Development of antiviral therapies for chronic hepatitis B virus infection

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To my mother and father

for giving me the opportunity to be who I am and for your boundless love, care and support

&

To my brothers and sisters

for your love, care and for being such a strength
Publications and presentations resulting from this thesis

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Abstract

Acute hepatitis B virus (HBV) infection is self-limiting but leaves a residual infection that can become active in an individual under conditions of immunosuppression. In chronic HBV infection, the virus persistently replicates in hepatocytes and this leads to immune mediated hepatocyte damage. Chronic HBV infection, which occurs worldwide in more than 400 million people, is associated with liver disease, fibrosis, cirrhosis and hepatocellular carcinoma (HCC) (Hepatitis B Fact Sheet 2009). There is a significant need for treatment intervention in chronic HBV infection.

Despite the inability to remove the virus in more than 70% of patients, current treatments for chronic HBV infection, which include interferon alpha (IFN-α) and antiviral nucleotide/nucleoside analogues (NAs), aim to reduce levels of viral replication and to prevent or at least delay the progression of disease and the development of cirrhosis and HCC. Current NA therapy involves monotherapy with a conventional NA as a single antiviral agent (Sasadeusz et al. 2007). In the recent past, poor response to monotherapies with NAs and adverse effects to IFN-α have stimulated research into novel therapeutic strategies and enhancing the efficacy of existing NA therapy.

The duck HBV (DHBV) in its natural host, the Pekin duck (Anas domesticus platyrhynchos), has been used as an animal model to study treatment outcomes and antiviral studies at the pre-clinical level. Much of what is known about viral replication and outcomes of hepadnavirus infection has been discovered using the DHBV model (Schultz et al. 2004; Zoulim et al. 2008) and several immunotherapeutic and antiviral studies have been performed recently in our laboratory (Foster et al. 2003; Miller et al. 2004; Foster et al. 2005; Miller et al. 2006a; Miller et al. 2006b; Miller et al. 2008).

The studies described in this Ph.D. thesis focused on the development and testing of novel therapies for chronic HBV infection using the DHBV model. The first approach involved the use of novel amphipathic DNA polymers (APDPs) developed by REPLICor Inc. The second approach tested a combination of NAs developed by Gilead Sciences Pty. Ltd.
APDPs developed by REPLICor Inc. have been used as a novel therapeutic approach against human immunodeficiency virus type 1 (HIV-1) and have been shown to inhibit HIV-1-mediated membrane fusion and HIV-1 replication in a size dependent but sequence independent manner (Vaillant et al. 2006). HIV-1 entry is well characterised and involves fusion of the virus to its target cells using a type 1 fusion protein. APDPs are thought to inhibit HIV-1 infection by acting as fusion inhibitors that bind to the V3 loop of the HIV-1 gp41 domain preventing its interaction with the T cell receptor, CD4.

Phosphorothioation of oligonucleotides increases their hydrophobicity (amphipathicity) and also makes them more resistant to degradation by nucleases. The amphipathicity of APDPs plays a major role in their antiviral activity. Longer APDPs with lengths of ≥30 bases have a greater amphipathicity and were shown to be more potent in blocking the amphipathic interactions involved in the HIV-1 mediated membrane fusion than shorter APDPs with lengths of <30 bases (Vaillant et al. 2006). This novel antiviral mechanism of action of APDPs with ≥30 bases has future applications for therapy against infection with HIV-1 and other enveloped viruses.

In contrast to HIV, the entry mechanisms used by HBV are not well understood although HBV is thought to enter through receptor mediated endocytosis (RME). In RME fusion occurs between the virus and the cell membrane as a late event. We hypothesise that the late fusion of HBV with the cell membrane can be blocked by APDPs that are amphipathic.

As a first step in evaluating APDPs as a novel treatment for chronic HBV infection, the APDPs, REP 2006 and REP 2031 and a non-APDP, REP 2086, were tested in primary duck hepatocytes (PDH) for cytotoxicity and antiviral activity. REP 2006, a 40mer PS-ON with a completely degenerate sequence (random ATCG), REP 2031, a 40 mer PS-ON with a poly C sequence, and the non-APDP control, REP 2086, were all found to be non-cytotoxic in PDH. Treatment of PDH with REP 2006 inhibited DHBV infection at concentrations as low as 0.01 µM, while REP 2031 had a lower anti-DHBV activity. The antiviral activity of both APDPs, REP 2006 and 2031, was also found to be length and chemistry dependent and sequence independent.
Studies were then conducted to test the antiviral efficacy of REP 2006 and REP 2031 \textit{in vivo} using 14-day-old ducks infected with $5 \times 10^8$ DHBV DNA genomes. Ducks in 4 Groups were treated with either REP 2006 or REP 2031 or the Bristol-Myers Squibb NA, entecavir (ETV), or normal saline (NS) starting from 1 day prior to DHBV infection for 15 days. REP 2006 showed an excellent anti-DHBV activity but treatment cause some side effects. In contrast, treatment of ducks with REP 2031 was well tolerated. However, REP 2031 again showed less anti-DHBV activity than REP 2006. We hypothesised that the increased side effects in the REP 2006-treated ducks were due to CpG motifs present in the random ATCG sequence (Krieg 2000; Agrawal and Kandimalla 2001; Shen \textit{et al.} 2002; Isogawa \textit{et al.} 2005; Wilson \textit{et al.} 2006; Plitas \textit{et al.} 2008; Wang \textit{et al.} 2008). The lack of side effects with REP 2031 (which has no CpG motifs) was consistent with this hypothesis. The reason for the lower antiviral activity of REP 2031 is unclear. The subsequent testing of REP 2055 (a 40mer PS-ON with a poly AC sequence), which has no CpG motifs and also has an interrupted C nucleotide composition, showed an excellent recovery of antiviral activity without any observable side effects.

The antiviral efficacy of REP 2055 was tested \textit{in vivo} followed by dose optimisation studies using a range of dose regimens (0.5, 2, 3, 5 and 10 mg/kg). REP 2055 demonstrated excellent anti-DHBV activity with a dose as low as 2 mg/kg body weight.

The ability of REP 2055 to prevent the rebound of DHBV infection was next tested. Treatment with REP 2055 for 14 days prevented the rebound of DHBV infection after the cessation of treatment. This effect was observed if REP 2055 treatment was initiated one day prior to, or at an early phase (4 days p.i.) of DHBV infection, and continued for 14 days. In these 2 Groups, 4 out of 5 ducks were protected from the rebound of DHBV infection.

The therapeutic efficacy and the ability of REP 2055 to prevent the rebound of DHBV infection were then tested. REP 2055 treatment (10 mg/kg) was started at a late stage of DHBV infection when the liver was fully infected and treatment was continued for 28 days. A control Group of DHBV-infected ducks treated with NS was monitored for comparison. Liver enzymes and a complete blood evaluation (CBE) were performed prior to, during, at treatment endpoint and at the end of follow up, 16 weeks after the cessation of treatment. The results showed that 56% of ducks treated with REP 2055 were protected.
from rebound of DHBV infection and had developed an anti-DHBV surface antibody response, suggesting that they had resolved DHBV their infection. We concluded from this work that the APDP REP 2055 showed excellent anti-DHBV activity and has the ability to prevent the rebound of DHBV infection, making it suitable for further evaluation and possible clinical trials for the treatment of chronic HBV infection in humans.

Although the need for combination NA therapy has been suggested by many as a way to combat the development of antiviral resistance, very few studies have investigated the effectiveness of combination therapy using NAs. In a pre-clinical study using HepG2 hepatoma cells, the additive effect of adefovir (AFV) with ETV, emtricitabine (FTC), lamivudine (3TC) and telbivudine (TLB) has been reported (Delaney et al. 2004). AFV with all other NAs in dual combination provided an additive effect. AFV and 3TC combined had a better additive effect than other combinations (Delaney et al. 2004). We hypothesised that the combination of either tenofovir (TFV) or tenofovir disoproxil fumarate (TDF) and FTC is more likely to have a better therapeutic efficacy against chronic DHBV infection than either TDF or FTC alone.

As a first step the pharmacokinetics (PK) of TFV and TDF were investigated. This was followed by testing of the antiviral efficacy of TFV and FTC alone and in combination in ducks with persistent DHBV infection. PK studies of TFV and TDF showed that TFV has a half-life of 6 h when administered via the IP route whereas TDF had a half life of 4 h and required twice daily administration. TFV was chosen for the study for practical reasons of once daily administration. Next persistently DHBV infected ducks were treated daily with IP administration of 5 or 25 or 50 mg/kg of TFV or 100 or 200 mg/kg of FTC. The study showed that 5, 25 and 50 mg/kg of TFV suppressed serum levels of DHBV DNA by 3-logs compared to untreated ducks. FTC showed a 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.

In the next experiment, two different combinations of TFV and FTC were tested. The combination of 5 mg/kg TFV + 200 mg/kg FTC was able to suppress 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.
In conclusion, a combination of TFV and FTC was superior to either of these drugs alone in suppressing serum DHBV DNA levels in ducks with chronic DHBV infection. Further studies are warranted to test the ability of combinations of TFV and FTC to prevent the rebound of DHBV infection.
Declaration

NAME: Faseeha Noordeen               PROGRAM: Doctor of Philosophy (Ph.D.)

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>amino acid/s</td>
</tr>
<tr>
<td>AFV</td>
<td>adefovir</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine amino transference</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cells</td>
</tr>
<tr>
<td>APDP</td>
<td>amphipathic DNA polymer</td>
</tr>
<tr>
<td>ASHV</td>
<td>Arctic squirrel hepatitis virus</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>CBE</td>
<td>complete blood evaluation</td>
</tr>
<tr>
<td>cccDNA</td>
<td>covalently closed circular DNA</td>
</tr>
<tr>
<td>CDC</td>
<td>Centre for Disease Control</td>
</tr>
<tr>
<td>CHB</td>
<td>chronically HBV infected</td>
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<td>CHBV</td>
<td>crane hepatitis B virus</td>
</tr>
<tr>
<td>CMI</td>
<td>cell mediated immune response</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalo virus</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocytes</td>
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<tr>
<td>CV</td>
<td>cumulative variance</td>
</tr>
<tr>
<td>DHBV</td>
<td>duck hepatitis B virus</td>
</tr>
<tr>
<td>DHBcAg</td>
<td>duck hepatitis B virus core antigen</td>
</tr>
<tr>
<td>DHBeAg</td>
<td>duck hepatitis B virus e antigen</td>
</tr>
<tr>
<td>DHBsAg</td>
<td>duck hepatitis B virus surface antigen</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DR1</td>
<td>direct repeat 1</td>
</tr>
<tr>
<td>DR2</td>
<td>direct repeat 2</td>
</tr>
<tr>
<td>DW</td>
<td>distilled water</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene-diamine-tetra-acetic-acid disodium salt</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ETV</td>
<td>entecavir</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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FCS  foetal calf serum
FTC  emtricitabine
GGT  gamma glutamyl transferase
GMP  good manufacturing practice
GSHV  ground squirrel hepatitis virus
HBcAg  hepatitis B virus core antigen
HBeAg  hepatitis B virus e antigen
HBsAg  hepatitis B virus surface antigen
HBV  hepatitis B virus
HBV DNA  hepatitis B virus DNA
HCC  hepatocellular carcinoma
HCV  hepatitis C virus
HHBV  heron hepatitis B virus
HHV 6  human herpes virus 6
HIV  human immunodeficiency virus
HSV  herpes simplex virus
IFN-α  interferon alpha
IFN-β  interferon beta
IL-2  interleukin-2
IP  intraperitoneally
IMVS  Institute of Medical and Veterinary Science
IV  intravenously
L-FMAU  clevudine
MHC  major histocompatibility complex
MOI  multiplicity of infection
mRNA  messenger RNA
NA/s  nucleot/side analogue/s
ND  not detected
NDS  normal duck serum
NK  natural killer cells
NS  normal saline
NSS  normal sheep serum
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>O/N</td>
<td>overnight</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OPD</td>
<td>o-phenylenediamine</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PAMPs</td>
<td>pathogen specific molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDH</td>
<td>primary duck hepatocytes</td>
</tr>
<tr>
<td>PegIFN-α</td>
<td>pegylated interferon alpha</td>
</tr>
<tr>
<td>PEN</td>
<td>penciclovir</td>
</tr>
<tr>
<td>pgRNA</td>
<td>pregenomic RNA</td>
</tr>
<tr>
<td>PHH</td>
<td>primary human hepatocytes</td>
</tr>
<tr>
<td>PK</td>
<td>pharmacokinetics</td>
</tr>
<tr>
<td>Pol</td>
<td>polymerase</td>
</tr>
<tr>
<td>PolyA</td>
<td>polyadenylene signal</td>
</tr>
<tr>
<td>pre-S/S</td>
<td>small and large envelope proteins</td>
</tr>
<tr>
<td>PS-ON/s</td>
<td>phosphorothioated oligodeoxynucleotide/s</td>
</tr>
<tr>
<td>rcDNA</td>
<td>relaxed circular DNA</td>
</tr>
<tr>
<td>RGHBV</td>
<td>Ross goose hepatitis B virus</td>
</tr>
<tr>
<td>RME</td>
<td>receptor mediated endocytosis</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDH</td>
<td>sorbitol dehydrogenase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SGHBV</td>
<td>snow goose hepatitis B virus</td>
</tr>
<tr>
<td>SS</td>
<td>sodium salts</td>
</tr>
<tr>
<td>STHBV</td>
<td>stork hepatitis B virus</td>
</tr>
<tr>
<td>SVR</td>
<td>sustained virological response</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate EDTA buffer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TDF</td>
<td>tenofovir disoproxil fumarate</td>
</tr>
<tr>
<td>TFV</td>
<td>tenofovir</td>
</tr>
<tr>
<td>TLB</td>
<td>telbivudine</td>
</tr>
<tr>
<td>3TC</td>
<td>lamivudine</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>Tyr-63</td>
<td>tyrosine residue in 63rd position</td>
</tr>
<tr>
<td>Tyr-96</td>
<td>tyrosine residue in 96th position</td>
</tr>
<tr>
<td>UC</td>
<td>untreated control</td>
</tr>
<tr>
<td>VLP</td>
<td>virus-like particles</td>
</tr>
<tr>
<td>VZV</td>
<td>varicella zoster virus</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WHV</td>
<td>woodchuck hepatitis virus</td>
</tr>
<tr>
<td>WMHBV</td>
<td>woolly monkey hepatitis B virus</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>ε</td>
<td>stem looped encapsidation signal</td>
</tr>
<tr>
<td>μg</td>
<td>microgram/s</td>
</tr>
<tr>
<td>μL/μl</td>
<td>microlitre/s</td>
</tr>
<tr>
<td>μm</td>
<td>micrometre/s</td>
</tr>
<tr>
<td>μM</td>
<td>micromoles</td>
</tr>
<tr>
<td>g</td>
<td>relative centrifugal force</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>gL⁻¹</td>
<td>grams per litre</td>
</tr>
<tr>
<td>h</td>
<td>hour/s</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>sulfuric acid</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase/s</td>
</tr>
<tr>
<td>kDA</td>
<td>kilodalton/s</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram/s</td>
</tr>
<tr>
<td>L</td>
<td>litre/s</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>lethal dose to 50% of the population</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>--------</td>
<td>-----------------</td>
</tr>
<tr>
<td>mg</td>
<td>milligram/s</td>
</tr>
<tr>
<td>min</td>
<td>minute/s</td>
</tr>
<tr>
<td>mL</td>
<td>millilitre/s</td>
</tr>
<tr>
<td>mM</td>
<td>millimoles</td>
</tr>
<tr>
<td>ng</td>
<td>nanograms</td>
</tr>
<tr>
<td>nm</td>
<td>nanometres</td>
</tr>
</tbody>
</table>
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