DHBV infection. DHBV DNA detected from the liver was in agreement with the percentage of DHBsAg-positive hepatocytes detected in all ducks in Experiment III (Figures 8.5 and 8.6). Only <2.5 copies of DHBV DNA were detected per hepatocyte in the first combination regimen and the copy

number per hepatocyte decreased further to 0.07 in the second combination regimen. Ducks treated with a combination of 5 mg/kg TFV and 200 mg/kg FTC achieved SVR faster than the ducks treated with a combination of 5 mg/kg TFV and 100 mg/kg FTC with regard to serum and hepatic virological markers. Ducks treated with NS had >95% DHBsAg-positive hepatocytes and DHBV DNA of 17 copies/cell in the liver. These markers of NS-treated ducks are significantly higher ($p<0.05$) than what was detected in the ducks treated with TFV and FTC combinations in both protocols.

Overall, these findings suggest that the combination therapy regimens of TFV and FTC were superior to the monotherapy regimens of TFV or FTC alone in suppressing the serum and hepatic virological markers. Moreover, combination therapy with TFV and FTC in ducks produced SVR (Figures 8.5 and 8.6), without producing any observable changes in duck health, body weight, haematological and biochemical markers when compared with treatment with NS.

8.4 Discussion

The scientific and the medical community has long understood the benefits of combining antimicrobial agents since the introduction of multiple antibiotics for tuberculosis treatment in order to combat the emergence of resistance (Reichman 1994; Bass 1995). Lessons from anti-tuberculosis treatment using combinations of antibiotics were utilised for treating HIV/AIDS with antiviral agents and this contributed significantly in the battle against emerging antiviral resistance by HIV (Wong *et al.* 2000). HIV has different drug targets and as a consequence antiviral agents with different acting mechanisms are combined to block varying aspects of HIV replication. One of the common drug targets of HIV is the reverse transcription step which is targeted by NAs. HBV replication also involves reverse transcription and therefore NAs are active against HBV. Experts stress the importance of combination therapy as a means of combating the emerging problem of multi-drug resistant HBV (Sasadeusz *et al.* 2007; Zoulim and Perrillo 2008; Seetharam and Lisker-Melman 2009).

However, HBV due to its small size genome and dependence on cellular enzyme for its life cycle does not have many currently known or potential drug targets that are being investigated (Ghany and Liang 2007; Muller and Krausslich 2009). As HBV Pol performs

the major enzymatic function(s) during HBV replication, it is a well studied target for the antiviral action of NAs (Ghany and Liang 2007; Muller and Krausslich 2009). Thus investigating the newer NAs such as TFV in combination with other NAs for their potential to produce a better antiviral therapeutic efficacy is warranted in order to minimize the emergence of antiviral resistance against commonly used NA monotherapies.

In clinical practice, the first line of antiviral therapy for chronic HBV infection is 3TC, a well known NA. Once HBV becomes resistant to 3TC and then adefovir dipivoxil (ADV), a nucleotide analogue is added on or ADV is administered as a monotherapy. It is hypothesised that *de novo* combination of a nucleotide and a nucleoside analogue will provide a better SVR than a sequential combination therapy that is introduced after the HBV had developed resistance. In this study, TFV was chosen as nucleotide analogue candidate because TFV is superior to ADV in terms of antiviral efficacy (Marcellin *et al.* 2008) against HBV. FTC was chosen as a nucleoside analogue for a *de novo* combination therapy against HBV using persistent DHBV infection model.

PK of TFV showed a dose-related increase in AUC_{inf} that was observed after oral administration of TDF. Such a dose-related increase in AUC_{inf} was not observed on IP TFV, indicating that the lowest dose of TFV can be used for IP administration. This effect appears to be due to low levels of TFV absorbed into the circulation from its prodrug TDF to establish a mean AUC_{inf} of 1125.1. When the dose of the TDF was increased the potential for the AUC_{inf} to increase to a mean level of 4399 still existed. With IP administration of TFV, 5 mg/kg of the drug was able to achieve a mean AUC_{inf} of 11801.9 but increasing the dose of TFV to 15 mg/kg did not change the mean AUC_{inf} to a higher level than that achieved for 5 mg/kg. Thus IP route of administration produced a significantly higher mean of AUC_{inf} in both doses compared with oral TDF (Tables 8.3 and 8.4). An IP dose of 5 mg/kg TFV once daily was expected to achieve a better means of C_{max} (12X), C_{min} (0.5X) and AUC_{inf} (4.5X) than an oral dosing of 5 mg/kg twice daily regimen of TDF with C_{max} (2X), C_{min} (3X) and AUC_{inf} (0.9X) compared to values achieved in humans at therapeutic doses (Yadav *et al.* 2009). Hence 5, 25 and 50 mg/kg dose regimens of TFV were chosen for the monotherapy experiment.

It has been shown previously that a dose of 100 mg/kg of FTC successfully decreased the serum DHBV DNA levels by 1-3 logs when it was given IP from 3 days p.i. for 7 days.

All the ducks, however, rebounded once the treatment was stopped. A dose of 50 mg/kg of FTC was unable to produce an effect (Seigneres *et al.* 2003). Hence, 100 and 200 mg/kg of FTC were used in the current experiments.

A widespread DHBV infection was observed in Experiments II and III when the 14-day-old ducks were inoculated with a dose of 5 x 10^8 DHBV DNA genomes that has been shown to produce a widespread persistent infection (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 high levels of viraemia as shown by DHBsAg and DHBV DNA in the serum 14 days p.i. and a widespread DHBV infection with more than 95% DHBsAg-positive hepatocytes was noted at autopsy (42 day p.i.) in the liver. This finding shows the effect of anti-DHBV activity of TFV and FTC in suppressing the serum and hepatic DHBsAg and DHBV DNA in ducks treated with TFV and FTC either drug alone or in combination (Figures 8.1, 8.2 and 8.3).

In the monotherapy experiment, TFV was able to reduce the mean serum DHBV DNA levels by 3 logs in all three dose regimens. This shows the superior antiviral effects of TFV over FTC that was only able to produce 1-2 log reduction in the mean serum DHBV DNA levels against persistent DHBV infection. Furthermore, the serum DHBsAg levels were in agreement with the serum DHBV DNA profile in that TFV could suppress DHBV antigenaemia more effectively than FTC. Hepatic virological markers were well correlated with serum based virological markers. The percentage of DHBsAg-positive hepatocytes closely represented DHBV copy numbers tested by qPCR. Conversely, TFV-treated ducks had relatively low levels of DHBV DNA copy numbers per cell when compared with the hepatic levels of DHBV DNA in FTC-treated ducks.

In the combination therapy protocol, TFV and FTC were able to suppress the serum DHBsAg and DHBV DNA more effectively than either of them did in the monotherapy regimen. In the hepatic virological profile, 25 mg/kg TFV regimen was able to bring liver DHBV DNA levels to <1.4 copies/cell. The best combination regimen was able to reduce the copy numbers to 0.07 copies/cell, thus demonstrating the superior effect of TFV and FTC under an optimal regimen. However, the first combination regimen was able to reduce the copy numbers to 2.34 copies/cell. Overall, the second combination therapy regimen (Group 8.H) showed the best effect on serum based and hepatic virological markers. A 4 log

reduction in mean serum DHBV DNA was achieved by reducing DHBsAg-positive hepatocytes and DHBV DNA to 5% and 0.07 copies/cell. This highlights the potential of the combination therapy. In a recent study by Menne *at al.* (2008), the application of combination therapy using oral TDF and FTC once daily against persistent WHV infection was able to bring serum DHBV DNA levels down by 6 logs after 48 weeks of treatment compared with placebo-treated woodchucks. Moreover, other WHV markers in serum and liver were also suppressed more effectively in woodchucks treated with combination therapy using TDF and FTC than those treated with mono therapy with TDF or FTC alone (Menne *et al.* 2008), supporting the effectiveness of the combination of TFV and FTC against persistent hepadnavirus infection.

No observable indication of toxicity emerged in terms of changes in clinical or biochemical or haematological parameters. Furthermore, no pathological changes in external orifices or internal organs at autopsy in all 3 dose regimens of TFV or 2 dose regimens of FTC or both of these drugs in combination, supported the safety profile of these two drugs either in mono or combination regimens. Conversely, no toxicity has been observed in woodchucks treated with TDF and FTC combination therapy in terms of changes in clinical or biochemical or haematological parameters (Menne *et al.* 2008).

The level of DHBV DNA in the liver of NS-treated ducks varied markedly between the Groups 8.F (NS-treated control for the monotherapy experiment) and 8.I (NS-treated control for the combination therapy experiment). In this respect, Group 8.F ducks had a group mean of 144 copies/cell, whereas Group 8.I had a group mean of 16.94 copies/cell, which is an 8.5 fold decrease. The reason for this discrepancy between the controls of 2 different groups of NS-treated ducks is not clear. It appears, however, the 25 mg of liver tissue that was used to extract the cellular and viral DNA of Group 8.I ducks did not have high level of DHBV DNA like the liver tissues of Group 8.F ducks. Nonetheless, for Experiment III Group 8.I ducks provided a good internal control as the group mean of total DHBV DNA in Group 8.I ducks was 16.94 copies/cell. This was 7.2 and 242 fold higher than the Groups 8.G and 8.H ducks (treated with TFV and FTC combination therapy regimens), respectively.

In conclusion, the results of combination therapy are promising in terms of producing a better SVR than mono therapy regimens. The antiviral efficacy of TFV appeared to be superior to FTC. The most effective TFV monotherapy regimen was able to bring the serum

DHBV DNA from 10^{10}copies/mL to 10^{6}copies/mL at treatment endpoint. At treatment endpoint, DHBV DNA in the serum decreased to 10^{2}-10^{3}copies/mL from 10^{9}copies/mL of pre-treatment levels in both combination regimens. However, DHBV DNA levels of 10^{2}-10^{3}copies/mL suggested the potential for rebound if the treatment was ceased. What would have happened if the treatment had continued for another 28 days and the ducks were followed up for 28-56 days after ceasing the treatment? Answers to these questions are very important if we are to understand the dynamics of viral suppression and clearance following combination therapy. Analysis of liver biopsy material collected prior to and during treatment, at treatment endpoint and end of follow-up will provide a clear picture about the levels of DHBsAg, DHBV DNA including cccDNA in the liver. Thus the effect of combination therapy on hepatic viral dynamics will be better understood.

Table 8.1: Experiment I-Distribution of TFV in plasma following oral treatment with TDF

Dose of TDF mg/kg[a]	Duck No[b]	Plasma TFV concentration (ng/mL) at a given time (h)[c]								
		0	**0.5**	**1**	**2**	**4**	**6**	**8**	**12**	**24**
5	176	0	502.7	178.5	72.3	44.9	26.8	20.7	10.3	0
	177	0	784.3	450.5	80.6	77.4	51.3	34.7	13.8	0
	Mean[d]	0	643.5	314.5	126.4	61.2	39.0	27.7	12.1	0
	SD[e]	0	199.1	192.3	76.6	23.0	17.3	9.9	2.5	-
	CV%[f]	-	31	61	61	38	44	36	21	-
15	178	0	2774.2	1629.5	441.1	210.1	128.2	90.5	40.8	0
	179	0	265.3	1384.1	874.5	403.8	165.4	05.5	52.9	11.2
	Mean[g]	0	1519.7	1506.8	658.1	306.9	146.8	98.0	46.8	5.6
	SD[h]	0	1774.1	173.5	306.0	137.0	26.3	10.6	8.6	7.9
	CV%[i]	-	117	12	47	45	18	11	18	141

Table 8.2: Experiment I-Distribution of TFV in plasma following IP treatment with TVF

Dose of TFV mg/kg[a]	Duck No[b]	Plasma TFV concentration (ng/mL) at a given time (h)[c]								
		0	**0.5**	**1**	**2**	**4**	**6**	**8**	**12**	**24**
5	265	0	3693.8	4740.3	3274.7	305.7	748.7	281.7	74.5	34.3
	267	0	2838.5	2324.4	1263.8	470.0	50.5	93.1	01.7	14.4
	Mean[d]	0	3266.1	3532.4	2269.2	887.8	499.6	187.4	138.1	24.3
	SD[e]	0	604.8	1708.2	1421.9	590.9	352.3	133.4	51.5	14.1
	CV%[f]	-	19	48	63	67	71	71	37	58
15	263	0	2022.6	3111.8	3374.6	1678.5	978.8	466.5	277.8	22.7
	264	0	7054.2	3858.9	1254.6	404.3	211.8	121.2	64.5	12.2
	Mean[g]	0	4538.4	3485.4	2314.6	1041.4	595.3	293.9	171.1	17.4
	SD[h]	0	3557.9	528.3	1499.1	901.0	542.4	244.2	150.8	7.4
	CV%[f]	-	78	15	65	87	91	83	88	43

[a] TDF (Table 8.1) and TFV (Table 8.2) were administered in doses 5 and 15 mg/kg;

[b] 8-months-old DHBV negative healthy ducks 176, 177, 178 and 179 were treated orally with a single dose of 5 or 15 mg/kg of TDF in Groups of two as shown in Table 8.1;

[b] 8-months-old DHBV negative healthy ducks 265, 267, 263 and 264 were treated intraperitoneally (IP) with a single dose of 5 or 15 mg/kg of TFV in Groups of two as shown in Table 8.2;

[c] Blood samples were collected at pre-treatment or at 0 h and 0.5, 1, 2, 4, 6, 8, 12 and 24 h after TDF (Table 8.1) or TFV treatment (Table 8.2);

Mean TFV concentration with oral treatment of ducks with [d]5 and [g]15 mg/kg of TDF (Table 8.1) or IP treatment of TFV (Table 8.2);

Standard deviation (SD) of TFV concentration in with oral treatment of ducks with [e]5 and [h]15mg/kg TDF or IP treatment of TFV;

Cumulative variance (CV%) of TFV concentration with oral treatment of ducks with [f]5 and [i]15 mg/kg TDF or IP treatment of TFV.

Table 8.3: Experiment I-PK of TFV in ducks following oral treatment with TDF

Dose mg/kg	Duck No	T_{max} (h)[a]	C_{max} (ng/mL)[b]	AUC h (ng/mL)[c]	λz (h-1)[d]	$T_{1/2}$[e]
5	176	0.7	502.7	792.3	0.168	4.13
	177	0.85	784.3	1457.8	0.216	3.20
	Mean[f]	0.77	643.5	1125.1	0.192	3.67
	SD[g]	0.11	199.1	470.6	0.034	0.66
	CV%[h]	14	31	42	18	18
15	178	0.62	2774.2	4450.0	0.196	3.54
	179	1.10	1384.1	4348.0	0.138	5.03
	Mean[f]	0.86	2079.2	4399.0	0.167	4.29
	SD[g]	0.34	983.0	72.2	0.041	1.05
	CV%[h]	39	47	2	25	25

Table 8.4: Experiment I-PK of TFV in ducks following IP treatment with TFV

Dose mg/kg	Duck No	T_{max} (h)[a]	C_{max} (ng/mL)[b]	AUC_h (ng/mL)[c]	λz (h-1)[d]	$T^{1/2e}$
5	265	1.00	4740.3	16244.2	0.133	5.23
	267	0.60	2838.5	7359.6	0.144	4.81
	Mean[f]	0.8	3789.4	11801.9	0.138	5.02
	SD[g]	0.28	1344.8	6282.4	0.008	0.29
	CV%[h]	35	35	53	6	6
15	263	2.07	3374.6	16577.2	0.194	3.58
	264	0.62	7054.2	9798.1	0.139	4.98
	Mean[f]	1.34	5214.4	13187.6	0.166	4.28
	SD[g]	1.03	2601.9	4793.5	0.039	0.99
	CV%[h]	76	50	36	23	23

[a] Time taken to achieve maximum drug concentration {T_{max}(h)};

[b] Maximum drug concentration {C_{max} (ng/mL)};

[c] Area under the curve {AUC_h (ng/mL)};

[d] Drug elimination kinetics {λz (h-1)};

[e] Plasma half life of TFV after administering TDF orally or TFV IP {$T^{½}$}.

Table 8.5: Experiment II-Duck body weights during TFV or FTC monotherapy

Treatment Group	Duck No	Body weight (g)									
		Age (days)									
		14	**17**	**20**	**23**	**27**	**30**	**33**	**37**	**46**	**55**
8.A 5 mg/kg TFV IP[a] daily	1	350	470	620	945	1400	1750	2050	2290	2650	3010
	2	345	555	720	1050	1420	1730	2100	2370	2700	3090
	3	405	605	805	1165	1525	1830	2160	2400	2690	2900
Mean body weight		**367[b]**	**543[c]**	**715[d]**	**1053[e]**	**1448[f]**	**1770[g]**	**2103[h]**	**2353[i]**	**2680[j]**	**3000[k]**
8.B 25 mg/kg TFV IP[a] daily	4	335	395	515	950	1250	1550	1900	2200	2625	3000
	5	370	425	635	995	1195	1640	1975	2240	2675	2900
	6	365	515	705	1100	1405	1710	2045	2390	2720	3100
Mean body weight		**357[b]**	**445[c]**	**618[d]**	**1015[e]**	**283[f]**	**1633[g]**	**1973[h]**	**2487[i]**	**2800[j]**	**3187[k]**
8.C 50 mg/kg TFV IP[a] daily	7	355	515	810	1190	1525	1810	2110	2420	2745	3190
	8	395	605	840	1195	1445	1860	2170	2490	2790	3200
	9	410	605	945	1205	1685	1945	2200	2550	2865	3170
Mean body weight		**387[b]**	**575[c]**	**865[d]**	**1197[e]**	**1552[f]**	**1872[g]**	**2160[h]**	**2487[i]**	**2800[j]**	**3187[k]**
8.D 100 mg/kg FTC IP[a] daily	10	355	465	665	995	1295	1615	1885	2090	2460	2900
	11	365	465	745	1050	1275	1490	1975	2260	2690	3000
	12	415	545	775	1100	1465	1730	2110	2410	2700	3100
Mean body weight		**378[b]**	**492[c]**	**728[d]**	**1048[e]**	**1345[f]**	**1612[g]**	**1990[h]**	**2362[i]**	**2706[j]**	**3062[k]**
8.E 200 mg/kg FTC IP[a] daily	13	325	435	705	985	1375	1600	1830	2100	2630	3010
	14	375	465	725	990	1305	1670	1925	2210	2700	3090
	15	365	475	745	995	1325	1680	1980	2290	2730	3190
Mean body weight		**355[b]**	**458[c]**	**725[d]**	**990[e]**	**1335[f]**	**1650[g]**	**1912[h]**	**2200[i]**	**2687[j]**	**3097[k]**
8.F NS IP[a] daily	16	405	535	850	1150	1475	1870	2190	2490	2795	3200
	17	345	505	815	1130	1465	1790	2045	2310	2680	2990
	18	395	525	835	1110	1495	1750	2020	2415	2770	3015
Mean body weight		**382[b]**	**522[c]**	**833[d]**	**1130[e]**	**1478[f]**	**1803[g]**	**2085[h]**	**2405[i]**	**2748[j]**	**3068[k]**

[a] IP - intraperitoneal injection;

Mean body weight on [b] = 14, [c] = 17, [d] = 20, [e] = 23, [f] = 27, [g] = 30, [h] = 33, [i] = 37, [j] = 46 and [k] = 55 days of age;

Differences in mean body weight of Groups 8.A, 8.B, 8.C, 8.D and 8.E on 14, 17, 20, 23, 27, 30, 33, 37, 46 and 55 days of age were not statistically significant to the mean body weight of Group 8.F ($p>0.05$).

Table 8.6: Experiment III-Duck body weight during TFV & FTC combination therapy

Treatment Group	Duck No	Body weight (g)									
		Age (days)									
		14	**17**	**20**	**23**	**27**	**30**	**33**	**37**	**46**	**55**
8.G 5 mg/kg TFV + 100 mg/kg FTC IP[a] daily	76	335	400	480	780	1050	1265	1750	2000	2495	2890
	77	360	450	660	985	1195	1395	1800	2100	2505	3030
	78	365	535	720	1180	1305	1610	2060	2360	2790	3100
	79	405	600	815	1265	1555	1820	2100	2410	2800	3290
	80	395	470	720	1025	1205	1480	1905	2220	2670	2995
Mean body weight		**372[b]**	**491[c]**	**679[d]**	**1047[e]**	**1262[f]**	**1514[g]**	**1923[h]**	**2330[i]**	**2753[j]**	**3128[k]**
8.H 5 mg/kg TFV + 200 mg/kg FTC IP[a] daily	82	415	625	930	1260	1660	1850	2145	2375	2700	3090
	83	375	425	760	1005	1260	1510	1910	2270	2690	3015
	84	345	415	525	960	1255	1490	1970	2295	2725	3200
	85	350	495	655	1025	1205	1590	2000	2310	2760	3115
	86	375	515	735	1035	1275	1525	1990	2385	2790	3210
Mean body weight		**372[b]**	**495[c]**	**721[d]**	**1057[e]**	**1331[f]**	**1593[g]**	**2003[h]**	**2327[i]**	**2733[j]**	**3126[k]**
8.I NS IP[a] daily	81	385	545	810	1120	1295	1565	1905	2310	2705	2995
	87	375	575	850	1260	1375	1620	2200	2475	2895	3105
	88	395	625	870	1295	1465	1715	1990	2310	2715	3215
	89	415	700	1045	1305	1525	1835	2225	2480	2800	3110
	90	355	505	665	1010	1290	1575	1995	2305	2690	3000
Mean body weight		**385[b]**	**590[c]**	**848[d]**	**1198[e]**	**1390[f]**	**1662[g]**	**2063[h]**	**2344[i]**	**2748[j]**	**3113[k]**

[a] IP - intraperitoneal injection;

Mean body weights on [b] = 14, [c] = 17, [d] = 20, [e] = 23, [f] = 27, [g] = 30, [h] = 33, [i] = 37, [j] = 46 and [k] = 55 days of age;

Differences in mean body weight of Groups 8.G and 8.H on 14, 17, 20, 23, 27, 30,,33, 37, 46 and 55 days of age were not statistically significant to the mean body weight of Group 8.I ($p>0.05$).

Table 8.7: Experiment II-CBE including total RBC, WBC and platelet counts of ducks treated with TFV or FTC monotherapy at autopsy

Experimental Group	Duck No	CBE[a] including total RBC, WBC and platelet counts		
		Total RBC/L[b]	Total WBC/L[c]	Total platelets/L[d]
8.A TFV 5 mg/kg	1	2.45×10^{12}	8.4×10^{9}	3×10^{9}
	2	2.55×10^{12}	7.5×10^{9}	4×10^{9}
	3	2.48×10^{12}	9.4×10^{9}	3×10^{9}
Mean RBC or WBC or platelet/L		**2.49×10^{12}[e]**	**8.43×10^{9}[f]**	**3.33×10^{9}[g]**
8.B TFV 25 mg/kg	4	2.81×10^{12}	9.2×10^{9}	3×10^{9}
	5	2.38×10^{12}	9.4×10^{9}	3×10^{9}
	6	2.52×10^{12}	6.4×10^{9}	3×10^{9}
Mean RBC or WBC or platelet /L		**2.57×10^{12}[e]**	**8.33×10^{9}[f]**	**3.00×10^{9}[g]**
8.C TFV 50 mg/kg	7	2.38×10^{12}	8.4×10^{9}	4×10^{9}
	8	2.31×10^{12}	9.4×10^{9}	3×10^{9}
	9	2.58×10^{12}	7.4×10^{9}	4×10^{9}
Mean RBC or WBC or platelet/L		**2.42×10^{12}[e]**	**8.40×10^{9}[f]**	**3.67×10^{9}[g]**
8.D FTC 100 mg/kg	10	2.40×10^{12}	8.2×10^{9}	3×10^{9}
	11	2.61×10^{12}	9.2×10^{9}	5×10^{9}
	12	2.36×10^{12}	7.4×10^{9}	3×10^{9}
Mean RBC or WBC or platelet/L		**2.46×10^{12}[e]**	**8.70×10^{9}[f]**	**3.67×10^{9}[g]**
8.E FTC 200 mg/kg	13	2.62×10^{12}	11.4×10^{9}	3×10^{9}
	14	2.33×10^{12}	10.4×10^{9}	6×10^{9}
	15	2.75×10^{12}	4.8×10^{9}	3×10^{9}
Mean RBC or WBC or platelet/L		**2.57×10^{12}[e]**	**8.87×10^{9}[f]**	**4.00×10^{9}[g]**
8.F NS	16	2.44×10^{12}	9.4×10^{9}	5×10^{9}
	17	2.64×10^{12}	6.4×10^{9}	2×10^{9}
	18	2.76×10^{12}	10.4×10^{9}	3×10^{9}
Mean RBC or WBC or platelet/L		**2.61×10^{12}[e]**	**8.73×10^{9}[f]**	**3.33×10^{9}[g]**

[a] Complete blood evaluation;

[b] RBC/L: RBCs per litre;

[c] WBC/L: WBCs per litre;

[d] Platelet/L: Platelets per litre;

[e] Mean RBC count of TFV- or FTC-treated ducks;

[f] Mean WBC count of TFV- or FTC-treated ducks;

[g] Mean platelet count of TFV- or FTC-treated ducks;

Differences in mean RBC, WBC and platelet counts of Groups 8.A, 8.B, 8.C, 8.D and 8.E at autopsy were analysed. The differences were not statistically significant to the mean RBC, WBC and platelet counts of Group 8.F ($p>0.05$).

Table 8.8: Experiment III-CBE including total RBC, WBC and platelet counts of ducks treated with TFV and FTC combination therapy at autopsy

Experimental Group	Duck No	CBE[a] including total RBC, WBC and platelet counts		
		Total RBC/L[b]	Total WBC/L[c]	Total platelet/L[d]
8.G TFV 5 mg/kg + FTC 100 mg/kg	76	2.10×10^{12}	7.2×10^{9}	4×10^{9}
	77	2.18×10^{12}	8.4×10^{9}	3×10^{9}
	78	2.07×10^{12}	6.4×10^{9}	3×10^{9}
	79	2.18×10^{12}	9.2×10^{9}	3×10^{9}
	80	1.99×10^{12}	9.4×10^{9}	5×10^{9}
Mean RBC or WBC or platelet/L		**2.10×10^{12}[e]**	**8.12×10^{9}[f]**	**3.60×10^{9}[g]**
8.H TV 5 mg/kg + FTC 200 mg/kg	81	2.35×10^{12}	6.4×10^{9}	4×10^{9}
	82	2.10×10^{12}	8.2×10^{9}	4×10^{9}
	83	2.16×10^{12}	9.2×10^{9}	3×10^{9}
	84	2.20×10^{12}	10.4×10^{9}	3×10^{9}
	85	1.95×10^{12}	11.4×10^{9}	2×10^{9}
Mean RBC or WBC or platelet/L		**2.16×10^{12}[e]**	**9.12×10^{9}[f]**	**3.20×10^{9}[g]**
8.I NS	86	2.41×10^{12}	10.4×10^{9}	2×10^{9}
	87	2.18×10^{12}	6.8×10^{9}	4×10^{9}
	88	2.75×10^{12}	9.4×10^{9}	3×10^{9}
	89	2.79×10^{12}	8.4×10^{9}	3×10^{9}
	90	2.34×10^{12}	7.4×10^{9}	3×10^{9}
Mean RBC or WBC or platelet/L		**2.49×10^{12}[e]**	**8.48×10^{9}[f]**	**3.00×10^{9}[g]**

[a] Complete blood evaluation;

[b] RBC/L: RBCs per litre;

[c] WBC/L: WBCs per litre;

[d] Platelet/L: Platelets per litre;

[e] Mean RBC count of (TFV+FTC)- and NS-treated ducks;

[f] Mean WBC count of (TFV+ FTC)- and NS-treated ducks;

[g] Mean platelet count of (TFV+FTC)- and NS-treated ducks;

Differences in mean RBC, WBC and platelet counts of ducks in Groups 8.G and 8.H at autopsy were not statistically significant to the mean RBC, WBC and platelet counts of Group 8.I ($p>0.05$).

Table 8.9: Experiment II-Liver enzyme analysis of ducks treated with TFV or FTC monotherapy at autopsy

Experimental Group	Duck No	Serum liver enzyme levels at autopsy					
		GGT[a]	Mean GGT[a]	ALT[b]	Mean ALT[b]	AST[c]	Mean AST[c]
8.A TFV 5 mg/kg	1	3	**3[d]**	32	**27.67[e]**	141	**60.67[f]**
	2	3		28		17	
	3	3		23		24	
8.B TFV 25 mg/kg	4	2	**2.33[d]**	49	**43.67[e]**	30	**30.33[f]**
	5	3		29		29	
	6	2		53		32	
8.C TFV 50 mg/kg	7	3	**2.67[d]**	35	**36[e]**	18	**31.33[f]**
	8	3		33		16	
	9	2		40		60	
8.D FTC 100 mg/kg	10	4	**3.67[d]**	27	**28.67[e]**	17	**18.67[f]**
	11	5		32		17	
	12	2		27		22	
8.E FTC 200 mg/kg	13	4	**2.67[d]**	30	**31.67[e]**	17	**25.33[f]**
	14	3		30		34	
	15	1		35		25	
8.F NS	16	1	**0.67[d]**	32	**39.33[e]**	56	**47.67[f]**
	17	1		38		39	
	18	0		48		48	

[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± SD) 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 of ducks in Groups 8.A, 8.B, 8.C, 8.D and 8.E were analysed and differences were not statistically significant to the mean liver enzyme levels of Group 8.F ($p>0.05$).

Table 8.10: Experiment III-Liver enzyme analysis of ducks treated with TFV or FTC combination therapy at autopsy

Experimental Group	Duck No	Serum liver enzyme levels at autopsy					
		GGT[a]	Mean GGT[a]	ALT[b]	Mean ALT[b]	AST[c]	Mean AST[c]
8.G TFV 5 mg/kg + FTC 100 mg/kg	76	1	**1.6[d]**	146	**68.8[e]**	78	**42.4[f]**
	77	2		33		37	
	78	2		35		25	
	79	2		38		28	
	80	1		92		44	
8.H TFV 5 mg/kg + FTC 200 mg/kg	81	1	**1[d]**	29	**39[e]**	15	**28.8[f]**
	82	0		47		43	
	83	1		35		27	
	84	1		49		25	
	85	2		35		34	
8.I NS	86	1	**1.4[d]**	33	**47.2[e]**	63	**41.4[f]**
	87	1		59		40	
	88	1		59		63	
	89	1		50		21	
	90	3		35		20	

[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± SD) 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 of Groups 8.G and 8.H were analysed and differences were not statistically significant to the mean liver enzyme levels of Group 8.I ($p>0.05$).

Table 8.11: Experiment II-The antiviral effect of TFV or FTC monotherapy on persistent DHBV infection (autopsy livers were tested for DHBsAg-positive hepatocytes)

Treatment Groups	% DHBsAg-positive hepatocytes 28 days after treatment		
	Duck No	Path No	% DHBsAg-positive hepatocytes
8.A 5 mg/kg TFV	1	5213	7.21
	2	5215	11.29
	3	5217	11.5
Mean % DHBsAg-positive hepatocytes			***10*** [a]
8.B 25 mg/kg TFV	4	5219	8.5
	5	5221	7.5
	6	5223	15
Mean % DHBsAg-positive hepatocytes			***10*** [a]
8.C 50 mg/kg TFV	7	5225	15.63
	8	5227	11.7
	9	5229	17.67
Mean % DHBsAg-positive hepatocytes			***15*** [a]
8.D 100 mg/kg FTC	10	5231	34.47
	11	5233	32.5
	12	5235	39.67
Mean % DHBsAg-positive hepatocytes)			***35.54*** [a]
8.E 200 mg/kg FTC	13	5237	25.76
	14	5239	34.92
	15	5241	31.73
Mean % DHBsAg-positive hepatocytes			***30.8*** [a]
8.F NS	16	5243	>95
	17	5245	>95
	18	5247	>95
Mean % DHBsAg-positive hepatocytes			***>95*** [a]

[a] Mean %DHBsAg-positive hepatocytes 28 days after treatment with TFV (8.A-8.C) or FTC (8.D-8.E) or NS (8.F);

Mean %DHBsAg-positive hepatocyte counts of Groups 8.A, 8.B, 8.C, 8.D and 8.E were analysed. Differences were statistically significant to the mean %DHBsAg-positive hepatocyte counts of Group 8.F ($p<0.05$).

Figure 8.1: Experimental outline of Experiment I (Panel A), Experiment II (Panel B) and Experiment III (Panel C).

Panel A: Experiment I - PK of TDF through oral administration of 5 and 15 mg/kg of the drug.

PK of TFV through IP administration of 5 and 15 mg/kg of the drug.

Panel B: Experiment II - To investigate the antiviral efficacy of TFV using 3 dose regimens (5, 25 and 50 mg/kg) or FTC using 2 dose regimens (100 and 200 mg/kg).

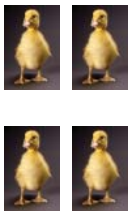
14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with TFV, FTC and NS from 14 day p.i. for 28 days.

Panel C: Experiment III - To investigate the antiviral efficacy of TFV and FTC using 2 combination dose regimens and NS.

14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with 5 mg/kg TFV + 100 mg/kg FTC or 5 mg/kg TFV + 200 mg/kg FTC or NS from 14 days p.i. for 28 days.

Black arrows indicate bleeding time points for testing for serum virological markers and the purple arrow indicates the autopsy time point at which blood was tested for CBE (Section 2.6), liver enzymes (Section 2.7) and virological markers. Liver samples were tested for DHBsAg-positive hepatocytes (Section 2.8.2) and DHBV DNA (Section 2.9.2).

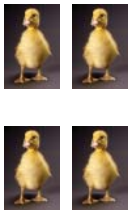
A **Experiment I: PK analysis of TFV/TDF (4 Groups of 2 ducks) = 8 Ducks**



5 mg/kg TDF

15 mg/kg TDF

Blood samples were collected 30 min, 1, 2, 4, 6, 8, 12 and 24 h after oral administration of TDF (5 and 15 mg/kg) for PK analysis

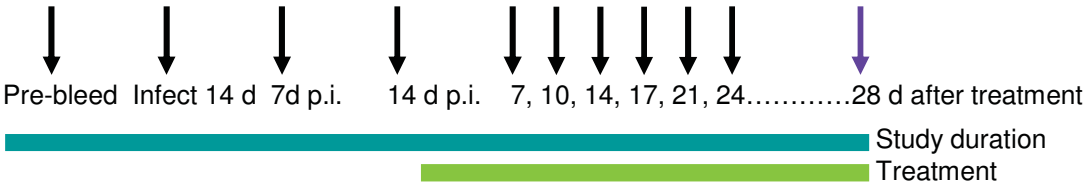


5 mg/kg TFV

15 mg/kg TFV

Blood samples were collected 30 min, 1, 2, 4, 6, 8, 12 and 24 h after IP administration of TFV (5 and 15 mg/kg) for PK analysis

B **Experiment II: Antiviral efficacy of TFV or FTC monotherapy against persistent DHBV infection (Groups 8.A- 8.F of 3 ducks) = 18 Ducks**



C **Experiment III: Antiviral efficacy of TFV and FTC combination therapy against persistent DHBV infection (Groups 8.G-8.I of 5 ducks) = 15 Ducks**

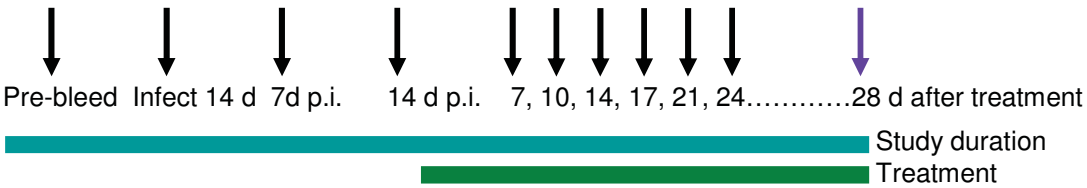


Figure 8.2: DHBsAg levels in the sera of TFV (Panels A, B and C), FTC (Panels D and E) and NS (Panel F) treated ducks. 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with TFV, FTC and NS from 14 day p.i. for 28 days.

Panel A: Group 8.A - Treated IP with 5 mg/kg TFV;

Panel B: Group 8.B - Treated IP with 25 mg/kg TFV;

Panel C: Group 8.C - Treated IP with 50 mg/kg TFV;

Panel D: Group 8.D - Treated IP with 100 mg/kg FTC;

Panel E: Group 8.E - Treated IP with 200 mg/kg FTC;

Panel F: Group 8.F - Treated IP with NS.

Serum samples were tested for DHBsAg levels using a quantitative enzyme linked immunosorbent assay (ELISA) described in Section 2.8.2.

DHBsAg levels are given in µg/ml of serum in the Y axis and days after DHBV infection or treatment are given in the X axis.

D p.i.: days post DHBV infection; **Dp Rx:** days post-treatment.

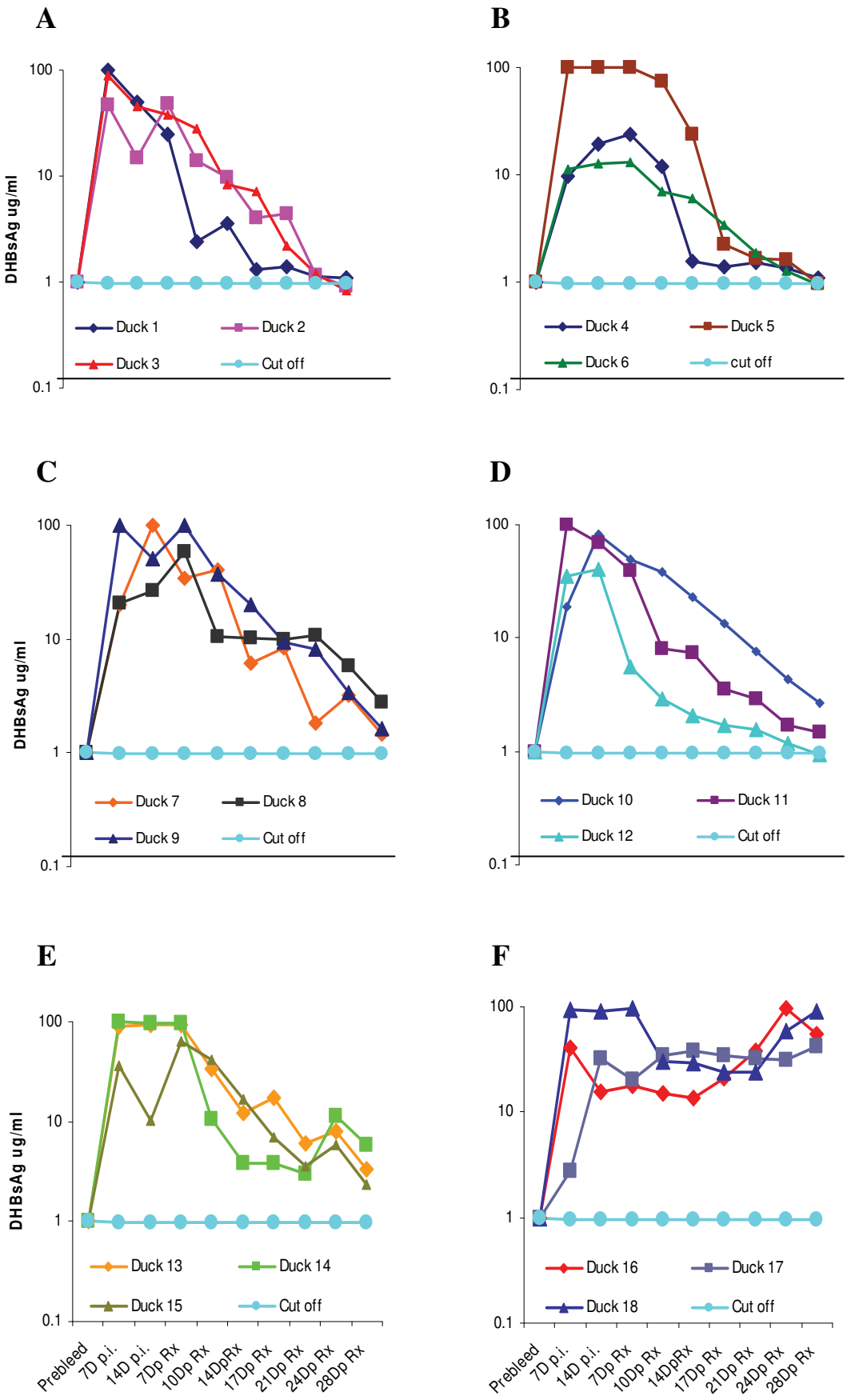

A
B
C
D
E
F
DHBsAg ug/ml
100
10
1
0.1
Duck 1
Duck 2
Duck 3
Cut off
Duck 4
Duck 5
Duck 6
cut off
Duck 7
Duck 8
Duck 9
Duck 10
Duck 11
Duck 12
Duck 13
Duck 14
Duck 15
Duck 16
Duck 17
Duck 18
Prebleed
7D p.i.
14D p.i.
7Dp Rx
10Dp Rx
14DpRx
17Dp Rx
21Dp Rx
24Dp Rx
28Dp Rx

Figure 8.3: DHBsAg levels in the sera of ducks treated with a combination of TFV and FTC (Panels G and H) or NS (Panel I). 14-day-old ducks were inoculated with 5 x 10^8 DHBV DNA genomes and treated with TFV and FTC in combination or NS from 14 day p.i. for 28 days.

Panel G: Group 8.G ducks treated IP with 5 mg/kg TFV + 100 mg/kg FTC;

Panel H: Group 8.H ducks treated IP with 5 mg/kg TFV + 200 mg/kg FTC;

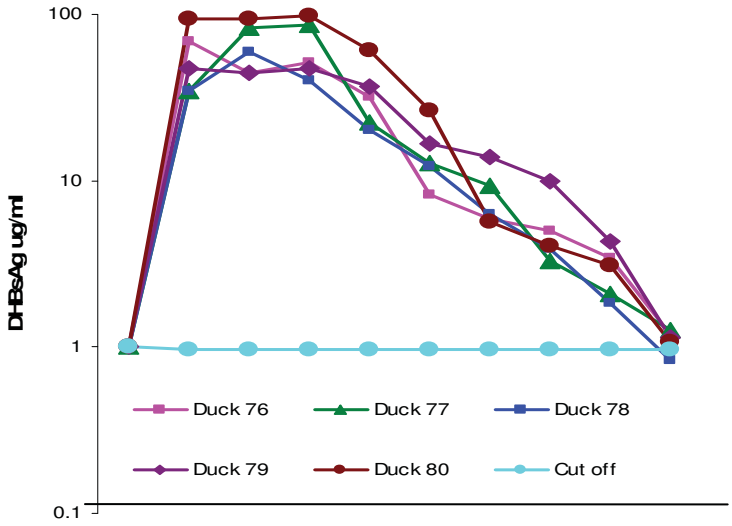
Panel I: Group 8.I ducks treated with IP NS.

Serum samples were tested for DHBsAg levels using a quantitative enzyme linked immunosorbent assay (ELISA) described in Section 2.8.2.

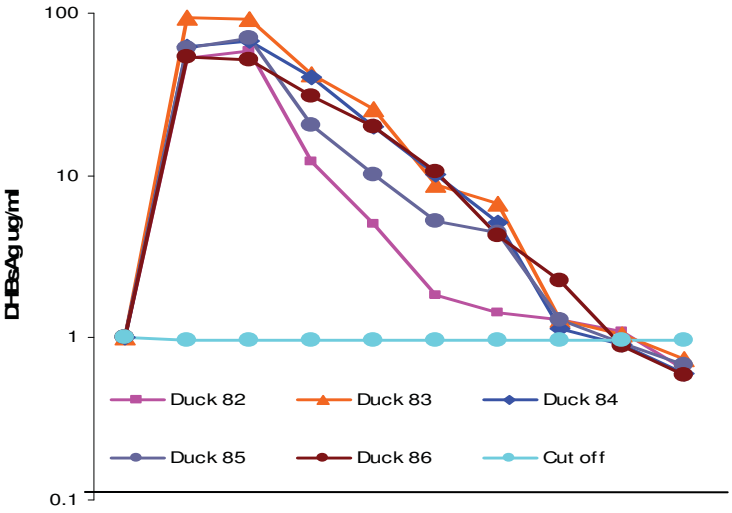
DHBsAg levels are given in µg/ml of serum in the Y axis and days after DHBV infection or treatment are given in the X axis.

D p.i.: days post DHBV infection; **Dp Rx:** days post-treatment.

G


100
10
1
0.1
DHBsAg ug/ml
Duck 76
Duck 77
Duck 78
Duck 79
Duck 80
Cut off

H


100
10
1
0.1
DHBsAg ug/ml
Duck 82
Duck 83
Duck 84
Duck 85
Duck 86
Cut off

I

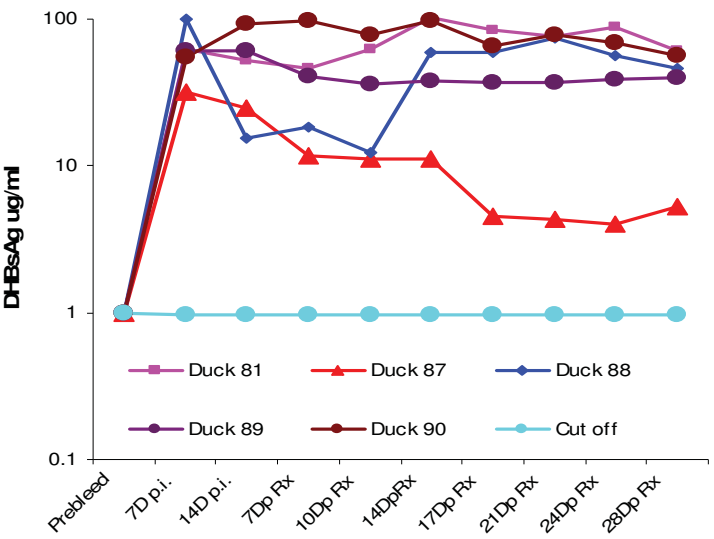
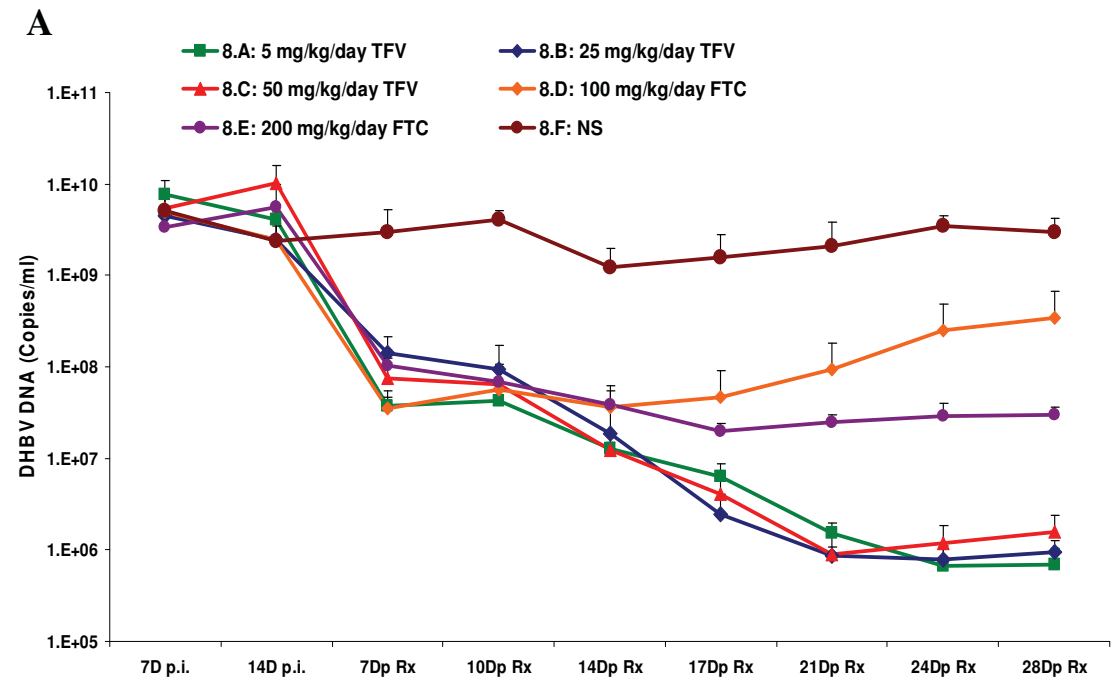

100
10
1
0.1
DHBsAg ug/ml
Duck 81
Duck 87
Duck 88
Duck 89
Duck 90
Cut off
Prebleed
7D p.i.
14D p.i.
7Dp Rx
10Dp Rx
14DpRx
17Dp Rx
21Dp Rx
24Dp Rx
28Dp Rx

Figure 8.4: Serum DHBV DNA levels detected by qPCR as described in Section 2.10.4 in ducks in 6 experimental Groups (8.A-8.F) treated with TFV in 3 different doses (5, 25 and 50 mg/kg) or FTC in 2 different doses (100 and 200 mg/kg) of monotherapy regimens or NS **(A)**.

Serum DHBV DNA levels in ducks in 3 experimental Groups (8.G-8.I) treated with TFV and FTC in 2 different combination dose regimens or NS **(B)**.

Group means of DHBV DNA copy numbers are given per ml of serum in the Y axis of the graph. Days after the DHBV infection or treatment are given in the X axis.

D p.i.: days post DHBV infection; **Dp Rx:** days post-treatment.


A
8.A: 5 mg/kg/day TFV
8.B: 25 mg/kg/day TFV
8.C: 50 mg/kg/day TFV
8.D: 100 mg/kg/day FTC
8.E: 200 mg/kg/day FTC
8.F: NS
DHBV DNA (Copies/ml)
1.E+11
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
7D p.i.
14D p.i.
7Dp Rx
10Dp Rx
14Dp Rx
17Dp Rx
21Dp Rx
24Dp Rx
28Dp Rx

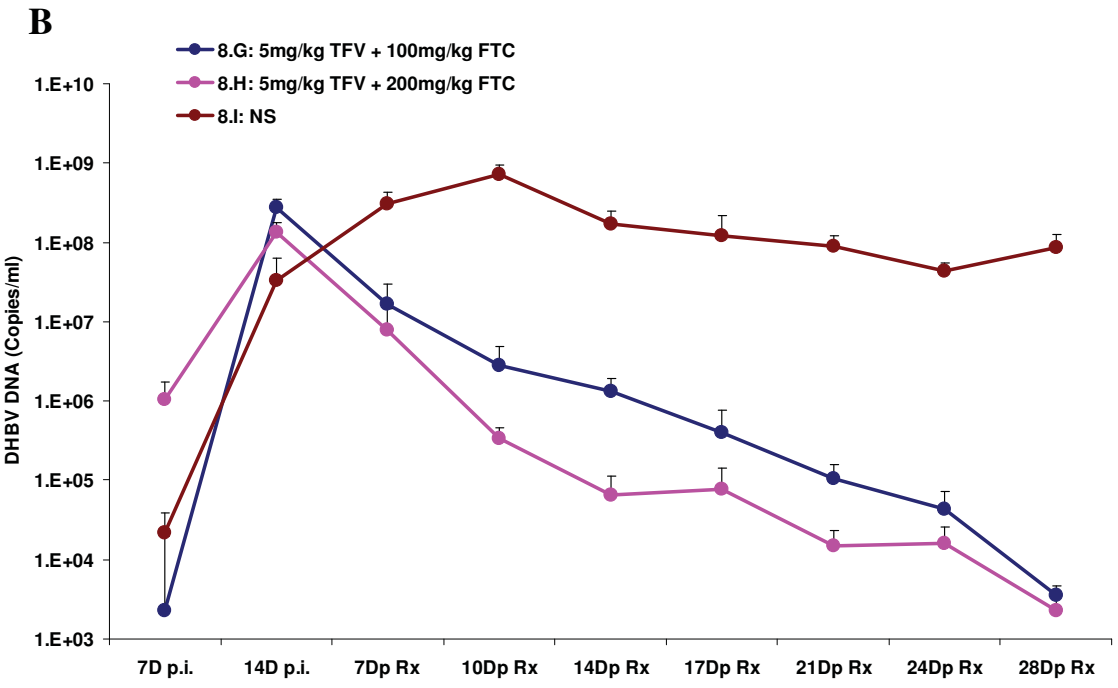

B
8.G: 5mg/kg TFV + 100mg/kg FTC
8.H: 5mg/kg TFV + 200mg/kg FTC
8.I: NS
DHBV DNA (Copies/ml)
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
7D p.i.
14D p.i.
7Dp Rx
10Dp Rx
14Dp Rx
17Dp Rx
21Dp Rx
24Dp Rx
28Dp Rx

Figure 8.5: Panels A-F: Hepatic DHBsAg levels in ducks in 6 experimental Groups treated with TFV in 3 different doses or FTC in 2 different doses or NS in monotherapy experiment. **Panels G-I:** Hepatic DHBsAg levels in ducks in 3 experimental Groups treated with TFV and FTC in 2 different combination dose regimens or NS.

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.

Group mean percentages of DHBsAg-positive hepatocytes are given in the photomicrographs. 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.

Experiment II: Liver sections of ducks in monotherapy

Panel A: Group 8.A ducks treated with 5 mg/kg TFV;

Panel B: Group 8.B ducks treated with 25 mg/kg TFV;

Panel C: Group 8.C ducks treated with 50 mg/kg TFV;

Panel D: Group 8.D ducks treated with 100 mg/kg FTC;

Panel E: Group 8.E ducks treated with 200 mg/kg FTC;

Panel F: Group 8.F ducks treated with NS.

Experiment III: Liver sections of ducks in combination therapy

Panel G: Group 8.G ducks treated with 5 mg/kg TFV + 100 mg/kg FTC;

Panel H: Group 8.H ducks treated with 5 mg/kg TFV + 200 mg/kg FTC;

Panel I: Group 8.I ducks treated with NS.

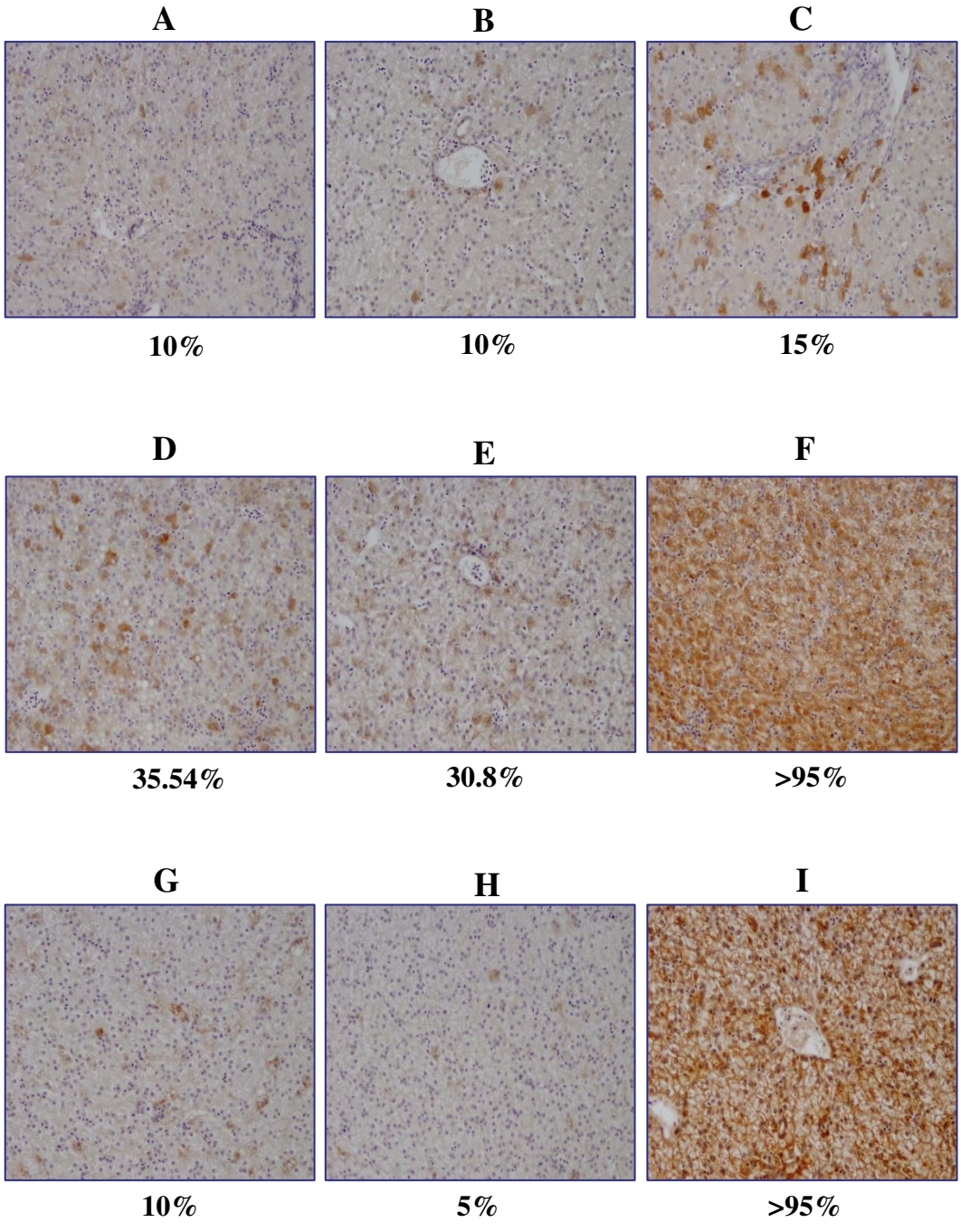

A
B
C
10%
10%
15%
D
E
F
35.54%
30.8%
>95%
G
H
I
10%
5%
>95%

Figure 8.6: Hepatic DHBV DNA levels as detected by qPCR using methods described in Section 2.10.5.

Experiment II: Ducks in 6 experimental Groups were treated with TFV in 3 different doses or FTC in 2 different doses or NS **(Panel A)**.

8.A: Group 8.A ducks treated IP with 5 mg/kg TFV;

8.B: Group 8.B ducks treated IP with 5 mg/kg TFV;

8.C: Group 8.C ducks treated IP with 5 mg/kg TFV;

8.D: Group 8.D ducks treated IP with 100 mg/kg FTC;

8.E: Group 8.E ducks treated IP with 200 mg/kg FTC;

8.F: Ducks treated with IP NS.

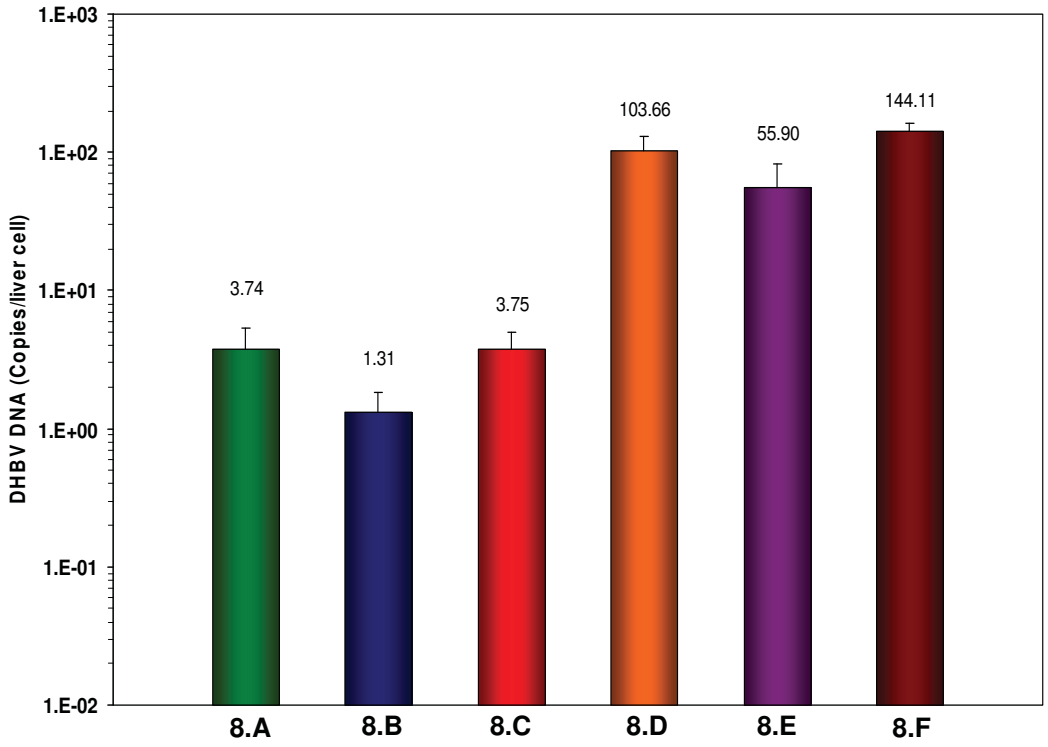
Experiment III: Ducks in 3 experimental Groups were treated with TFV and FTC in 2 different combination therapy regimens or NS **(Panel B)**.

8.G: Ducks treated IP with 5 mg/kg TFV + 100 mg/kg FTC;

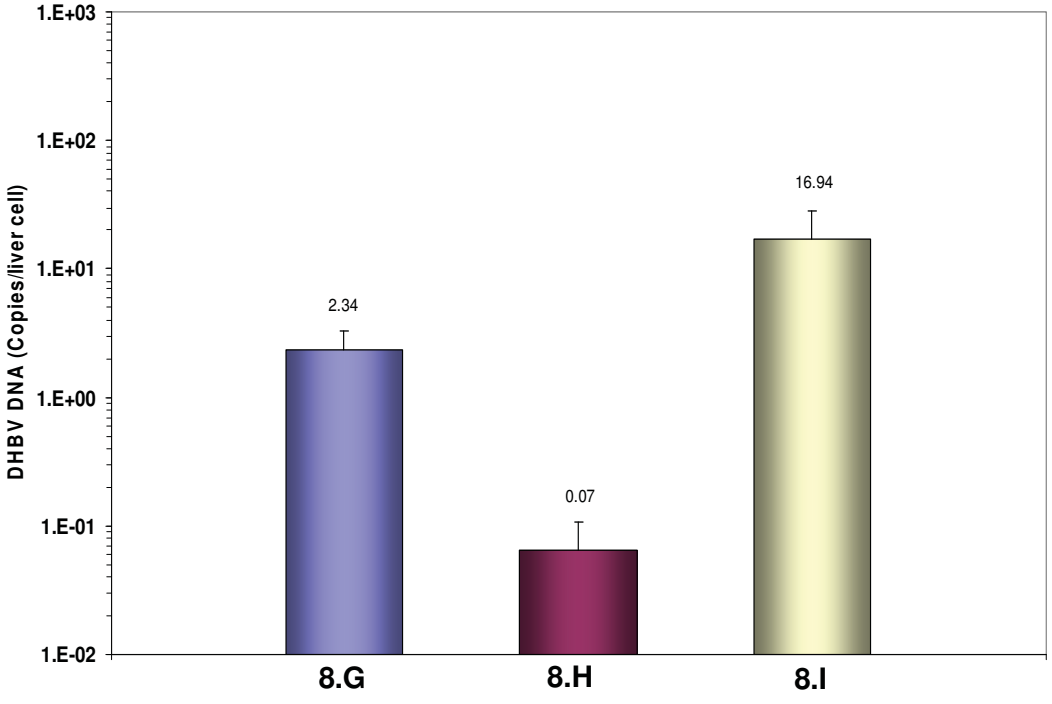
8.H: Ducks treated IP with 5 mg/kg TFV + 200 mg/kg FTC;

8.I: Ducks treated IP with NS.

A


1.E+03
1.E+02
1.E+01
1.E+00
1.E-01
1.E-02
DHBV DNA (Copies/liver cell)
3.74
1.31
3.75
103.66
55.90
144.11
8.A
8.B
8.C
8.D
8.E
8.F

B


1.E+03
1.E+02
1.E+01
1.E+00
1.E-01
1.E-02
DHBV DNA (Copies/liver cell)
2.34
0.07
16.94
8.G
8.H
8.I

Chapter 9: Discussion

9.1 Introduction

Studies described in this Ph.D. thesis focused on the development and testing of novel therapies for chronic HBV infection using the DHBV-duck model. Firstly, the novel antiviral agents, APDPs, developed by REPLICor Inc, were comprehensively tested against DHBV infection *in vitro* and *in vivo.* This project aimed to examine the ability of APDPs to act as prophylactic and therapeutic antiviral agents. Secondly, a combination of NAs developed by Gilead Sciences Pty. Ltd. was tested for their antiviral efficacy against persistent DHBV infection *in vivo*, in order to study the advantages of combination therapy over monotherapy of NAs. Each of these areas of work is summarised below.

9.2 Antiviral activity of APDPs *in vitro* including the role of APDP chemistry on antiviral activity

The pharmacokinetic and pharmacodynamic properties of APDPs is believed to be similar to several PS-ONs (Yu *et al.* 2009a; Yu *et al.* 2009b), which have been researched and tested to improve existing therapeutic options for various diseases including cancer (Badros *et al.* 2005; Morris *et al.* 2005). Extensive clinical experience with many different oligonucleotides that are similar in nucleotide length and chemistry to APDPs has demonstrated that parenteral use of these compounds in humans is safe, in terms of: toxicity and carcinogenicity; independent of nucleotide sequence; and presence or absence of phosphorothioation (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).

Phosphorothioation of oligonucleotides increases their hydrophobicity (amphipathicity) and also makes them resistant to degradation by nucleases. The amphipathic nature of APDPs plays a major role in their antiviral activity. For example, longer APDPs with lengths of ≥30 bases have a greater amphipathicity and are more potent in blocking the amphipathic interactions involved in the HIV-1 gp41 six-helix bundle formation and inhibiting HIV-1-mediated cell-cell fusion than shorter APDPs with lengths of <30 bases (Vaillant *et al.* 2006). This novel antiviral mechanism of APDPs' action with >30 bases has

future applications in antiviral therapy against HIV-1 and other enveloped viruses. Moreover, the amphipathic nature and polymer size-related antiviral activity has been shown as working against HCV infection *in vitro,* using the HCV replicon system. It also works *in vivo* using uPA/SCID mice engrafted with human hepatocytes (Matsumura *et al.* 2009).

In order to study the effect of amphipathicity independently of stability on the antiviral activity of APDPs against DHBV infection, a 2' ribose modified non-APDP was used. 2' ribose modification maintains stability without amphipathicity. Furthermore, the effect of oligonucleotide size on the antiviral activity of APDPs against DHBV infection was studied using PS-ON polymers of 10-80 bases to determine the effect of oligonucleotide size on antiviral activity using PDH.

As a first step in investigating the activity of APDPs against DHBV infection, APDPs REP 2006 and REP 2031 and a control non-APDP REP 2086 were tested in PDH and found to be non-cytotoxic. Subsequently, REP 2006, REP 2031, REP 2055, and REP 2086 were investigated for anti-DHBV activity *in vitro.* REP 2006 is a 40mer PS-ON with a completely degenerate sequence, REP 2031 is a 40mer poly C PS-ON, REP 2055 is a 40mer poly AC PS-ON and REP 2086 is a non-APDP control only used in a preliminary experiment.

The antiviral activity of APDPs against DHBV infection was nucleotide length and chemistry dependent but sequence independent, a characteristic that is consistent with the studies of APDPs' antiviral activity against HIV-1 and HCV (Vaillant *et al.* 2006; Matsumura *et al.* 2009). Moreover, it was evident that the antiviral activity of REP 2031 was weaker than REP 2006 and REP 2055. The weaker antiviral activity of REP 2031 may be due to the fact that this compound undergoes a conformational change and is unable to elicit an effective antiviral activity at the acidic pH (Manzini *et al.* 1994; Kanehara *et al.* 1997; Kanaori *et al.* 2004) that prevails in the microenvironment in which the fusion, entry and the early events of DHBV replication occur. REP 2086, a non-APDP control, did not show anti-DHBV activity that is comparable to the level of anti-DHBV activity shown by REP 2006 and REP 2055, supporting the role of amphipathicity on the antiviral activity of APDPs. Conversely, more effective uptake of CY3-labelled REP 2006 by DHBV-infected and uninfected PDH than uptake of CY3-labelled REP 2086 showed that the amphipathic nature is an important property. This is needed for REP 2006 to accumulate in the PDH where the action of the compound is needed to elicit its anti-viral activity.

9.3 Antiviral activity of REP 2006 and REP 2031 *in vivo*

Having characterised the nucleotide size and chemistry dependent anti-DHBV activity of APDPs *in vitro*, further studies were conducted to test the anti-viral efficacy of REP 2006 and REP 2031 *in vivo*. DHBV infection was initiated using a dose of virus that has been shown to produce persistent infection in two week-old ducks. Ducks in 4 experimental Groups were treated with either REP 2006 or REP 2031 or ETV or NS starting from 1 day prior to DHBV infection for 15 days. REP 2006 showed an effective anti-DHBV activity, however, REP 2006-treated ducks experienced some adverse effects that included pain on abdominal palpation and IP injection and moderate to severe bleeding at the surgical incision site at the time of biopsy. The bleeding was manageable and the surgical biopsy was completed without complications or post-surgical mortality. In contrast, REP 2031 treatment in ducks was well tolerated, but it failed to show anti-DHBV activity against DHBV infection despite a minimal activity at the early stage when compared with NS treatment.

It was hypothesised that the side effects in the REP 2006-treated ducks were due to CpG motifs present in REP 2006 sequence. CpG may have activated the innate immune response and associated pro-inflammatory activities (Isogawa *et al.* 2005). The lack of these side effects with REP 2031 (which has no CpG motifs) was consistent with this hypothesis. The lack of antiviral activity of REP 2031 *in vivo* agreed with *in vitro* studies; as noted earlier, poly C oligonucleotides are known to form homo-tetramers at less acidic pH (Manzini *et al.* 1994; Huertas and Azorin 1996; Kanehara *et al.* 1997; Pataskar *et al.* 2001; Kanaori *et al.* 2004), which would negate the amphipathic activity present in REP 2031. Thus the lack of antiviral activity with REP 2031 suggests that target interaction with REP 2031 was not optimal in an acidic environment.

Subsequently, REP 2055, which does not have CpG motifs but has an interrupted C nucleotide sequence composition with alternate C and A nucleotides, was tested against DHBV infection. An excellent antiviral activity without any alterations in clinicopathological, haematological and biochemical markers resulted in no observable side effects. These studies investigated the dose range of REP 2055, which proved to be efficacious against DHBV infection.

9.4 Antiviral activity of REP 2055 *in vivo*

The antiviral efficacy of REP 2055 was investigated *in vivo* using a range of dose regimens (0.5, 2, 3, 5 and 10 mg/kg). REP 2055 treatment was administered from 1 day prior to DHBV infection for 15 days to young ducks infected with a dose of DHBV, which has been shown to produce persistent DHBV infection (Jilbert *et al.* 1998; Foster *et al.* 2005). Treatment prevented extracellular spread of DHBV and development of persistent infection with doses as low as 0.5-2 mg/kg without producing observable adverse reactions.

These early experiments with REP 2055 were done to investigate its antiviral efficacy when administration was initiated prior to DHBV inoculation. The ability of REP 2055 to prevent development and spread of DHBV when treatment began after viral infection was investigated in the next group of studies. These subsequent experiments also investigated the ability of REP 2055 to prevent rebound of DHBV infection after treatment withdrawal. In these studies, REP 2055 treatment was started 1 day prior to and 4 and 12 days after DHBV infection. Ducks were monitored for ~50 days after stopping REP 2055 therapy.

9.5 The ability of REP 2055 to prevent the rebound of DHBV

Ducks infected with a DHBV dose that is capable of producing persistent infection were treated with REP 2055 from 1 day prior to infection for 15 days. In this Group of ducks, ~80% lacked detectable levels of serum and hepatic virological markers when examined at the end of follow-up, 49 or 53 days after stopping REP 2055 treatment. In a different antiviral study (Foster *et al.* 2005), treatment with ETV 24 h prior to DHBV infection protected ~50% ducks from the development of persistent infection with the same dose of DHBV and the age of ducks used in the current study.

Moreover, REP 2055 treatment (10 mg/kg) was also able to prevent the rebound of DHBV infection when started at an early phase of DHBV infection (day 4 after infection with <10% of DHBsAg-positive hepatocytes in the liver). In this experiment, infection did not rebound in 75% of the ducks, as determined 49 days after stopping treatment. In contrast, a dose of 2 mg/kg REP 2055 prevented the rebound of infection in only 20% of ducks.

REP 2055 treatment was further delayed by 12 days after infection to test the activity of REP 2055 against a widespread DHBV infection, with >95% DHBsAg-positive hepatocytes. In this Group of ducks, REP 2055 treatment was administered (10 mg/kg) for 7 days, followed by 7 weekly doses. This treatment reduced viraemia by 2-3 logs in 100% of the ducks. However, only 20% of the ducks achieved a true SVR while 80% had fluctuating levels of DHBsAg and DHBV DNA in the serum. Moreover, autopsy liver of these latter ducks revealed >55-95% DHBsAg-positive hepatocytes and detectable levels DHBV DNA, indicating a failure of this treatment protocol. The inability of REP 2055 to protect 80% of the ducks in this treatment protocol implied that the treatment regimen was suboptimal against an established infection. In particular, a 7-day interval between drug injections, following the initial daily treatments, appears to be too long to allow the build up of a therapeutic intracellular drug level. PS-ONs that are similar to REP 2055 have a short intrahepatic half-life in ducks, which could explain the very low or zero levels of REP 2055 in the plasma and in the liver (Soni *et al.* 1998). Thus it failed to elicit an effective antiviral response when given at 7-day intervals.

9.6 The antiviral efficacy of REP 2055 against persistent DHBV infection and its ability to prevent the rebound of infection

Based on the above findings, it was obvious that daily treatment with REP 2055 with a dose of 10 mg/kg can protect against rebound of DHBV infection when the treatment was administered 1 day prior to or at an early stage of DHBV infection. However, daily dosing with 2 mg/kg of REP 2055 for 14 days during an early stage or 10 mg/kg of REP 2055 with 7 days of daily doses, followed by 7 weekly doses, were not effective in preventing persistent infection.

Thus a large *in vivo* experiment was done to determine a more optimal REP 2055 treatment regimen against persistent DHBV infection. In this study, daily REP 2055 treatment (10 mg/kg) was carried out beginning at 14 days post-infection (>95% DHBsAg-hepatocytes positive in the liver) for 28 days (a longer treatment duration than in the earlier studies). A control group of ducks treated with NS was monitored for comparison. Haematological (CBE) and biochemical (liver enzymes) markers were tested prior to, during and at treatment endpoint and at the end of a 16 week follow-up after cessation of treatment.

Using this protocol, approximately 56% of the ducks were protected from rebound of infection. This is the first study of this nature in which antiviral therapy provided a high level of protection against rebound of a persistent DHBV infection. Furthermore, the 56% of ducks that showed an SVR had undetectable levels of serum and hepatic virological markers together with a high level of anti-DHBs antibodies. Similar outcome in CHB patients will be the most preferable one as seroconversion to anti-HBs antibodies is the accepted marker of recovery from persistent infections in patients who are chronically infected with HBV.

9.7 The antiviral efficacy of TFV and FTC against persistent DHBV infection as mono and combination therapies

HBV clearance or a decrease in HBV load in HIV and HBV co-infected patients that were treated with anti-HIV NAs was the trigger for NAs such as 3TC, FTC, ADF and TFV to be introduced for HBV therapy. HBV and HIV-1 share a reverse transcription step in their replication strategy and combination chemotherapy has been widely used against HIV. Moreover, a combination of TDF and FTC is licensed to treat HIV infection in humans (Gazzard 2006; Munoz de Benito and Arribas Lopez 2006). Hence, it was hypothesised that a combination of TDF and FTC would have better therapeutic efficacy against chronic HBV infection than either TDF or FTC alone. This hypothesis was tested using the duck model for HBV infection. Having studied the PK of TFV and TDF in DHBV-negative healthy ducks, the antiviral efficacy of TFV and FTC was tested as mono and combination therapies against persistent DHBV infection in two large *in vivo* experiments.

Based on PK analysis, TFV was chosen for the study instead of TDF for practical reasons of once daily administration. TFV had a half-life of 6 h when administered IP whereas TDF had a half life of 4 h and required twice daily administration. Persistently DHBV-infected ducks were treated from 14 days p.i. with daily IP administration of 5, 25, or 50 mg/kg of TFV or 100 or 200 mg/kg of FTC as monotherapies.

The study showed that 5, 25 and 50 mg/kg of TFV suppressed serum levels of DHBV DNA by 3 logs compared to NS-treated ducks. FTC showed dose-dependent serum DHBV DNA suppression with 1 log reduction for a dose of 100 mg/kg, and a 2 log reduction for a dose of 200 mg/kg. Subsequently, two different combinations of TFV and FTC were tested in ducks with persistent DHBV infection. A combination of 5 mg/kg TFV + 200 mg/kg FTC

suppressed levels of serum DHBV DNA by 5 logs whereas the combination of 5 mg/kg TFV + 100 mg/kg FTC reduced the levels of serum DHBV DNA by 3 logs. Serum and hepatic virological markers in all Groups tested in these experiments were in agreement with each other. A combination therapy approach with TFV and FTC produced superior anti-DHBV activity to monotherapies with either NA alone in suppressing serum and hepatic virological markers in ducks with persistent DHBV infection. This study also produced a platform for future antiviral studies using TFV and FTC in ducks for experimental applications. This may help to establish successful combination therapy regimens using NAs for chronic HBV infection. Future studies should investigate the effect of FTC and FTC on cccDNA levels and this combination's ability to prevent rebound of persistent DHBV infection.

Furthermore, TFV and FTC combination therapy has been shown to be more effective in decreasing the HBV load without inducing resistance in HIV and HBV for a relatively longer duration than NA combinations in HIV/HBV coinfected patients (Soriano *et al.* 2005; Soriano *et al.* 2006; Soriano *et al.* 2007; Thio and Locarnini 2007). According to a recent study, TFV and FTC in combination have shown additive effects when tested using AD38 cell line that expresses wild-type HBV (Zhu *et al.* 2009). Findings from these clinical studies in humans and the latter *in vitro* study already support the applicability of our data of TFV and FTC combination therapy approach for treating HBV infection in humans.

It can also be speculated from our results that there could be additive effects of TFV and FTC combination therapy on HBV-infected carriers when compared to TFV or FTC alone in reducing the virus load and possibilities of inducing resolution of infection. On this line treating HBV carrier mothers to reduce the virus load prior to delivery may minimise the chance of passing the HBV to the neonate during delivery.

9.8 Conclusions and future directions

In conclusion, the antiviral activity of APDPs developed by REPLICor Inc. appears to be mediated through targeting of multiple stages of DHBV replication including virus entry and production of progeny virus. This conclusion is supported by the fact that APDPs showed antiviral activity when treatment was started prior to or at an early or late stage of DHBV infection with 0-95% DHBsAg-positive hepatocytes in the liver. The chemistry and nucleotide length dependent anti-DHBV activity of APDPs was evident when they were

administered pre- and post-DHBV infection in both *in vitro* and *in vivo* studies. However, the exact mechanism through which APDPs inhibit DHBV infection is yet to be identified.

Moreover, APDPs including REP 2055 may also have the ability to induce the immune clearance of DHBV, as shown by 56% of ducks that achieved SVR and developed anti-DHBsAg antibodies (Chapter 7). The presence of anti-DHBs antibodies in these ducks is indicative of resolution of DHBV infection, as anti-DHBs antibodies are serological markers of immune clearance of DHBV if the liver is free of DHBsAg and DHBV DNA. Furthermore, the lack of rebound of DHBV infection in those ducks that achieved SVR is an indication of either reduction of cccDNA to residual levels or clearance of cccDNA from the liver. According to studies on the mechanisms of removal of cccDNA, the elimination of cccDNA requires killing of hepatocytes and dilution by cell division. Cytokines can contribute to the curing of infected hepatocytes. Perhaps APDPs induce various mechanisms that dilute the cccDNA pool to control hepdnavirus infection. However, evidence of apoptosis and cell division was not investigated in our study to support this postulate.

In summary, the APDP, REP 2055 showed excellent anti-DHBV activity and the ability of this APDP to prevent rebound of DHBV infection, making it a new class of DNA-based antiviral agent. This finding and the safe toxicology data for REP 2055 like compounds have now opened the avenue for evaluating this novel anti-HBV agent against chronic HBV infection. Results of a preliminary investigation show that two CHB patients that received weekly tratement of REP 2055 lost HBsAg from the serum and developed an anti-HBs response (Vaillant *et al.* unpublished). This data is very promising as HBsAg loss and seroconversion to anti-HBs antibodies are only rarely achieved with current NA monotherapy regimens even after 2 years of therapy in >90% patients. Further studies in other CHB patients are underway (Vaillant *et al.* unpublished).

Furthermore, the novel RELICor antiviral REP 2055 might be equally effective against different genotypes of HBV due to the ability to target structural domains irrespective of sequence homology. In order to understand these aspects this molecular antiviral agent has to be tested in CHB patients that are infected with different genotypes of HBV especially in Southeast Asia and sub Saharan Africa where HBV is hyperendemic.

In this respect, pharmacokinetic and pharmacodynamic properties of APDPs including their specific safety data for REP 2055 with long term use and effective therapeutic doses needs to be studied in humans to make this novel approach acceptable among medical and scientific communities. Progressing to a phase II clinical trial may take time and effort with sufficient capital and if done this new class of drugs (APDPs) might change the future management of CHB patients.

APDPs also have future clinical applications in preventing rebound of HBV infection following liver transplantation. APDPs target the amphipathic domain(s) that is/are structurally conserved in enveloped viruses, independent of their sequence relatedness. This feature may make APDPs an attractive broad spectrum antiviral against many viruses including HBV, HCV, HIV and herpes viruses. APDPs appeared to interact with sequence independent but structurally conserved targets which would make the multiple mutations that occur during the emergence of antiviral resistance less likely. Hence, they would minimise the problems associated with antiviral resistance.

Promising results of a combination regimen of TFV and FTC against persistent DHBV infection provides support for this approach in treating CHB patients, as suggested by studies in which this combination was used to treat HIV/HBV co-infected patients. More studies are needed to test the ability of TFV-FTC combination therapy to induce cccDNA clearance and prevent the rebound that commonly occurs following the cessation of NA monotherapy.

Another possible approach to treat CHB patients will be combining REP 2055 therapy with conventional NA therapy. This will help to target multiple stages of HBV replication and can overcome the problems of antiviral resistance with better treatment outcomes in CHB patients. For example, such a combination antiviral therapy might be used in transplant recipients to effectively eliminate the risk of escape mutants and thus terminate HBV replication.

Chapter 10: References

Act, Medicine (1968). "The UK Statute Law Databases".

Agrawal, S., J. Temsamani and J. Y. Tang (1991). "Pharmacokinetics, biodistribution, and stability of oligodeoxynucleotide phosphorothioates in mice." Proc Natl Acad Sci U S A **88**(17): 7595-9.

Agrawal, S. and E. R. Kandimalla (2001). "Antisense and/or immunostimulatory oligonucleotide therapeutics." Curr Cancer Drug Targets **1**(3): 197-209.

Ancell, C. D., J. Phipps and L. Young (2001). "Thymosin alpha-1." Am J Health Syst Pharm **58**(10): 879-85; quiz 886-8.

Akbar, S. M., M. Abe, T. Masumoto, N. Horiike and M. Onji (1999). "Mechanism of action of vaccine therapy in murine hepatitis B virus carriers: vaccine-induced activation of antigen presenting dendritic cells." J Hepatol **30**(5): 755-64.

Alberti, A., S. Diana, G. H. Scullard, W. F. Eddleston and R. Williams (1978). "Full and empty Dane particles in chronic hepatitis B virus infection: relation to hepatitis B e antigen and presence of liver damage." Gastroenterology **75**(5): 869-74.

Anderson, A. L., K. E. Banks, M. Pontoglio, M. Yaniv and A. McLachlan (2005). "Alpha/beta interferon differentially modulates the clearance of cytoplasmic encapsidated replication intermediates and nuclear covalently closed circular hepatitis B virus (HBV) DNA from the livers of hepatocyte nuclear factor 1alpha-null HBV transgenic mice." J Virol **79**(17): 11045-52.

Badros, A. Z., O. Goloubeva, A. P. Rapoport, B. Ratterree, N. Gahres, B. Meisenberg, N. Takebe, M. Heyman, J. Zwiebel, H. Streicher, C. D. Gocke, D. Tomic, J. A. Flaws, B. Zhang and R. G. Fenton (2005). "Phase II study of G3139, a Bcl-2 antisense oligonucleotide, in combination with dexamethasone and thalidomide in relapsed multiple myeloma patients." J Clin Oncol **23**(18): 4089-99.

Balsano, C. and A. Alisi (2008). "Viral hepatitis B: established and emerging therapies." Curr Med Chem **15**(9): 930-9.

Bartenschlager, R. and H. Schaller (1992). "Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome." Embo J **11**(9): 3413-20.

Bartlett, D. W. and M. E. Davis (2008). "Impact of tumor-specific targeting and dosing schedule on tumor growth inhibition after intravenous administration of siRNA-containing nanoparticles." Biotechnol Bioeng **99**(4): 975-85.

Bartlett, D. W., H. Su, I. J. Hildebrandt, W. A. Weber and M. E. Davis (2007). "Impact of tumor-specific targeting on the biodistribution and efficacy of siRNA nanoparticles measured by multimodality in vivo imaging." Proc Natl Acad Sci U S A **104**(39): 15549-54.

Bass, J. B., Jr. (1995). "Tuberculosis in the 1990s." Alcohol Clin Exp Res **19**(1): 3-5.

Bernstein, D. I., N. Goyette, R. Cardin, E. R. Kern, G. Boivin, J. Ireland, J. M. Juteau and A. Vaillant (2008). "Amphipathic DNA polymers exhibit antiherpetic activity in vitro and in vivo." Antimicrob Agents Chemother **52**(8): 2727-33.

Bertoletti, A. and A. J. Gehring (2006). "The immune response during hepatitis B virus infection." J Gen Virol **87**(Pt 6): 1439-49.

Bertram, E. (1997). Characterisation of duck lymphoid cell populations and their role in immunity to duck hepatitis B virus. Microbiology and Immunology. Adelaide, The University of Adelaide**:** 218.

Block, T. M., H. Guo and J. T. Guo (2007). "Molecular virology of hepatitis B virus for clinicians." Clin Liver Dis **11**(4): 685-706, vii.

Blumberg, B. S. (1977). "Australia antigen and the biology of hepatitis B." Science **197**(4298): 17-25.

Blumberg, B. S., H. J. Alter and S. Visnich (1965). "A "New" Antigen in Leukemia Sera." Jama **191**: 541-6.

Bodkin, D. K. and D. L. Knudson (1985). "Sequence relatedness of Palyam virus genes to cognates of the Palyam serogroup viruses by RNA-RNA blot hybridization." Virology **143**(1): 55-62.

Bowden, S. (2008). Laboratory diagnosis of hepatitis B. Hepatitis B virus. S. Locarnini and C. L. Lai. London, Atlanta, Singapore, International Medical Press. **1:** 11.1-11.16.

Breiner, K. M. and H. Schaller (2000). "Cellular receptor traffic is essential for productive duck hepatitis B virus infection." J Virol **74**(5): 2203-9.

Brunello, F., G. Emanuelli and G. Camussi (1979). "Markers of type B viral hepatitis." Minerva Med **70**(41): 2791-800.

Bruss, V. (2004). "Envelopment of the hepatitis B virus nucleocapsid." Virus Res **106**(2): 199-209.

Bruss, V. (2007). "Hepatitis B virus morphogenesis." World J Gastroenterol **13**(1): 65-73.

Bruss, V., E. Gerhardt, K. Vieluf and G. Wunderlich (1996). "Functions of the large hepatitis B virus surface protein in viral particle morphogenesis." Intervirology **39**(1-2): 23-31.

Cardin, R. D., F. J. Bravo, A. P. Sewell, J. Cummins, L. Flamand, J. M. Juteau, D. I. Bernstein and A. Vaillant (2009). "Amphipathic DNA polymers exhibit antiviral activity against systemic murine Cytomegalovirus infection." Virol J **6**: 214

Chang, J. S., K. C. Wang, H. W. Liu, M. C. Chen, L. C. Chiang and C. C. Lin (2007). "Sho-saiko-to (Xiao-Chai-Hu-Tang) and crude saikosaponins inhibit hepatitis B virus in a stable HBV-producing cell line." Am J Chin Med **35**(2): 341-51.

Chi, K. N., A. Zoubeidi and M. E. Gleave (2008). "Custirsen (OGX-011): a second-generation antisense inhibitor of clusterin for the treatment of cancer." Expert Opin Investig Drugs **17**(12): 1955-62.

Chiang, L. C., L. T. Ng, L. T. Liu, D. E. Shieh and C. C. Lin (2003). "Cytotoxicity and anti-hepatitis B virus activities of saikosaponins from Bupleurum species." Planta Med **69**(8): 705-9.

Colledge, D., G. Civitico, S. Locarnini and T. Shaw (2000). "In vitro antihepadnaviral activities of combinations of penciclovir, lamivudine, and adefovir." Antimicrob Agents Chemother **44**(3): 551-60.

Cooksley, W. G., T. Piratvisuth, S. D. Lee, V. Mahachai, Y. C. Chao, T. Tanwandee, A. Chutaputti, W. Y. Chang, F. E. Zahm and N. Pluck (2003). "Peginterferon alpha-2a (40 kDa): an advance in the treatment of hepatitis B e antigen-positive chronic hepatitis B." J Viral Hepat **10**(4): 298-305.

Cote, H. C., B. Yip, J. J. Asselin, J. W. Chan, R. S. Hogg, P. R. Harrigan, M. V. O'Shaughnessy and J. S. Montaner (2003). "Mitochondrial:nuclear DNA ratios in peripheral blood cells from human immunodeficiency virus (HIV)-infected patients who received selected HIV antiretroviral drug regimens." J Infect Dis **187**(12): 1972-6.

Council of Australian Government and National Health and Medical Research (2004). Australain code of practice for the care and use of animals for scientific purpose. Animal welfare organisations and National and Medical Research Council, Australian Government 2004**:** 84.

Cova, L. (2005). "DNA-designed avian IgY antibodies: novel tools for research, diagnostics and therapy." J Clin Virol **34 Suppl 1**: S70-4.

Cray, C., D. Gautier, D. J. Harris and K. L. Arheart (2008). "Changes in clinical enzyme activity and bile acid levels in psittacine birds with altered liver function and disease." J Avian Med Surg **22**(1): 17-24.

Dai, C. Y., W. L. Chuang, J. F. Huang and M. L. Yu (2009). "Hepatitis B e antigen-negative patients with persistently normal alanine aminotransferase levels and hepatitis B virus DNA > 2000 IU/mL." Hepatology **49**(2): 704-5; author reply 705-6.

Dandri, M. and J. Petersen (2005). "Hepatitis B virus cccDNA clearance: killing for curing?" Hepatology **42**(6): 1453-5.

De Clercq, E. and H. J. Field (2008). "Antiviral Chemistry & Chemotherapy's current antiviral agents FactFile (2nd edition): retroviruses and hepadnaviruses." Antivir Chem Chemother **19**(2): 75-105.

Dejean, A., C. Brechot, P. Tiollais and S. Wain-Hobson (1983). "Characterization of integrated hepatitis B viral DNA cloned from a human hepatoma and the hepatoma-derived cell line PLC/PRF/5." Proc Natl Acad Sci U S A **80**(9): 2505-9.

Delaney, W. E. t. and K. Borroto-Esoda (2008). "Therapy of chronic hepatitis B: trends and developments." Curr Opin Pharmacol **8**(5): 532-40.

Delaney, W. E. t., H. Yang, M. D. Miller, C. S. Gibbs and S. Xiong (2004). "Combinations of adefovir with nucleoside analogs produce additive antiviral effects against hepatitis B virus in vitro." Antimicrob Agents Chemother **48**(10): 3702-10.

Dryden, K. A., S. F. Wieland, C. Whitten-Bauer, J. L. Gerin, F. V. Chisari and M. Yeager (2006). "Native hepatitis B virions and capsids visualized by electron cryomicroscopy." Mol Cell **22**(6): 843-50.

Fabrizi, F., P. Martin, V. Dixit, S. Bunnapradist and G. Dulai (2004). "Meta-analysis: the effect of age on immunological response to hepatitis B vaccine in end-stage renal disease." Aliment Pharmacol Ther **20**(10): 1053-62.

Fattovich, G., F. Bortolotti and F. Donato (2008). "Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors." J Hepatol **48**(2): 335-52.

Fleet, J. C., K. A. Golemboski, R. R. Dietert, G. K. Andrews and C. C. McCormick (1990). "Induction of hepatic metallothionein by intraperitoneal metal injection: an associated inflammatory response." Am J Physiol **258**(6 Pt 1): G926-33.

Foster, W. K., D. S. Miller, P. L. Marion, R. J. Colonno, I. Kotlarski and A. R. Jilbert (2003). "Entecavir therapy combined with DNA vaccination for persistent duck hepatitis B virus infection." Antimicrob Agents Chemother **47**(8): 2624-35.

Foster, W. K., D. S. Miller, C. A. Scougall, I. Kotlarski, R. J. Colonno and A. R. Jilbert (2005). "Effect of antiviral treatment with entecavir on age- and dose-related outcomes of duck hepatitis B virus infection." J Virol **79**(9): 5819-32.

Fournier, C. and F. Zoulim (2008). "Combination therapy in chronic hepatitis B." Gastroenterol Clin Biol **32**(1 Pt 2): S42-9.

Franke, C., U. Matschl and M. Bruns (2007). "Enzymatic treatment of duck hepatitis B virus: topology of the surface proteins for virions and noninfectious subviral particles." Virology **359**(1): 126-36.

Freiman, J. S., S. M. Murray, K. Vickery, D. Lim and Y. E. Cossart (1990). "Postexposure treatment of experimental DHBV infection: a new therapeutic strategy." J Med Virol **30**(4): 272-6.

Funk, A., H. Hohenberg, M. Mhamdi, H. Will and H. Sirma (2004). "Spread of hepatitis B viruses in vitro requires extracellular progeny and may be codetermined by polarized egress." J Virol **78**(8): 3977-83.

Funk, A., M. Mhamdi, H. Hohenberg, J. Heeren, R. Reimer, C. Lambert, R. Prange and H. Sirma (2008). "Duck hepatitis B virus requires cholesterol for endosomal escape during virus entry." J Virol **82**(21): 10532-42.

Funk, A., M. Mhamdi, H. Will and H. Sirma (2007). "Avian hepatitis B viruses: molecular and cellular biology, phylogenesis, and host tropism." World J Gastroenterol **13**(1): 91-103.

Gazzard, B. G. (2006). "Use of tenofovir disoproxil fumarate and emtricitabine combination in HIV-infected patients." Expert Opin Pharmacother **7**(6): 793-802.

Geary, R. S., R. Z. Yu, T. Watanabe, S. P. Henry, G. E. Hardee, A. Chappell, J. Matson, H. Sasmor, L. Cummins and A. A. Levin (2003). "Pharmacokinetics of a tumor necrosis factor-alpha phosphorothioate 2'-O-(2-methoxyethyl) modified antisense oligonucleotide: comparison across species." Drug Metab Dispos **31**(11): 1419-28.

Ghany, M. and T. J. Liang (2007). "Drug targets and molecular mechanisms of drug resistance in chronic hepatitis B." Gastroenterology **132**(4): 1574-85

Glebe, D., H. Lorenz, W. H. Gerlich, S. D. Butler, I. A. Tochkov, B. C. Tennant, P. Cote and S. Menne (2009). "Correlation of virus and host response markers with circulating immune complexes during acute and chronic woodchuck hepatitis virus infection." J Virol **83**(4): 1579-91.

Glebe, D. and S. Urban (2007). "Viral and cellular determinants involved in hepadnaviral entry." World J Gastroenterol **13**(1): 22-38.

Guidotti, L. G. and F. V. Chisari (2001). "Noncytolytic control of viral infections by the innate and adaptive immune response." Annu Rev Immunol **19**: 65-91.

Guidotti, L. G. and F. V. Chisari (2006). "Immunobiology and pathogenesis of viral hepatitis." Annu Rev Pathol **1**: 23-61.

Guidotti, L. G., B. Matzke, C. Pasquinelli, J. M. Shoenberger, C. E. Rogler and F. V. Chisari (1996). "The hepatitis B virus (HBV) precore protein inhibits HBV replication in transgenic mice." J Virol **70**(10): 7056-61.

Guidotti, L. G., R. Rochford, J. Chung, M. Shapiro, R. Purcell and F. V. Chisari (1999). "Viral clearance without destruction of infected cells during acute HBV infection." Science **284**(5415): 825-9.

Guo, H., D. Jiang, T. Zhou, A. Cuconati, T. M. Block and J. T. Guo (2007). "Characterization of the intracellular deproteinized relaxed circular DNA of hepatitis B virus: an intermediate of covalently closed circular DNA formation." J Virol **81**(22): 12472-84.

Guzman, E. M., N. Cheshenko, V. Shende, M. J. Keller, N. Goyette, J. M. Juteau, G. Boivin, A. Vaillant and B. C. Herold (2007). "Amphipathic DNA polymers are candidate vaginal microbicides and block herpes simplex virus binding, entry and viral gene expression." Antivir Ther **12**(8): 1147-56.

Haasnoot, J. and B. Berkhout (2009). "Nucleic acids-based therapeutics in the battle against pathogenic viruses." Handb Exp Pharmacol(189): 243-63.

Hadziyannis, S. J., G. V. Papatheodoridis and D. Vassilopoulos (2003). "Treatment of HBeAg-negative chronic hepatitis B." Semin Liver Dis **23**(1): 81-8.

Han, Y. Q., Z. M. Huang, X. B. Yang, H. Z. Liu and G. X. Wu (2008). "In vivo and in vitro anti-hepatitis B virus activity of total phenolics from Oenanthe javanica." J Ethnopharmacol **118**(1): 148-53.

He, B. G., J. L. Melnick, A. Siddiqui, W. S. Robinson, S. W. Law and E. C. Lai (1985). "Molecular cloning and characterization of the cDNA coding for hepatitis B virus surface antigen." Sci Sin (Act) **28**(1): 49-59.

Heermann, K. H., U. Goldmann, W. Schwartz, T. Seyffarth, H. Baumgarten and W. H. Gerlich (1984). "Large surface proteins of hepatitis B virus containing the pre-s sequence." J Virol **52**(2): 396-402.

Henry, S. P., M. V. Templin, N. Gillett, J. Rojko and A. A. Levin (1999). "Correlation of toxicity and pharmacokinetic properties of a phosphorothioate oligonucleotide designed to inhibit ICAM-1." Toxicol Pathol **27**(1): 95-100.

Hepatitis B Fact Sheet and Hepatitis B Foundation. (2009). "Hepatitis B Fact Sheet".

Hu, J. and C. Seeger (1996). "Expression and characterization of hepadnavirus reverse transcriptases." Methods Enzymol **275**: 195-208.

Huertas, D. and F. Azorin (1996). "Structural polymorphism of homopurine DNA sequences. d(GGA)n and d(GGGA)n repeats form intramolecular hairpins stabilized by different base-pairing interactions." Biochemistry **35**(40): 13125-35.

Hui, A. Y., H. L. Chan, A. Y. Cheung, G. Cooksley and J. J. Sung (2005). "Systematic review: treatment of chronic hepatitis B virus infection by pegylated interferon." Aliment Pharmacol Ther **22**(6): 519-28.

Isogawa, M., M. D. Robek, Y. Furuichi and F. V. Chisari (2005). "Toll-like receptor signaling inhibits hepatitis B virus replication in vivo." J Virol **79**(11): 7269-72.

Janssen, H. L., G. Gerken, V. Carreno, P. Marcellin, N. V. Naoumov, A. Craxi, H. Ring-Larsen, G. Kitis, J. van Hattum, R. A. de Vries, P. P. Michielsen, F. J. ten Kate, W. C. Hop, R. A. Heijtink, P. Honkoop and S. W. Schalm (1999). "Interferon alfa for chronic hepatitis B infection: increased efficacy of prolonged treatment. The European Concerted Action on Viral Hepatitis (EUROHEP)." Hepatology **30**(1): 238-43.

Jilbert, A. R., J. A. Botten, D. S. Miller, E. M. Bertram, P. M. Hall, J. Kotlarski and C. J. Burrell (1998). "Characterization of age- and dose-related outcomes of duck hepatitis B virus infection." Virology **244**(2): 273-82.

Jilbert, A. R. and I. Kotlarski (2000). "Immune responses to duck hepatitis B virus infection." Dev Comp Immunol **24**(2-3): 285-302.

Marcellin, P., T. Asselah and N. Boyer (2005). "Treatment of chronic hepatitis B." J Viral Hepat **12**(4): 333-45.

Marcellin, P., E. J. Heathcote, M. Buti, E. Gane, R. A. de Man, Z. Krastev, G. Germanidis, S. S. Lee, R. Flisiak, K. Kaita, M. Manns, I. Kotzev, K. Tchernev, P. Buggisch, F. Weilert, O. O. Kurdas, M. L. Shiffman, H. Trinh, M. K. Washington, J. Sorbel, J. Anderson, A. Snow-Lampart, E. Mondou, J. Quinn and F. Rousseau (2008). "Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B." N Engl J Med **359**(23): 2442-55.

Marion, P. L., F. H. Salazar, M. A. Winters and R. J. Colonno (2002). "Potent efficacy of entecavir (BMS-200475) in a duck model of hepatitis B virus replication." Antimicrob Agents Chemother **46**(1): 82-8.

Martin, P., H. W. Hann, S. Westerberg, S. J. Munoz, R. Rubin and W. C. Maddrey (1998). "Interferon-alpha2b therapy is efficacious in Asian-Americans with chronic hepatitis B infection: a prospective controlled trial." Dig Dis Sci **43**(4): 875-9.

Mast, E. E., H. S. Margolis, A. E. Fiore, E. W. Brink, S. T. Goldstein, S. A. Wang, L. A. Moyer, B. P. Bell and M. J. Alter (2005). "A comprehensive immunization strategy to eliminate transmission of hepatitis B virus infection in the United States: recommendations of the Advisory Committee on Immunization Practices (ACIP) part 1: immunization of infants, children, and adolescents." MMWR Recomm Rep **54**(RR-16): 1-31.

Matsumura, T., Z. Hu, T. Kato, M. Dreux, Y. Y. Zhang, M. Imamura, N. Hiraga, J. M. Juteau, A. Vaillant and T. J. Liang (2009). "Amphipathic DNA polymers inhibits hepatitis C virus infection by blocking entry." Gastroenterology **137**(2): 673-81.

Matthews, S. J. (2007). "Telbivudine for the management of chronic hepatitis B virus infection." Clin Ther **29**(12): 2635-53.

Mazzucco, C. E., R. K. Hamatake, R. J. Colonno and D. J. Tenney (2008). "Entecavir for treatment of hepatitis B virus displays no in vitro mitochondrial toxicity or DNA polymerase gamma inhibition." Antimicrob Agents Chemother **52**(2): 598-605.

McMahon, B. J. (2005). "Epidemiology and natural history of hepatitis B." Semin Liver Dis **25 Suppl 1**: 3-8.

Meier, P., C. A. Scougall, H. Will, C. J. Burrell and A. R. Jilbert (2003). "A duck hepatitis B virus strain with a knockout mutation in the putative X ORF shows similar infectivity and in vivo growth characteristics to wild-type virus." Virology **317**(2): 291-8.

Menne, S., S. D. Butler, A. L. George, I. A. Tochkov, Y. Zhu, S. Xiong, J. L. Gerin, P. J. Cote and B. C. Tennant (2008). "Antiviral effects of lamivudine, emtricitabine, adefovir dipivoxil, and tenofovir disoproxil fumarate administered orally alone and in combination to woodchucks with chronic woodchuck hepatitis virus infection." Antimicrob Agents Chemother **52**(10): 3617-32.

Menne, S., C. A. Roneker, M. Roggendorf, J. L. Gerin, P. J. Cote and B. C. Tennant (2002). "Deficiencies in the acute-phase cell-mediated immune response to viral antigens are associated with development of chronic woodchuck hepatitis virus infection following neonatal inoculation." J Virol **76**(4): 1769-80.

Mhamdi, M., A. Funk, H. Hohenberg, H. Will and H. Sirma (2007). "Assembly and budding of a hepatitis B virus is mediated by a novel type of intracellular vesicles." Hepatology **46**(1): 95-106.

Miller, D. S., E. M. Bertram, C. A. Scougall, I. Kotlarski and A. R. Jilbert (2004). "Studying host immune responses against duck hepatitis B virus infection." Methods Mol Med **96**: 3-25.

Miller, D. S., D. Boyle, F. Feng, G. Y. Reaiche, I. Kotlarski, R. Colonno and A. R. Jilbert (2008). "Antiviral therapy with entecavir combined with post-exposure "prime-boost" vaccination eliminates duck hepatitis B virus-infected hepatocytes and prevents the development of persistent infection." Virology **373**(2): 329-41.

Miller, D. S., M. Halpern, I. Kotlarski and A. R. Jilbert (2006). "Vaccination of ducks with a whole-cell vaccine expressing duck hepatitis B virus core antigen elicits antiviral immune responses that enable rapid resolution of de novo infection." Virology **348**(2): 297-308 (a).

Miller, D. S., I. Kotlarski and A. R. Jilbert (2006). "DNA vaccines expressing the duck hepatitis B virus surface proteins lead to reduced numbers of infected hepatocytes and protect ducks against the development of chronic infection in a virus dose-dependent manner." Virology **351**(1): 159-69 (b).

Montaner, J. S., H. C. Cote, M. Harris, R. S. Hogg, B. Yip, J. W. Chan, P. R. Harrigan and M. V. O'Shaughnessy (2003). "Mitochondrial toxicity in the era of HAART: evaluating venous lactate and peripheral blood mitochondrial DNA in HIV-infected patients taking antiretroviral therapy." J Acquir Immune Defic Syndr **34 Suppl 1**: S85-90.

Morris, M. J., C. Cordon-Cardo, W. K. Kelly, S. F. Slovin, K. Siedlecki, K. P. Regan, R. S. DiPaola, M. Rafi, N. Rosen and H. I. Scher (2005). "Safety and biologic activity of intravenous BCL-2 antisense oligonucleotide (G3139) and taxane chemotherapy in patients with advanced cancer." Appl Immunohistochem Mol Morphol **13**(1): 6-13.

Moucari, R., V. Mackiewicz, O. Lada, M. P. Ripault, C. Castelnau, M. Martinot-Peignoux, A. Dauvergne, T. Asselah, N. Boyer, P. Bedossa, D. Valla, M. Vidaud, M. H. Nicolas-Chanoine and P. Marcellin (2009). "Early serum HBsAg drop: a strong predictor of sustained virological response to pegylated interferon alfa-2a in HBeAg-negative patients." Hepatology **49**(4): 1151-7.

Moulder, S. L., W. F. Symmans, D. J. Booser, T. L. Madden, C. Lipsanen, L. Yuan, A. M. Brewster, M. Cristofanilli, K. K. Hunt, T. A. Buchholz, J. Zwiebel, V. Valero, G. N. Hortobagyi and F. J. Esteva (2008). "Phase I/II study of G3139 (Bcl-2 antisense oligonucleotide) in combination with doxorubicin and docetaxel in breast cancer." Clin Cancer Res **14**(23): 7909-16.

Muller, B. and H. G. Krausslich (2009). "Antiviral strategies." Handb Exp Pharmacol(189): 1-24.

Munoz de Benito, R. M. and J. R. Arribas Lopez (2006). "Tenofovir disoproxil fumarate-emtricitabine coformulation for once-daily dual NRTI backbone." Expert Rev Anti Infect Ther **4**(4): 523-35.

Nash, K. (2009). "Telbivudine in the treatment of chronic hepatitis B." Adv Ther **26**(2): 155-69.

Nassal, M. (1996). "Hepatitis B virus morphogenesis." Curr Top Microbiol Immunol **214**: 297-337.

Nassal, M. and H. Schaller (1996). "Hepatitis B virus replication--an update." J Viral Hepat **3**(5): 217-26.

Neurath, A. R., S. B. Kent, N. Strick and K. Parker (1986). "Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus." Cell **46**(3): 429-36.

Newbold, J. E., H. Xin, M. Tencza, G. Sherman, J. Dean, S. Bowden and S. Locarnini (1995). "The covalently closed duplex form of the hepadnavirus genome exists in situ as a heterogeneous population of viral minichromosomes." J Virol **69**(6): 3350-7.

Nicoll, A. J., D. L. Colledge, J. J. Toole, P. W. Angus, R. A. Smallwood and S. A. Locarnini (1998). "Inhibition of duck hepatitis B virus replication by 9-(2-phosphonylmethoxyethyl)adenine, an acyclic phosphonate nucleoside analogue." Antimicrob Agents Chemother **42**(12): 3130-5.

Ocama, P., C. K. Opio and W. M. Lee (2005). "Hepatitis B virus infection: current status." Am J Med **118**(12): 1413.

Offensperger, W. B., S. Offensperger, E. Walter, H. E. Blum and W. Gerok (1993). "Suramin prevents duck hepatitis B virus infection in vivo." Antimicrob Agents Chemother **37**(7): 1539-42.

Okabe, M., M. Enomoto, H. Maeda, K. Kuroki and K. Ohtsuki (2006). "Biochemical characterization of suramin as a selective inhibitor for the PKA-mediated phosphorylation of HBV core protein in vitro." Biol Pharm Bull **29**(9): 1810-4.

Olkowski, A. A. and H. L. Classen (1998). "Safety of isoflurane anaesthesia in high risk avian patients." Vet Rec **143**(3): 82-3.

Osiowy, C., D. Gordon, J. Borlang, E. Giles and J. P. Villeneuve (2008). "Hepatitis B virus genotype G epidemiology and co-infection with genotype A in Canada." J Gen Virol **89**(Pt 12): 3009-15.

Pastor-Anglada, M., A. Felipe and F. J. Casado (1998). "Transport and mode of action of nucleoside derivatives used in chemical and antiviral therapies." Trends Pharmacol Sci **19**(10): 424-30.

Pataskar, S. S., D. Dash and S. K. Brahmachari (2001). "Progressive myoclonus epilepsy (Pataskar *et al.*) repeat d(CCCCGCCCCGCG)n forms folded hairpin structures at physiological pH." J Biomol Struct Dyn **19**(2): 293-305.

Petcu, D. J., C. E. Aldrich, L. Coates, J. M. Taylor and W. S. Mason (1988). "Suramin inhibits in vitro infection by duck hepatitis B virus, Rous sarcoma virus, and hepatitis delta virus." Virology **167**(2): 385-92.

Petit, M. A., D. Buffello-Le Guillou, B. Roche, E. Dussaix, J. C. Duclos-Vallee, C. Feray and D. Samuel (2001). "Residual hepatitis B virus particles in liver transplant recipients receiving lamivudine: PCR quantitation of HBV DNA and ELISA of preS1 antigen." J Med Virol **65**(3): 493-504.

Plitas, G., B. M. Burt, H. M. Nguyen, Z. M. Bamboat and R. P. DeMatteo (2008). "Toll-like receptor 9 inhibition reduces mortality in polymicrobial sepsis." J Exp Med **205**(6): 1277-83.

Pugh, J. C., Q. Di, W. S. Mason and H. Simmons (1995). "Susceptibility to duck hepatitis B virus infection is associated with the presence of cell surface receptor sites that efficiently bind viral particles." J Virol **69**(8): 4814-22.

Rabe, B., D. Glebe and M. Kann (2006). "Lipid-mediated introduction of hepatitis B virus capsids into nonsusceptible cells allows highly efficient replication and facilitates the study of early infection events." J Virol **80**(11): 5465-73.

Rapti, I., E. Dimou, P. Mitsoula and S. J. Hadziyannis (2007). "Adding-on versus switching-to adefovir therapy in lamivudine-resistant HBeAg-negative chronic hepatitis B." Hepatology **45**(2): 307-13.

Reaiche, G. (2008). Characterisation of the events involved in the resolution of acute duck hepatitis B virus infection. Microbiology and Immunology. Adelaide, The University of Adelaide**:** 190.

Rehermann, B. (2000). "Intrahepatic T cells in hepatitis B: viral control versus liver cell injury." J Exp Med **191**(8): 1263-8.

Rehermann, B. and M. Nascimbeni (2005). "Immunology of hepatitis B virus and hepatitis C virus infection." Nat Rev Immunol **5**(3): 215-29.

Reichman, L. B. (1994). "Multidrug-resistant tuberculosis: meeting the challenge." Hosp Pract (Off Ed) **29**(5): 85-8, 91, 95-6.

Rieger, A. and M. Nassal (1995). "Distinct requirements for primary sequence in the 5'- and 3'-part of a bulge in the hepatitis B virus RNA encapsidation signal revealed by a combined in vivo selection/in vitro amplification system." Nucleic Acids Res **23**(19): 3909-15.

Rieger, A. and M. Nassal (1996). "Specific hepatitis B virus minus-strand DNA synthesis requires only the 5' encapsidation signal and the 3'-proximal direct repeat DR1." J Virol **70**(1): 585-9.

Roh, S. and K. Kim (2003). "Overcoming tolerance in hepatitis B virus transgenic mice: a possible involvement of regulatory T cells." Microbiol Immunol **47**(6): 453-60.

Rosenthal, K. L. (2006). "Tweaking Innate Immunity: the Promise of Innate Immunologicals As Anti-infectives." Can J Infect Dis Med Microbiol **17**(5): 307-14.

Rossin, R., S. Muro, M. J. Welch, V. R. Muzykantov and D. P. Schuster (2008). "In vivo imaging of 64Cu-labeled polymer nanoparticles targeted to the lung endothelium." J Nucl Med **49**(1): 103-11.

Sakamoto, Y., G. Yamada, M. Mizuno, T. Nishihara, S. Kinoyama, T. Kobayashi, T. Takahashi and H. Nagashima (1983). "Full and empty particles of hepatitis B virus in hepatocytes from patients with HBsAg-positive chronic active hepatitis." Lab Invest **48**(6): 678-82.

Sangfelt, P., I. Uhnoo, A. Hollander, G. Lindh and O. Weiland (2002). "Lamivudine and famciclovir combination therapy with or without addition of interferon-alpha-2b for HBeAg-positive chronic hepatitis B: a pilot study." Scand J Infect Dis **34**(7): 505-11.

Sasadeusz, J. J., S. L. Locarnini and G. Macdonald (2007). "Why do we not yet have combination chemotherapy for chronic hepatitis B?" Med J Aust **186**(4): 204-6.

Schildgen, O., M. Fiedler, U. Dahmen, J. Li, B. Lohrengel, M. Lu and M. Roggendorf (2006). "Fluctuation of the cytokine expression in the liver during the chronic woodchuck hepatitis virus (WHV) infection is not related to viral load." Immunol Lett **102**(1): 31-7.

Schreiber, A., G. Harter, A. Schubert, D. Bunjes, T. Mertens and D. Michel (2009). "Antiviral treatment of cytomegalovirus infection and resistant strains." Expert Opin Pharmacother **10**(2): 191-209.

Schultz, J. R., F. R. Nichol, G. L. Elfring and S. D. Weed (1973). "Multiple-stage procedures for drug screening." Biometrics **29**(2): 293-300.

Schultz, U., E. Grgacic and M. Nassal (2004). "Duck hepatitis B virus: an invaluable model system for HBV infection." Adv Virus Res **63**: 1-70.

Schultz, U., J. Summers, P. Staeheli and F. V. Chisari (1999). "Elimination of duck hepatitis B virus RNA-containing capsids in duck interferon-alpha-treated hepatocytes." J Virol **73**(7): 5459-65.

Scully, L. J., D. Brown, C. Lloyd, R. Shein and H. C. Thomas (1990). "Immunological studies before and during interferon therapy in chronic HBV infection: identification of factors predicting response." Hepatology **12**(5): 1111-7.

Seetharam, A. and M. Lisker-Melman (2009). "Treatment of chronic hepatitis B: are we ready for combination therapy?" Curr Gastroenterol Rep **11**(1): 22-7.

Seigneres, B., P. Martin, B. Werle, O. Schorr, C. Jamard, L. Rimsky, C. Trepo and F. Zoulim (2003). "Effects of pyrimidine and purine analog combinations in the duck hepatitis B virus infection model." Antimicrob Agents Chemother **47**(6): 1842-52.

Shaw, T., P. Amor, G. Civitico, M. Boyd and S. Locarnini (1994). "In vitro antiviral activity of penciclovir, a novel purine nucleoside, against duck hepatitis B virus." Antimicrob Agents Chemother **38**(4): 719-23.

Shen, W., M. Waldschmidt, X. Zhao, T. Ratliff and A. M. Krieg (2002). "Antitumor mechanisms of oligodeoxynucleotides with CpG and polyG motifs in murine prostate cancer cells: decrease of NF-kappaB and AP-1 binding activities and induction of apoptosis." Antisense Nucleic Acid Drug Dev **12**(3): 155-64.

Skrastina, D., A. Bulavaite, I. Sominskaya, L. Kovalevska, V. Ose, D. Priede, P. Pumpens and K. Sasnauskas (2008). "High immunogenicity of a hydrophilic component of the hepatitis B virus preS1 sequence exposed on the surface of three virus-like particle carriers." Vaccine **26**(16): 1972-81.

Soni, P. N., D. Brown, R. Saffie, K. Savage, D. Moore, G. Gregoriadis and G. M. Dusheiko (1998). "Biodistribution, stability, and antiviral efficacy of liposome-entrapped phosphorothioate antisense oligodeoxynucleotides in ducks for the treatment of chronic duck hepatitis B virus infection." Hepatology **28**(5): 1402-10.

Soriano, V., P. Barreiro and M. Nunez (2006). "Management of chronic hepatitis B and C in HIV-coinfected patients." J Antimicrob Chemother **57**(5): 815-8.

Soriano, V., M. Puoti, M. Bonacini, G. Brook, A. Cargnel, J. Rockstroh, C. Thio and Y. Benhamou (2005). "Care of patients with chronic hepatitis B and HIV co-infection: recommendations from an HIV-HBV International Panel." Aids **19**(3): 221-40.

Soriano, V., E. Vispo, L. Martin-Carbonero, P. Labarga, J. Garcia-Samaniego and P. Barreiro (2007). "Management and therapy of chronic hepatitis C in HIV." Curr Opin HIV AIDS **2**(6): 482-8.

Sprengel, R., C. Kuhn, H. Will and H. Schaller (1985). "Comparative sequence analysis of duck and human hepatitis B virus genomes." J Med Virol **15**(4): 323-33.

Sprengers, D. and H. L. Janssen (2005). "Immunomodulatory therapy for chronic hepatitis B virus infection." Fundam Clin Pharmacol **19**(1): 17-26.

Sprengers, D. and H. L. Janssen (2005). "Immunomodulatory therapy for chronic hepatitis B virus infection in children." Fundam Clin Pharmacol **19**(4): 447.

Summers, J., A. R. Jilbert, W. Yang, C. E. Aldrich, J. Saputelli, S. Litwin, E. Toll and W. S. Mason (2003). "Hepatocyte turnover during resolution of a transient hepadnaviral infection." Proc Natl Acad Sci U S A **100**(20): 11652-9.

Suzuki, F., J. Toyoda, Y. Katano, M. Sata, M. Moriyama, F. Imazeki, M. Kage, T. Seriu, M. Omata and H. Kumada (2008). "Efficacy and safety of entecavir in lamivudine-refractory patients with chronic hepatitis B: randomized controlled trial in Japanese patients." J Gastroenterol Hepatol **23**(9): 1320-6.

Takehara, T., T. Suzuki, K. Ohkawa, A. Hosui, M. Jinushi, T. Miyagi, T. Tatsumi, Y. Kanazawa and N. Hayashi (2006). "Viral covalently closed circular DNA in a non-transgenic mouse model for chronic hepatitis B virus replication." J Hepatol **44**(2): 267-74.

Tang, H. and A. McLachlan (2002). "A pregenomic RNA sequence adjacent to DR1 and complementary to epsilon influences hepatitis B virus replication efficiency." Virology **303**(1): 199-210.

Tang, T. J., J. Kwekkeboom, S. Mancham, R. S. Binda, R. A. de Man, S. W. Schalm, J. G. Kusters and H. L. Janssen (2005). "Intrahepatic CD8+ T-lymphocyte response is important for therapy-induced viral clearance in chronic hepatitis B infection." J Hepatol **43**(1): 45-52.

Thermet, A., T. Buronfosse, B. Werle-Lapostolle, M. Chevallier, P. Pradat, C. Trepo, F. Zoulim and L. Cova (2008). "DNA vaccination in combination or not with lamivudine treatment breaks humoral immune tolerance and enhances cccDNA clearance in the duck model of chronic hepatitis B virus infection." J Gen Virol **89**(Pt 5): 1192-201.

Thimme, R., S. Wieland, C. Steiger, J. Ghrayeb, K. A. Reimann, R. H. Purcell and F. V. Chisari (2003). "CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection." J Virol **77**(1): 68-76.

Thio, C. L. and S. Locarnini (2007). "Treatment of HIV/HBV coinfection: clinical and virologic issues." AIDS Rev **9**(1): 40-53.

Thomas, M., R. L. White and R. W. Davis (1976). "Hybridization of RNA to double-stranded DNA: formation of R-loops." Proc Natl Acad Sci U S A **73**(7): 2294-8.

Thorpe, M. (2007). Studies on the survival of duck hepatitis B virus cccDNA through hepatocyte mitosis *in vivo*. Microbiology and Immunology. Adelaide, The University of Adelaide**:** 48.

Tolcher, A. W., J. Kuhn, G. Schwartz, A. Patnaik, L. A. Hammond, I. Thompson, H. Fingert, D. Bushnell, S. Malik, J. Kreisberg, E. Izbicka, L. Smetzer and E. K. Rowinsky (2004). "A Phase I pharmacokinetic and biological correlative study of oblimersen sodium (genasense, g3139), an antisense oligonucleotide to the bcl-2 mRNA, and of docetaxel in patients with hormone-refractory prostate cancer." Clin Cancer Res **10**(15): 5048-57.

Treichel, U., K. H. Meyer zum Buschenfelde, H. P. Dienes and G. Gerken (1997). "Receptor-mediated entry of hepatitis B virus particles into liver cells." Arch Virol **142**(3): 493-8.

Triyatni, M., P. Ey, T. Tran, M. Le Mire, M. Qiao, C. Burrell and A. Jilbert (2001). "Sequence comparison of an Australian duck hepatitis B virus strain with other avian hepadnaviruses." J Gen Virol **82**(Pt 2): 373-8.

Triyatni, M., A. R. Jilbert, M. Qiao, D. S. Miller and C. J. Burrell (1998). "Protective efficacy of DNA vaccines against duck hepatitis B virus infection." J Virol **72**(1): 84-94.

Tseng, Y. P., Y. H. Kuo, C. P. Hu, K. S. Jeng, D. Janmanchi, C. H. Lin, C. K. Chou and S. F. Yeh (2008). "The role of helioxanthin in inhibiting human hepatitis B viral replication and gene expression by interfering with the host transcriptional machinery of viral promoters." Antiviral Res **77**(3): 206-14.

Vaillant, A., J. M. Juteau, H. Lu, S. Liu, C. Lackman-Smith, R. Ptak and S. Jiang (2006). "Phosphorothioate oligonucleotides inhibit human immunodeficiency virus type 1 fusion by blocking gp41 core formation." Antimicrob Agents Chemother **50**(4): 1393-401.

Valsamakis, A. (2007). "Molecular testing in the diagnosis and management of chronic hepatitis B." Clin Microbiol Rev **20**(3): 426-39.

Van Dyke, R. B., L. Wang and P. L. Williams (2008). "Toxicities associated with dual nucleoside reverse-transcriptase inhibitor regimens in HIV-infected children." J Infect Dis **198**(11): 1599-608.

Vickery, K., R. Tohidi-Esfahani, J. Pouliopoulos, R. Welschinger, R. Dixon, A. Deva and Y. Cossart (2006). "The effect of surgical immunomodulation on liver inflammation and clearance of DHBV infection." J Med Virol **78**(12): 1572-8.

Villeneuve, J. P. (2005). "The natural history of chronic hepatitis B virus infection." J Clin Virol **34 Suppl 1**: S139-42.

Visvanathan, K., N. A. Skinner, A. J. Thompson, S. M. Riordan, V. Sozzi, R. Edwards, S. Rodgers, J. Kurtovic, J. Chang, S. Lewin, P. Desmond and S. Locarnini (2007). "Regulation of Toll-like receptor-2 expression in chronic hepatitis B by the precore protein." Hepatology **45**(1): 102-10.

Wang, D., L. Bhagat, D. Yu, F. G. Zhu, J. X. Tang, E. R. Kandimalla and S. Agrawal (2008). "Oligodeoxyribonucleotide-Based Antagonists for Toll-Like Receptors 7 and 9." J Med Chem.

Wang, Y., S. Menne, B. H. Baldwin, B. C. Tennant, J. L. Gerin and P. J. Cote (2004). "Kinetics of viremia and acute liver injury in relation to outcome of neonatal woodchuck hepatitis virus infection." J Med Virol **72**(3): 406-15.

Waxman, L. and P. L. Darke (2000). "The herpesvirus proteases as targets for antiviral chemotherapy." Antivir Chem Chemother **11**(1): 1-22.

Weber, F., G. Kochs and O. Haller (2004). "Inverse interference: how viruses fight the interferon system." Viral Immunol **17**(4): 498-515.

Wilson, H. L., A. Dar, S. K. Napper, A. Marianela Lopez, L. A. Babiuk and G. K. Mutwiri (2006). "Immune mechanisms and therapeutic potential of CpG oligodeoxynucleotides." Int Rev Immunol **25**(3-4): 183-213.

Wong, T., M. A. Chiasson, A. Reggy, R. J. Simonds, J. Heffess and V. Loo (2000). "Antiretroviral therapy and declining AIDS mortality in New York City." J Urban Health **77**(3): 492-500.

Yadav, M., T. Mishra, P. Singhal, S. Goswami and P. S. Shrivastav (2009). "Rapid and specific liquid chromatographic tandem mass spectrometric determination of tenofovir in human plasma and its fragmentation study." J Chromatogr Sci **47**(2): 140-8.

Yang, P. L., A. Althage, J. Chung and F. V. Chisari (2002). "Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection." Proc Natl Acad Sci U S A **99**(21): 13825-30.

Yim, H. J., M. Hussain, Y. Liu, S. N. Wong, S. K. Fung and A. S. Lok (2006). "Evolution of multi-drug resistant hepatitis B virus during sequential therapy." Hepatology **44**(3): 703-12.

Younger, H. M., A. J. Bathgate and P. C. Hayes (2004). "Review article: Nucleoside analogues for the treatment of chronic hepatitis B." Aliment Pharmacol Ther **20**(11-12): 1211-30.

Yu, R. Z., R. S. Geary, J. D. Flaim, G. C. Riley, D. L. Tribble, A. A. Vanvliet and M. K. Wedel (2009). "Lack of Pharmacokinetic Interaction of Mipomersen Sodium (ISIS 301012), a 2'-O-Methoxyethyl Modified Antisense Oligonucleotide Targeting Apolipoprotein B-100 Messenger RNA, with Simvastatin and Ezetimibe." Clin Pharmacokinet **48**(1): 39-50 (a).

Yu, R. Z., T. W. Kim, A. Hong, T. A. Watanabe, H. J. Gaus and R. S. Geary (2007). "Cross-species pharmacokinetic comparison from mouse to man of a second-generation antisense oligonucleotide, ISIS 301012, targeting human apolipoprotein B-100." Drug Metab Dispos **35**(3): 460-8.

Yu, R. Z., K. M. Lemonidis, M. J. Graham, J. E. Matson, R. M. Crooke, D. L. Tribble, M. K. Wedel, A. A. Levin and R. S. Geary (2009). "Cross-species comparison of in vivo PK/PD relationships for second-generation antisense oligonucleotides targeting apolipoprotein B-100." Biochem Pharmacol **77**(5): 910-9 (b).

Yuen, M. F. and C. L. Lai (2008). The natural histroy of chronic hepatitis B. Hepatitis B virus. S. Locarnini and C. L. Lai. London, Atlanta, Singapore, International Medical Press. **1:** 12.1-12.11.

Zhao, Q., J. T. Ernst, A. D. Hamilton, A. K. Debnath and S. Jiang (2002). "XTT formazan widely used to detect cell viability inhibits HIV type 1 infection in vitro by targeting gp41." AIDS Res Hum Retroviruses **18**(14): 989-97.

Zhou, T., H. Guo, J. T. Guo, A. Cuconati, A. Mehta and T. M. Block (2006). "Hepatitis B virus e antigen production is dependent upon covalently closed circular (ccc) DNA in HepAD38 cell cultures and may serve as a cccDNA surrogate in antiviral screening assays." Antiviral Res **72**(2): 116-24.

Zhu, Y., M. Curtis, X. Qi, M. D. Miller and K. Borroto-Esoda (2009). "Anti-hepatitis B virus activity in vitro of combinations of tenofovir with nucleoside/nucleotide analogues." Antivir Chem Chemother **19**(4): 165-76.

Zoulim, F. (2004). "Antiviral therapy of chronic hepatitis B: can we clear the virus and prevent drug resistance?" Antivir Chem Chemother **15**(6): 299-305.

Zoulim, F. (2005). "Combination of nucleoside analogues in the treatment of chronic hepatitis B virus infection: lesson from experimental models." J Antimicrob Chemother **55**(5): 608-11.

Zoulim, F. and R. Perrillo (2008). "Hepatitis B: reflections on the current approach to antiviral therapy." J Hepatol **48 Suppl 1**: S2-19.

Zoulim, F., F. Saadi, T. Buronfosse, F. Abdul and L. Cova (2008). Animal models for the study of infection. Hepatitis B virus. S. Locarnini and C. L. Lai. London, Atlanta, Singapore, International Medical Press. **1:** 6.1-6.20.