University of Alberta

The anti-HBV studies of a novel series of acyclic pyrimidine nucleoside analogs.

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the

requirements for the degree of Master of Science

in

Experimental Surgery

Department of *Surgery*

Edmonton, Alberta

Fall 2005

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I would like to dedicate this to my family (Dad, Salim, Soraya, Imane, and Amale), and friends who were always very supportive. But most importantly this thesis is dedicated to my mom, Fatna (aka Nina), who has always been an inspiration and one of my greatest teachers in life.

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Abstract

Presently, there are over 350 million chronic carriers of the hepatitis B virus (HBV). Chronic HBV infections lead to cirrhosis, liver failure, and hepatocellular carcinoma. Currently, lamivudine and adefovir are the most effective treatments for chronic HBV. However, viral rebound after the cessation of therapy and drug resistance upon continuous use are the two most significant limitations. Therefore, there is an increasing need for the development of novel antiviral treatments to augment the therapy, to avoid the emergence of drug resistance, or to use against the already established mutant forms. The aim of this project is to investigate two novel series of acyclic pyrimidine nucleoside analogs possessing various C-5 and/or C-6 substituents on the base moiety. It is our objective to synthesize and determine their anti-HBV effects, and establish the structure activity relationships (SARs) of these compounds, in the hopes of providing a possible novel treatment option.

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Abbreviations

3-TC	(-)-2',3'-Dideoxy-3'-thiacytidine
Abacavir	[1S,4R]-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol
ACV	Acyclovir, 9-[(2-hydroxyethoxy)methyl]guanine
AIDS	Acquired immunodeficiency syndrome
ALT	Alanine aminotransferase
aq.	Aqueous
ASHV	Arctic squirrel hepatitis virus
Asp	Aspartic acid
AST	Aspartate aminotransferases
ATP	Adenosine triphosphate
ATPase	ATP hydrolysis enzyme
6-Azauracil	1,2,4-Triazine-3,5(2H,4H)-dione
bp	Base pairs
Br	Bromine
C	Carbon
°C	Degrees celsius
Ca^2	Calcium
CC_{50}	Cytotoxic concentration at which 50% of cells undergo cell death
cccDNA	Covalently closed circular DNA

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CH_3	Methyl
C_2H_5	Ethyl
$CHCl_3$	Chloroform
CH_2OH	Hydroxymethyl
Cl	Chlorine
CTL	Cytotoxic T lymphocyte
CO_2	Carbon dioxide
$COOCH_3$	Acetate
$COOC_2H_5$	Acetoxymethyl
DHBV	Duck hepatitis B virus
DHBsAg	Duck hepatitis B surface antigen
D-MEM	Dulbeco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DR1	Direct repeat 1
DR2	Direct repeat 2
EC_{50}	Effective concentration for a 50% reduction of viral replication
EDTA	Ethylenediamminetetraacetic Acid
EGTA	ethyleneglycol-bis (beta-aminoethylether)-N,N,N',N'-tetraacetic

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En1	Enhancer 1
En2	Enhancer 2
ER	Endoplasmic reticulum
EtOAc	Ethyl Acetate
FasL	Fas ligand
FBS	Fetal bovine serum
FHF	Fulminant hepatic failure
ft	Femtogram
FTC	(-)-ß-2',3'-dideoxy-5-fluoro-3'-thiacytidine
g	Gram
GCV	Gancyclovir, 9-[(2-hydroxy-1-hydroxymethyl)ethoxy)methyl]guanine
GSHV	Ground squirrel hepatitis B virus
H	Hydrogen
h	Hour
HAART	Highly active antiretroviral therapy
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBIG	Hepatitis B immune globulin
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid

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HCMV	Human Cytomegalovirus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMDS	Hexamethyldisilazane
HSV	Herpes simplex virus
I	Iodine
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
Ile	Isoleucine
IP	Intraperitoneal
kd	Kilodalton
KI	Potassium Iodide
kg	Kilogram
L-15	Leibovitz medium
L protein	Large protein
M	Molar
MEM	Eagle's minimum essential medium
MeOH	Methanol
Me_2SO	Dimethyl sulfoxide
Met	Methionine
mg	Milligram

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$MgCl_2$	Magnesium chloride
MHC	Major histocompatability complex
min	Minute
mL	Millilitre
mM	Millimolar
mmol	Millimole
mp	Melting point
M protein	Middle protein
mRNA	Messenger RNA
MTT	3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NDP	Nucleoside diphophate
NH_2	Amino group
NK	Natural killer cells
nm	Nanometre
NO_2	Nitro
NTP	Nucleotide triphosphate
O	Oxygen
OH	Hydroxy
ORF	Open reading frame

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PBS	Phosphate buffer solution
PEG	Polyethylene glycol
pg	Picogram
PKR	Protein kinase R
Pol	Polymerase
RBC	Red blood cell
rcDNA	Relaxed circular DNA
RNA	Ribonucleic acid
RNAse	Ribonuclease
Rpm	Revolutions per minute
RT	Reverse transcriptase
RT-PCR	Real-time polymerase chain reaction
SAR	Structure activity relationship
SDS	Sodium dodecyl sulfate
S Protein	Small/surface protein
Th	T helper lymphocyte
Thr	Threonine
TNF	Tumor necrosis factor
tRNA	Transfer RNA
Tyr	Tyrosine
µl	Microlitre
µg	Microgram
µm	Micrometre

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UV	Ultraviolet
Val	Valine
WHO	World Health Organization
WHV	Woodchuck hepatitis B virus
WMHBV	Wooly monkey hepatitis B virus

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Chapter One: Introduction.

1.1 Introduction.

One third of the world's population possesses serological evidence of a past or current hepatitis B viral (HBV) infection; this represents 2 billion people worldwide (1, 2). Of this group of people, the problem lies with the chronically infected patients. There are currently 350 million carriers of HBV, and this is despite the fact that there has been a preventative vaccine available since 1981 (3). It is this chronic HBV infection that can lead to severe liver conditions, such as cirrhosis, liver failure, and hepatocellular carcinoma (HCC) (4-7). For instance, a chronically infected person has 200-fold higher risk of developing HCC as compared to an uninfected person (8,9). In addition, liver disease is anticipated to develop in one fourth to one third of the population chronically infected with HBV (10). These liver diseases resulting from HBV infection account for over 1.2 million deaths worldwide annually (1,11). HBV virus infection results in a serious disease with a significant clinical impact. This introduction will provide a brief overview on many aspects of HBV including its classification, mode of transmission, geographic distribution, those at high risk for infection, clinical features, host immune response, genome structure, method of replication, and the current treatments available. The most common therapeutic options applied to treating chronic HBV infection include interferon, an immunomodulatory agent, and the antiviral nucleoside analogs, lamivudine (3-TC) and adefovir dipivoxil. The limitations on each of these current treatment options will be discussed, thus showing the necessity for new antiviral agents to combat HBVchronic infection.

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1.2 Hepadnaviridae family.

HBV is a DNA virus of the Hepadnaviridae family, the fact that it is a DNA virus is an exclusive feature of HBV amongst the hepatitis viruses (12). In addition, amongst animal viruses, hepadnaviruses consist of some of the smallest genome sizes (ranging from 3-3.3kb) (13). The Hepadnaviridae family includes multiple types of hepatitis B viruses, which infect different hosts. Some representative examples include human hepatitis B virus (HBV), which infects humans and chimpanzees. Duck hepatitis B virus (DHBV) infects Pekin ducks and geese, and other avian hepadnaviruses include heron hepatitis B virus and Ross's goose hepatitis virus (13-15). Eastern woodchucks are the natural hosts of woodchuck hepatitis B virus (WHV), and Beechey ground squirrels, woodchucks, and chipmunks can be infected by ground squirrel hepatitis B virus (GSHV) (14-18). A relatively new member of the Hepadnaviridae family is the arctic squirrel hepatitis virus (ASHV), which specifically infects arctic ground squirrels (13). The ASHV is similar to the GSHV and WHV, but is more closely related with the GSHV phylogentically (13). Another recent hepadnavirus isolated, came from the New World woolly monkeys and has been named WMHBV (19). WMHBV also has the potential to infect spider monkeys (19). The viruses that are able to infect mammals are categorized into the genus *Orthohepadnavirus*; this includes the woodchucks, ground squirrels, and humans. The genus *Avihepadnavirus* includes the duck hepatitis B virus, the heron hepatitis B virus and Ross's goose hepatitis virus (13,20).

The GSHV and WHV have a 70% sequence HBV homology and genome structure, and the DHBV shares similarities with HBV in terms of genome organization

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and means of replication (20). There is also limited cross reactivity between GSHV, WHV, and HBV surface antigen (13,16). Overall, virus morphology and structure, liver tropism, DNA, genomic organization and size, and replicative mode are common features amongst the different viral species (13,15-18,21-24). Within, the human HBV species, there are seven different genotypes, named a letter from A to G. The genotypes differ with respect to the different subdeterminants of the hepatitis B surface antigen (HBsAg). All genotypes have the same 'a'determinant (antibody target), but differ in either having a 'd' or 'y' and an 'r' or 'w' subdeterminant (25). These genotypes differ by nucleotide insertions or deletions accounting for a variance of approximately 8% across the whole genome. The different genotypes are associated with different pathogenesis (such as liver disease severity), and response profiles to antiviral therapy (26).

1.3 Mode of transmission.

Markers of an HBV infection include hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBcAg), and hepatitis B e antigen (HBeAg) (6). HBsAg is an envelope antigen, while HBcAg constitutes the viral nucleocapsid and is an intracellular particulate antigen, and HBeAg is a non-particulate peptide from the core gene that is secreted into serum (6,27,28). HBV virions may be present in blood serum, bodily fluid secretions such as semen and vaginal secretions, breast milk, saliva, and leukocytes. Thus, it can be transmitted through multiple routes including horizontally (parenterally, and non parenterally), and vertically (perinatally) (6,29-31). HBV infected serum typically has a high quantity of virions ranging from 10^8 to 10^{10} for very millilitre (6). As

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a result, one of the ways in which HBV enters the host is through a percutaneous route. This mode may include contaminated blood or blood product transfusions, needle stick injuries, health care equipment (insufficiently sterilized), and shared needles used by illicit drug users, tattooing, and acupuncture (1,32,33). A person who is injured with a needle previously used by an HBV infected individual with both HBsAg and HBeAg markers, has a 66% chance of being infected (29). Transfusions involving HBV contaminated blood is more of a problem in nations with a poorly developed healthcare infrastructure, and these individuals infected are not likely to be identified due to the lack of post transfusion follow ups (29,32). These individuals may then unknowingly continue to infect others in the population. A further problem is that HBV is stable and infectious for possibly greater than seven days on an environmental surface (1,34). As well in blood and blood products, HBV can remain stable for extended periods of time (32).

Sexual transmission is another method of transmission, which involves viral entry through the mucous membrane. There are many carriers who are unaware of their condition and continue infecting other sexual partners. Adults most often are infected with HBV through this route. Engaging in sexual activity that is considered high risk is based on the following factors: the number of partners, years of sexual activity, and a history of past sexually transmitted diseases. Homosexual men have been considered at a high risk due to the factors mentioned above (1,9,35). In a study done in Montreal in 2000 evaluating HBV prevalence amongst homosexual men, it was found that 41% of unvaccinated men had HBV markers (36). However, due to the spread of acquired immunodeficiency syndrome (AIDS) and AIDS awareness, homosexual transmission of

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HBV has been decreasing due to the same safety practices applied to prevent AIDS (29). In 1991, the most common route of HBV infection for North Americans was through sexual transmission, with 41% of cases of HBV infection attributed to heterosexual activity, and 14% due to homosexual activity. Thus, in North America most cases of HBV involved adults between the ages of 20-35 years old (37).

Sexual transmission may be a significant route for adults, but for infants the greatest risk results from perinatal transmission (2,38). Again, this is a result of exposure through the mucous membrane. Perinatal transmission may still ensue even if the mother is undergoing antiviral therapy and there is a significant reduction of HBV DNA levels (39). Typically, a mother infects her newborn postpartum, with a majority of cases occurring during delivery (40). In the Asia-Pacific region this is the most common route of HBV infection (38). Perinatal infection accounts for a minimum of 25% of persistent infections amongst the Asian adult population (35). The mothers who have the greatest risk of infecting their offspring are those that are HBeAg positive, since they have high viral titres, along with the presence of HBsAg markers (29,41,42). Furthermore, it has been proposed that *in utero*, HBeAg may provoke immunological (T cell) tolerance to HBV, thus leading to a chronic state of infection (27). Mothers who are HBeAg positive have a 70-90% chance of having an infant develop hepatitis B by six months of age, and 90% of these children remain chronically infected (43). Women who are HBeAg negative but are HBsAg positive have a 10 to 40% risk of infecting their offspring, and of these infants 40-70% develop a chronic infection (1). Mothers who are HBsAg negative, are considered at low risk for infecting their newborns (40). Intrauterine infection is a less frequent mode of transmission (27). However, it is the HBeAg that can be

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transmitted by intrauterine infection through either transplacental leakage or cell to cell transfer from the placenta, which can not be inhibited by the vaccine (43). Generally, the administration of HBV vaccination coupled with hepatitis B immune globulin (HBIG) is quite effective at preventing the mother to child transmission of HBV (44,45). If the newborn is administered the vaccination and HBIG within twelve hours after birth, there is only a 5-15 % risk of the newborn acquiring an HBV infection (46). The active immunization provided by the vaccination is in itself effective at blocking the transmission. However, the passive immunization mediated by the HBIG treatment, which features antibodies to HBV, can further decrease the risk of an HBV infection by eliciting an immediate immune response (44-46). In one study focusing on Chinese patients, when newborns of HBeAg mothers were given the vaccination and the HBIG treatment, 96-100% of the infants at one year of age were anti-HBs positive (47). The protection provided by the vaccination and HBIG treatment is invoked immediately and is long-lasting (48). HBV vaccinations are further discussed in a later section in the introduction (section 1.6). On another note, breastfeeding from a HBsAg carrier mother does not appear to be a valid route of HBV vertical transmission (49).

Transmission through saliva is another prospective route of infection. Although it has not been displayed in people, saliva transmission of HBV has been exhibited in chimpanzees (50). It has been postulated that the reason saliva is a limited mode of transmission, is because the saliva of an HBV carrier is only infective during the clinical phase of the disease. Neutralizing antibodies are produced early, locally and secreted with saliva, and that the quantity of HBsAg in saliva is inconsistent (31). Nonetheless, there is often a spread of hepatitis B among people in a nonsexual relationship in constant

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contact, such as in the case of family members of a chronically infected person. The possibilities may include broken skin or a mucous membrane exposed to blood, virions from inanimate objects, and/or saliva (1).

1.4 Prevalence of hepatitis B worldwide.

Areas with a high rate of infection include Southeast Asia, China, Philippines, Indonesia, Pacific Islands, Alaska, sub saharan Africa, Amazon Basin, the Caribbean, and some regions in the Middle East. These regions have an HBV prevalence greater than 8% of the population (4,6,10,35,51). These areas of high HBV prevalence, comprise 45% of the world's population (10). In these regions, serological evidence of a past hepatitis B infection exists in 70 to 90% of the population (1). In the Mediterranean basin, central South America, Central Asia, Southern and Eastern Europe, and Japan, the rate of prevalence is intermediate at 2-7% (4,10,35,51). These areas contain 43% of the world's population (10). There is low rate of infection associated with southern South America, Canada, United States, Western Europe, Australia, and New Zealand, where less than 2% of the population is affected by chronic HBV (4,6,10,35,51). In these countries the pervasiveness of HBV infection is strongly influenced by the immigration from regions with intermediate to high HBV endemicity (41).

The different prevalence rates are generally associated with typical modes of transmission. In the regions with high rates of HBV prevalence, HBV infections are acquired primarily through the perinatal route at infancy (4,6,10,51). In the regions of intermediate prevalence, the percutaneous route and early childhood infections are

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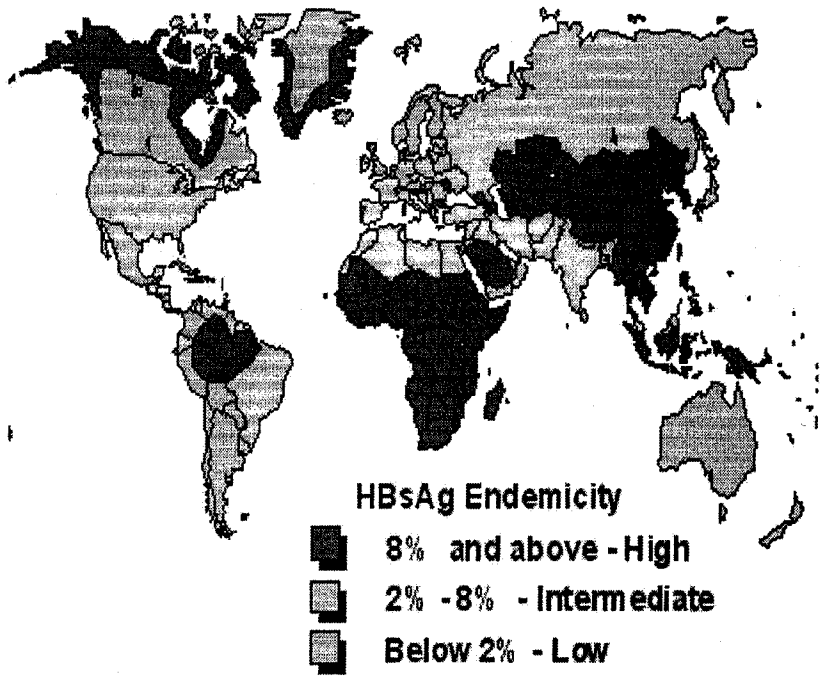

From: World Health Organization. Introduction of hepatitis B vaccine into childhood immunization services, Geneva, WHO, 2001 (unpublished document WHO/V&B/01.31; available on request from Department of Vaccines and Biologicals, World Health Organization, 1211 Geneva 27, Switzerland)[51]

Figure 1.1 Worldwide HBsAg endemicity. From ref. 52.

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responsible for most of hepatitis B transmission (4,46). Finally, in the regions of low prevalence, the infection is predominantly transmitted sexually and percutaneously, primarily as adults (4,10,41).

1.5 High-risk groups.

The high-risk groups for becoming HBV infected include those who travel to or are born in regions where there is a high prevalence of infection (4,35). Even in the United States where the prevalence of HBV is low, children born to immigrants from areas with a high occurrence of HBV have a 1-3% chance of being infected (53). Young infected children tend to be asymptomatic, and have a greater length of time to transmit and infect others in the population (53,54). Intravenous drug users, people in correctional facilities for long periods of time, homosexual men, and promiscuous heterosexuals are also considered high risk groups for HBV (4,9,35,53,55,56). Finally, health care and safety workers who are exposed to blood, family of mentally handicapped people in institutions and attendants of those people, patients who require frequent blood transfusions, and patients on hemodialyisis are also considered at a substantial risk for acquiring an HBV infection (4,53,55,56). An example of the distribution of the risk of an HBV in the United States is exemplified below in the pie chart.

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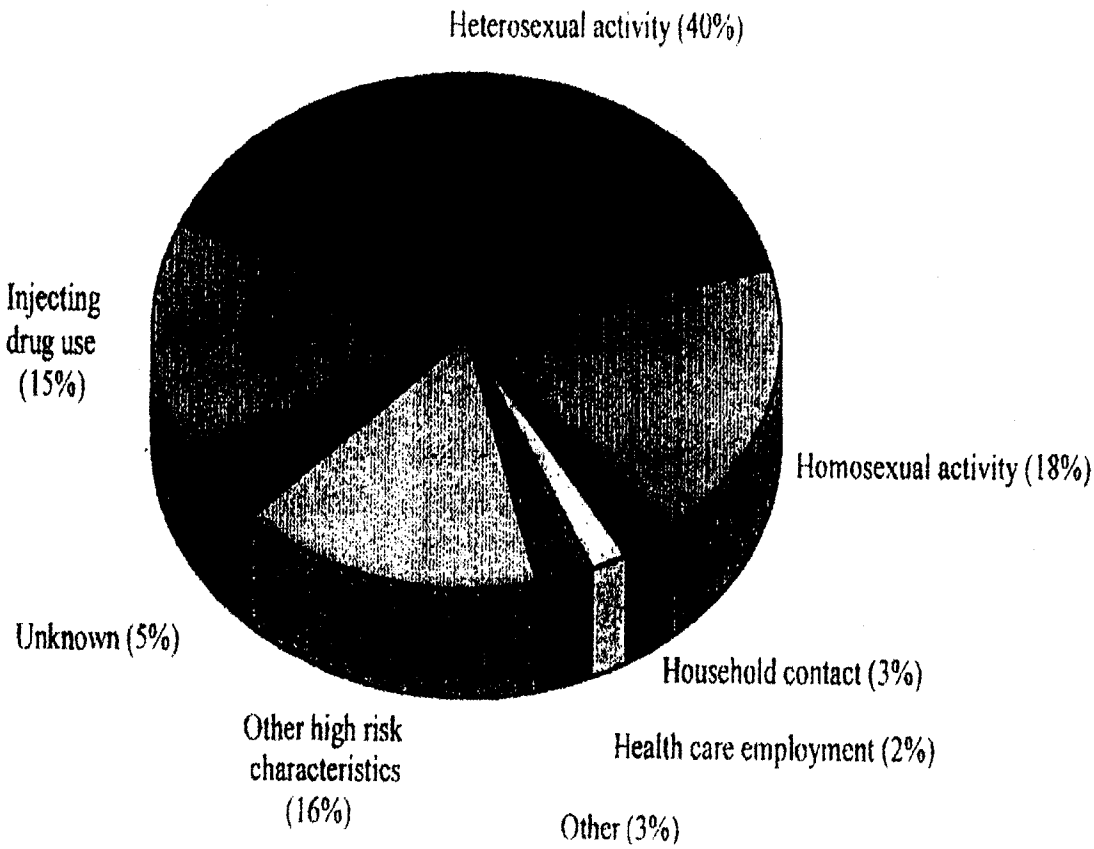

Figure 1.2 High-risk activities associated with acquiring an HBV infection. From ref. 53 (Original Source: Sentinel Countries Study of Viral Hepatitis, Centres for Disease Control and Prevention).

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1.6 Vaccine.

A preventative vaccine has been available since 1981, which utilizes HBsAg to elicit immunity against the virus (3,12,57). Current HBV vaccines feature HBsAg derived from yeast (*Saccharomyces cerevisae*) using recombinant DNA technology (12,57). However, vaccines were originally plasma derived, where the HBsAg was purified from human sera from asymptomatic chronic carriers of HBV (12,57,58). The plasma derived vaccines were not broadly used due to the supply limitations of human serum, high cost, insufficient quantity of chimpanzees for innocuity testing, and safety concerns and/or risk stigma of using human plasma (57-59). The yeast derived vaccines are pure, can be produced in high yields, and do not provoke any yeast hypersensitivity reactions (52,54). Furthermore, these vaccines are quite stable, being able to retain its functionality even after seven months at 37 ^{o}C (57).

Internationally, over 500 million people have been vaccinated (12). The vaccine is typically administered intramuscularly in three doses of 20 µg at zero, one, and six months (58,59). For children and neonates, 10 µg doses can be used (59). Anti-HBs antibodies levels of at least 10 mIU/mL indicate a protective state. Generally, as a result of the vaccination, infants and children have a seroprotection rate of at least 95%, and adults have rates of at least 90% (4,60). Overall, the HBV vaccination has proven quite effective (1,57,59,61). For instance in Taiwan, a mass HBV vaccination program has been utilized since July 1984, and it was deemed to be 84% effective in preventing chronic HBV infection (53). The related incidence of HCC in children was also studied (1,62). The number of chronically infected children under fifteen years of age was

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reduced by 93%, and subsequently the prevalence of HCC in children aged six to fourteen years old, decreased by 50% (1,62). As indicated earlier, the vaccination coupled with HBIG treatments is administered to newborns of HBV infected mothers to prevent the vertical transmission of HBV. This treatment is highly effective and if it is administered within twelve hours after birth, the risk of an infection is significantly reduced (44-48). The vaccine has proven to be highly effective in neonates. In a group of twenty three Chinese neonates, after the second dose of the vaccine, 91.3% of the neonates had anti-HBs antibodies with no adverse side effects. Furthermore, these neonates responded well to booster doses (after six months), which tended to increase antibody titre and seroconversion rates (61).

The vaccine is safe and has been sanctioned by multiple advisory committees, including the World Health Organization (WHO). Despite the fact that the vaccination has proven to be an effective method of prevention, in the year 2000 only 116 of 215 nations implemented a HBV childhood vaccination program. This accounts for only 31% of the world's birth cohort (1). A possibility for the lack of a global program may be the high cost of large scale vaccinations (32). Furthermore, the three dosage compliance and tracking of patients are challenges in vaccination programs (34). An additional factor, is that there is a fraction of the population (approximately 1-10% of individuals) that does not develop a protective state as a result of the vaccination, which could be attributed to host factors such as being immunocompromised, and due to major histocompatibility genes (12,60). Finally, another problem that is arising is that variants of HBV are developing through mutations in their amino acid sequences. Vaccinated patients infected with a mutant strain can still be infected, thus posing a problem in the future (1).

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1.7 Clinical features.

In general, the HBsAg in a patient's serum indicates an HBV infection, while the presence of HBeAg, and HBc, HBs, and HBe antibodies reflect the different stages of infection (2). For instance, the HBeAg is the antigen that associates with the nucleocapsid and dictates the level of viral replication. Positive levels of anti-HBe antibodies with negative HBeAg is linked to minimal to non existent replication, but positive levels of anti-HBe antibodies with positive HBeAg predicts viral replication (63). The association between HBeAg and viral replication may be related to the fact that when the virus replicates there is an increase in nucleocapsid protein for the newly developed virions (63). The exception to this rule is with respect to precore stop codon, core-promoter, and core-gene mutations, where there is a high level of viral replication despite the fact that the patient is HBeAg negative (4). Regardless if a patient has a mutation (such as in the precore region) and the patient is HBeAg negative and may be HBeAb positive, there is still the presence of HBV DNA in serum that serves as a reliable marker of an HBV infection. HBV DNA can be present when all other markers are absent, and the quantity of HBV DNA in serum is the most appropriate indicator of the level of viral replication (64,65).

HBV infection is typified by elevated alanine aminotransferases and aspartate aminotransferases (ALT and AST respectively) along with a varying increase of alkaline phosphatase and bilirubin levels. The resulting consequence of HBV infection is hepatic inflammation or hepatic cell necrosis of HBV infected hepatocytes (37,66). HBV is largely considered to be noncytopathic, but there is a correlation with an increased risk

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for hepatic decompensation and HCC with HBV replication (5,6,66). The host immune response in an attempt to clear the virus, often instigates liver disease by attacking infected hepatocytes (6,10,67). In some cases, integrated HBV DNA has been isolated from patients who are chronically infected with HBV and have HCC, further implying a relationship between HBV and HCC (68). Infections in neonates and infants, may not reflect the clinical features of HBV due to their less developed immune systems (10). In the majority of adult cases, (approximately 95% of the time), the primary infection can be cleared by the immune system and immunity prevents reinfection. But it is the minority, which accounts for the 350 million chronic carriers worldwide, that is responsible for the high morbidity and mortality associated with the disease, and for continuing to infect other members of the population (67).

An HBV infection can lead to either an acute hepatitis followed by immunity, a chronic infection, or an asymptomatic infection that may eventually lead to chronic HBV (49). An important factor in determining if an individual will acquire an acute versus a chronic infection is age (28,32,49,52). HBV in adults is more likely to result in an acute infection with clinical hepatitis (32). Meanwhile exposure to HBV at a younger age tends to result in a chronic infection. Of newborns infected with HBV, 70-90% become chronically infected, meanwhile for adults only 2-10% of infected adults develop chronic HBV (29,49). Again, the state of the host immune system is a factor. For instance, those who are immunocompromised are more likely to develop a chronic infection, rather than an acute infection that eventually resolves (14,52,69). The more specific role of the immune system with respect to a chronic infection will be described in more detail in a later section.

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In acute infections, incubation of the virus typically lasts six to twelve weeks long, and can persist for as long as six months (2). Sera from patients during this acute phase has at least 10^6 infectious virus particles per millilitre (70). During this time, arthralgia, discomfort in the abdomen, arthritis, and a skin rash may develop, then malaise, fatigue, anorexia, nausea, vomiting, and a mild fever may follow (4,37,71,72). Ten to twelve days after the appearance of these symptoms jaundice can occur, during which the other symptoms may subside (4,11,71). Neurological disorders such as ataxia, facial nerve palsy, or Guillain-Barre syndrome may also be associated with acute hepatitis B infections (71). Hematological, renal, gastroenterological, and cardiac complications may also occur (71,72). Fulminant hepatitis may arise when immunosuppressive agents are withdrawn, and a strong immune response results (6). Only in 0.1 to 0.5% of patients does the fatal fulminant hepatic failure (FHF) develop (4,11). Acute hepatitis B is usually indicated by a strong IgM antibody response to HBcAg and may be accompanied with anti-HBs antibodies (4,6,11,71). Without IgM HBc antibody, the acute hepatitis may have been caused by means other than by HBV (71). Typically in acute infections, clinical hepatitis resolves in one to three months and HBsAg is no longer detected. At the end of an acute infection, HBeAg antibody should be present without HBeAg. IgG anti-HBc antibody markers persist for life, to indicate previous infection (6).

Chronic infections develop in less than 5% of adults: 25-50% of children aged one through five, and 90% of infants under four years old (4,10,37). During chronic infection there is more than 10^5 copies of hepatitis B viral DNA per milliliter of serum, which still indicates significant viral replication. Chronic infection is defined by the

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presence of HBsAg for at least six months (4,73). However, during the carrier state there may be normal ALT levels, and minimal or absence of necro-inflammatory activity (74). Chronic infections can either be HBeAg positive or negative, there may be anti-HBe antibody markers, and a limited IgM anti-HBc antibody response may be stimulated (74,75).

1.8 Virology.

1.8.1 Viral genome and structure.

HBV is an enveloped virus consisting of a partially double stranded circular DNA, which is 3200 base pairs (bp) long (6,20,67,69). The circular structure of the double strands of DNA is maintained by the cohesive overlap of the direct repeats (DR1 and DR2) located at the 5' ends of the DNA strands (67,76). The HBV genome has four promoters, which are regulated by enhancers, En1 and En2 (20,77). The HBV minus strand is made up of four overlapping reading frames (ORF). Within one open reading frame, multiple proteins can be encoded through the use of numerous internal AUG start site codons (67).

Each open reading frame encodes specific viral proteins. The ORF P encodes for the HBV polymerase, which consists of the viral polymerase/reverse transcriptase, the ribonuclease H (RNAse H), the terminal protein, and a tether spacer region (20,67). The viral polymerase encoded by the ORF P is a reverse transcriptase (RT) covalently bound to the 5' end of the negative sense strand (67). In addition to its role in the replication cycle, the polymerase is involved in regulating core protein production (78).

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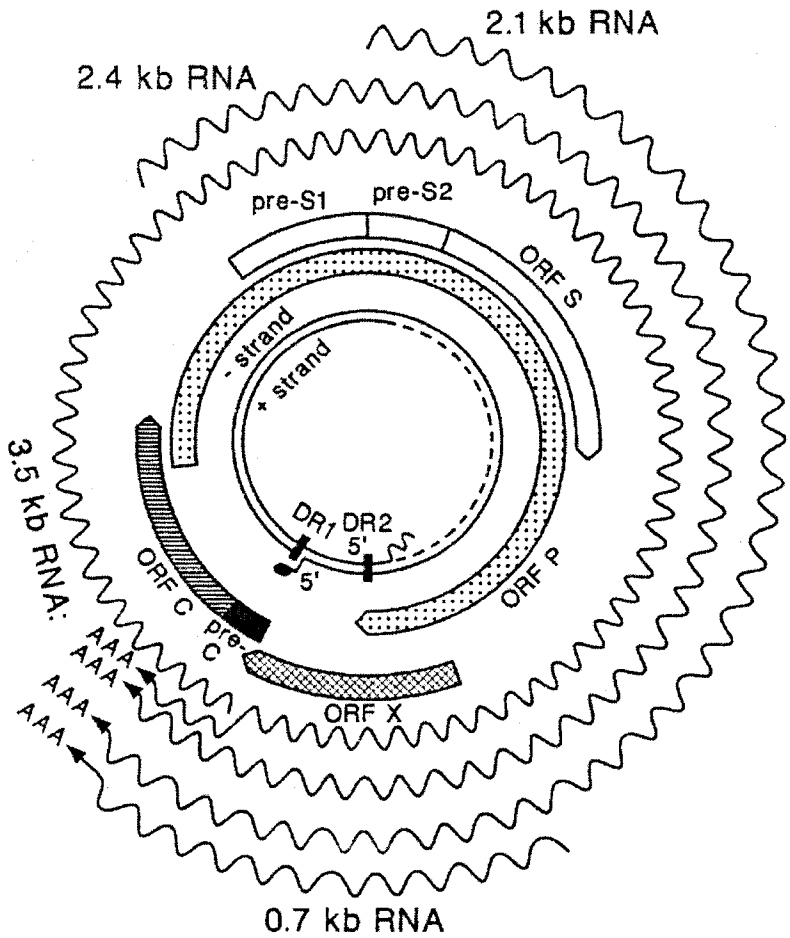

Figure 1.3 HBV genome. From ref. 14.

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The polymerase also recognizes the stem loop structure at the 5' end of the pregenomic RNA, which aids in its packaging into the nucleocapsid. The polymerase is essential in packaging the pregenomic RNA species in a nucleocapsid (78,79). The RNAse H is located at the carboxy end of the polymerase and degrades RNA template, assists in viral packaging, priming, and minus strand chain elongation. The terminal protein is at the amino end of the polymerase and is attached to the minus strand. This domain assists in packaging the pregenomic RNA and primes the minus strand DNA synthesis (14,20,67). The ORF C (and pre-C) is (are) the coding region(s) for nucleocapsid structural proteins, including HBcAg, and the pre-core domain produces HBeAg (14,20,67,69). Both the P and the C gene are necessary for virus production (80). The ORF S and pre-S region express glycoproteins on viral surfaces are involved in HBV attachment to host cell receptors (14,67,69). The pre-S region is upstream to the ORF S and is categorized into two subsets: pre-S1 and pre-S2 (6,14,67). The S gene and the pre-S1 and pre-S2 regions encode for three lipoprotein components of the viral envelope, all of which contain the HBsAg (6,69). The 39 kd L (large) protein is a product initiated by the start codon for pre-S1, thus containing the domains pre-S1, pre-S2, and S (14,67,81,82). The pre-S domain of the L protein can either be present in the cytosol or be translocated through the membrane of the endoplasmic reticulum (82). This L protein serves as an antigenic site for B and T cells, and is required for virion infectivity (67,81). The 31 kd M (medium) protein is initiated by the start codon for pre-S2 (14,67). The S (small/surface) protein, only including the S domain, is the main antigenic determinant HBsAg (14,67,82). Infectivity depends on the presence of both the L and S proteins (81). *Avihepadnaviruses* do not encode for an M type envelope protein, and the envelope

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proteins are not glycosylated as in the case of *Orthohepadnaviruses* (20). Finally, the ORF X (which is only present in mammalian hepadnaviruses) encodes for regulatory proteins, and may be a transcriptional transactivator (6,14,69). The HBx protein is necessary for *in vivo* infection, and may also have a role in HBV induced carcinogenesis (20,67,80).

The hepatitis B virus produces three types of particles: 20 nm spheres, 20 nm diameter filaments, and 42-47 nm Dane particles. The Dane particles are enveloped and comprise the infectious virion. The Dane envelope consists of HBsAg, while the HBcAg makes up the nucleocapsid, which encapsidates the viral genome and polymerase (6,67,70). The small spheres and the filament forms are noninfectious extra viral coat substance, and are normally more than one hundred times more frequent than the virions (6,20,67,70). All three HBV particles carry the HBsAg on their respective surfaces (14).

1.8.2 Viral replication cycle.

After viral entry into the host, the HBV travels to the hepatocytes, the primary site of infection, and spreads through out the liver (14,36,69). It is speculated that HBV may also infect bile ductile endothelial, pancreatic, kidney, or lymphoid cells (20). In humans infected with HBV, high HBsAg levels have been found in pancreatic and bile juices (83). In ducks congenitally infected with DHBV, viral antigen has been found in extrahepatic sites such as in the bile duct, kidney tubular epithelia, kidney glomeruli, and pancreatic acini in the α islets (84). Once the hepatocytes are infected, they shed virus into the bloodstream, which can then further infect other hepatocytes (20). The half life of

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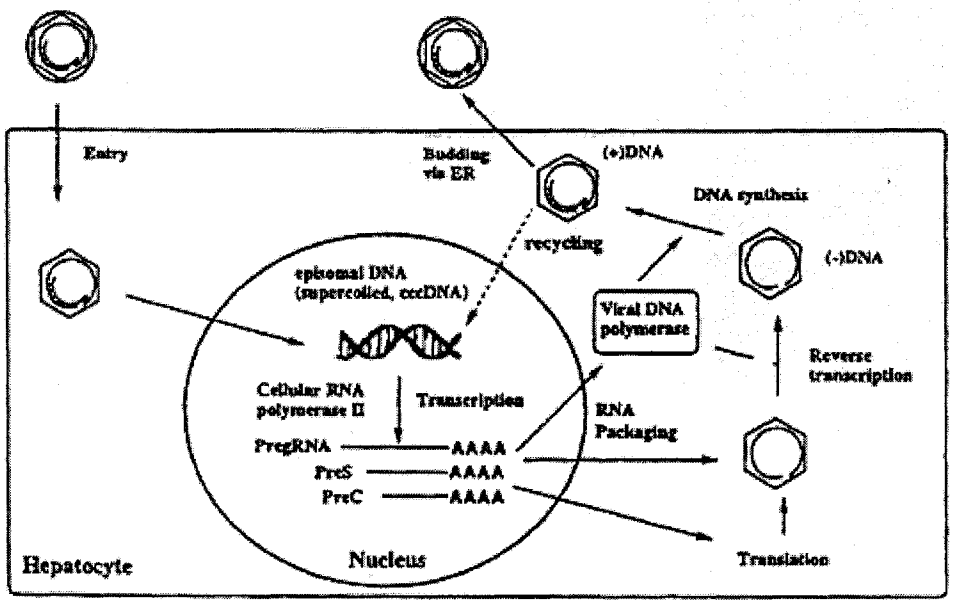

Figure 1.4 HBV viral replication cycle. From ref. 85.

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the HBV in plasma is approximately only a day, but the half life of infected hepatocytes is more dependent on the individual, and can vary from 10-100 days (86). Hepatocytes are non mitotic, and interact stably with HBV, thus facilitating a chronic infection in an immunocompromised individual (20).

The viral life cycle begins with the virus binding to the host cell receptor, followed by the fusion of the viral membrane into the host cell membrane, and the release of the virion nucleocapsid into the cytoplasm of the host cell (14). The virus and host receptor interaction contributes to the species and cell specificity of the virus (20,67,69). The pre-S1 protein may be responsible for binding to the plasma membrane of hepatocytes, specifically the host cell glycoprotein gp180, while the pre-S2 coding region may contribute to viral attachment, uptake, and uncoating (20,67,69,81). The nucleocapsid is then transported into the nucleus, where the genome is converted into covalently closed circular DNA (cccDNA) from relaxed circular DNA, rcDNA (20,67,79). The conversion involves the use of the cellular DNA repair system (an endogenous DNA polymerase) to fill in the single stranded gap area, the 5' terminal structures (such as the polymerase covalently linked on the minus strand and the capped oligoribonucleotide on the plus strand) are cleaved, and the eight to nine nucleotide long terminal redundancy on the negative sense strand is removed. After these processes, there is the covalent linkage of the two strands (20,67,77,87). The cccDNA functions as a viral transcriptional template, and it is transcribed by the cellular DNA dependent RNA polymerase II into four transcripts which share a common adenylation signal. One of the transcripts is a 3.5 kb viral pregenomic RNA. The pregenomic RNA is synthesized in the nucleus and is transported to the cytosol where it is packaged into the nucleocapsid and

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serves as an RNA intermediate for DNA synthesis (14,77,88). This genomic transcript also serves as a messenger RNA (mRNA) and translates to the core/nucleocapsid structural proteins, and the viral polymerase (67). Also inside the nucleus, the viral transcription of the cccDNA leads to the production of three sub genomic RNA transcripts, which are translocated to the cytoplasm. These sub genomic transcripts (either 0.7, 2.1 or 2.4 kb in size) are translated to various viral proteins in the cytoplasm. The S (envelope) proteins and X (regulatory) proteins are translated from sub genomic mRNA transcripts (14,20,67).

Reverse transcription is performed using the viral polymerase to produce a new circular DNA inside the capsid (3,88). The DNA synthesis involves using the pregenomic RNA template for reverse transcription to produce the rcDNA. This template also has an additional function of being the messenger RNA (mRNA) for translation to genomic protein products, which are required for further DNA synthesis (67,79,87,88). The HBV viral polymerase primes the synthesis with the hydroxyl (OH) belonging to the tyrosine residue found in the terminal protein at the 5' end of the minus strand (67,87,88,89). The priming reaction can only begin after the viral reverse transcriptase interacts with the HBV RNA signal, e, which is on the 5' end of the pregenomic RNA and dictates template sequence (89). The hydroxyl group of the tyrosine is joined through a phosphodiester bond to the 5' hydroxyl of the nucleic acid, and nucleic acid synthesis is then initiated (87). The negative strand is synthesized first, while the RNA template is degraded by RNAase H, and this is followed by positive strand synthesis where the minus strand is used as a template (67,88). Synthesis is complete when the plus strand is approximately 50-70% of the minus strand length (67).

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The plus strand of DHBV is completed to a greater degree (20). After DNA synthesis, capsids acquire a glycosylated protein envelope through budding from the endoplasmic reticulum (ER) or the Golgi apparatus. The virion is then secreted (14,20,67).

The persistent cccDNA pool is characteristic in chronic infections. The cccDNA pool can result from an intracellular conversion path whereby complete nucleocapsids return to the replication cycle instead of being exported, although this is rare in infections. Also, when the RNA primer does not transfer from DR1 to DR2, a double stranded linear DNA results, and the ends can recombine to form cccDNA (20). Regradless of the means, the result is that the virus has a reservoir of transcriptional templates at its disposal (67).

Due to the asymmetric reverse transcription of the pregenomic RNA, the gene is susceptible to mutations (66). The HBV mutation rate is ten fold greater than the mutation frequency associated with other DNA viruses (67). The mutated strains are a result of the replication method (since the viral polymerase does not have proof reading ability), the effect of the host immune interaction, and a result of antiviral treatment and/or the vaccine (66,67). These HBV mutations will be discussed to a greater extent in a later section.

1.9 Covalently closed circular DNA (cccDNA).

The cccDNA pool consists of a mixture of two types of cccDNA molecules. One of which, is a complete genome that can encode for all the viral proteins. The other is a defective molecule with deletions; this type of cccDNA is a result of nonhomologous

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recombination and joining of the ends of the linear double stranded DNA through a process of illegitimate replication (90,91). There is also evidence of two types of linear precursors, which may relate to the different cccDNA molecules (91). The cccDNA species differs from open circular DNA, in that the open form contains specific and consistent gaps in the genome and is covalently linked to the viral primer protein (92).

The cccDNA pool is an essential aspect of an HBV infection. HBV utilizes the process of cccDNA amplification (whereby the viral DNA produced during the infection is transported to the nucleus to be converted to cccDNA) to maintain a stock of transcriptional templates (93). The amplification occurs early in infection, and then is limited in order to prevent cytopathic effects (245). This cccDNA amplification is regulated through negative feedback by the presence of viral envelope proteins or protein products (such as the pre-S proteins) that can inhibit the conversion of relaxed circular DNA to cccDNA (79,93). Therefore, defective viral envelope proteins can lead to continuous high levels of cccDNA synthesis (245,246). The mean cccDNA copy number for each nucleus of hepatocytes tends to fluctuate during an infection. As an example, a duck infected with DHBV can contain between 2.9 to 8.6 cccDNA copy numbers in each hepatic nucleus and the infection can be sustained with as little as one cccDNA per cell (93).

The resilient factor of an HBV infection is the cccDNA. The cccDNA has a half life ranging from 35-57 days in the duck (94), and 33-50 days in the woodchuck (95). There is also the longevity of hepatocytes to consider, which as an example lives for six to twelve months in the woodchuck (96). Thus, in otherwise healthy chronic HBV carriers, hepatic turnover is slow with a half life of approximately a month, while in acute

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cases the hepatic turnover is greater than one week (96). However, there still remains a small quantity of cccDNA even after an acute infection (97). There is still some debate regarding whether or not the cccDNA is lost during cell division or is passed on to daughter cells (90,95,96). Nevertheless, it does appear to remain after antiviral therapy, and when treatment is suspended HBV replication rebounds (66). Although, it has been shown in a duck *in vivo* study that by continuing to suppress HBV replication with antiviral treatment, there can be either a gradual and continual decline of cccDNA copies or an initial decrease followed by a plateau and leveling off of the cccDNA population (94). Hence even if the use of an antiviral does not immediately deplete the cccDNA pool, it is still advantageous in controlling the cccDNA population. As an example, lamivudine inhibits viral DNA synthesis and thus limits the number of mature core viral particles traveling back to the nucleus to propagate more cccDNA production. An assay based on a human hepatoma cell line infected with HBV recombinant baculovirus (which is sensitive to cccDNA detection) has shown that lamivudine, can decrease this population (98). It has also been demonstrated that the use of the antiviral, adefovir, may not prevent the formation of cccDNA, but does have an inhibitory effect on cccDNA amplification (99). Overall, the use of an antiviral can aid in the decrease of cccDNA over time (96,246).

The stability of cccDNA is an important consideration, since the cccDNA pool indicates the continual production of HBeAg, maintains a stock of transcriptional template, and may be responsible for the relapse after treatment is stopped (94). The hope behind using an antiviral therapy is that it may inhibit further viral DNA synthesis to minimize the number of infected hepatocytes, the immune related cytokines may then

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be able to prevent *de novo* infection of uninfected hepatocytes, and the previously infected hepatocytes may be destroyed during hepatic cell turnover (90,95).

1.10 HBV mutations.

According to Pult et al. (100) viral variants arise as a result of replication errors, pressure from the environment, and due to genetic recombination (100,101). As a result, spontaneous mutations have been shown to occur (100,102). There are even cases where the mutation leads to a cytopathic variant of the virus. One such instance is exemplified by the DHBV-16 species, where at position 133 of the pre-S large envelope protein domain the amino acid glycine is replaced by glutamic acids (G133E mutation). However, the presence of a cytopathic virus variant is infrequent and transient. Due to its replicative disadvantage, its population is replaced spontaneously with the noncytopathic wildtype virus that is much more efficient at replication or other revertants that re-establish the regular pre-S protein function (100).

Other spontaneous mutations that occur in the HBV genome include the double mutation in the core promoter (A1762T/G1764A) and precore region (G1896A /C185T); these mutations tend to arise during HBeAg seroconversion (25,86,103,104). The core promoter mutation reduces precore mRNA transcription, while the precore mutation creates a stop codon and inhibits translation of this precore protein. Both mutations decrease HBeAg synthesis (25,103,104). HBeAg negative patients tend to possess a precore or core mutation; these patients develop more severe forms of HBV with continuous high viral loads, high ALT levels, and liver disease (103-106).

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In addition, due to HBV vaccinations and HBIG treatments, HBsAg mutants in the 'a' determinant have arisen, which typically involves a G145R mutation. A mathematical modeling study discussed by Zuckerman et al., discuss that with large scale HBV vaccinations and assuming the greater prevalence of wildtype HBV that the virus with the mutant surface antigens will predominate over the wildtype in four or five generations (107). The clinical effect of these mutations is still not established (25).

The drug-resistant mutations that occur have important clinical impacts. Drug resistance mutations will be discussed in this section, as well with reference to specific drug treatments in later sections. After a long term schedule of an antiviral treatment inducing continual suppression of viral replication and wildtype cccDNA depletion, often viral titters will suddenly increase due to the replacement of wildtype virus with the drug-resistant variety (102). Drug resistance appears quickly when β-L-dideoxy nucleosides and oxathiolane nucleosides, such as 3-TC, (-)-2',3'-Dideoxy-3'-thiacytidine, and FTC, (-)-ß-2',3'-dideoxy-5-fluoro-3'-thiacytidine, are used (108). 3-TC (lamivudine) resistance typically results in either a M552I (rtM204I) single mutation of the HBV polymerase or a double mutation of M552V/L528M (rtM204V/rtL180M) (108-112). Although uncommon, there can also be the combination of M552I/L528M or even M552I/M552V/L528M (108). The double mutation has proven to be more resistant to lamivudine than the single mutation (111). The M552I and M552V mutations reside in the conserved YMDD (tyrosine, methionine, aspartate, aspartate) catalytic motif of the C domain of the HBV polymerase, which is associated with the binding site for the nucleotides (109,111,113). The methionine to valine substitution in the YMDD motif alone can cause a 330 fold less sensitivity to 3-TC (114). Meanwhile the L528M

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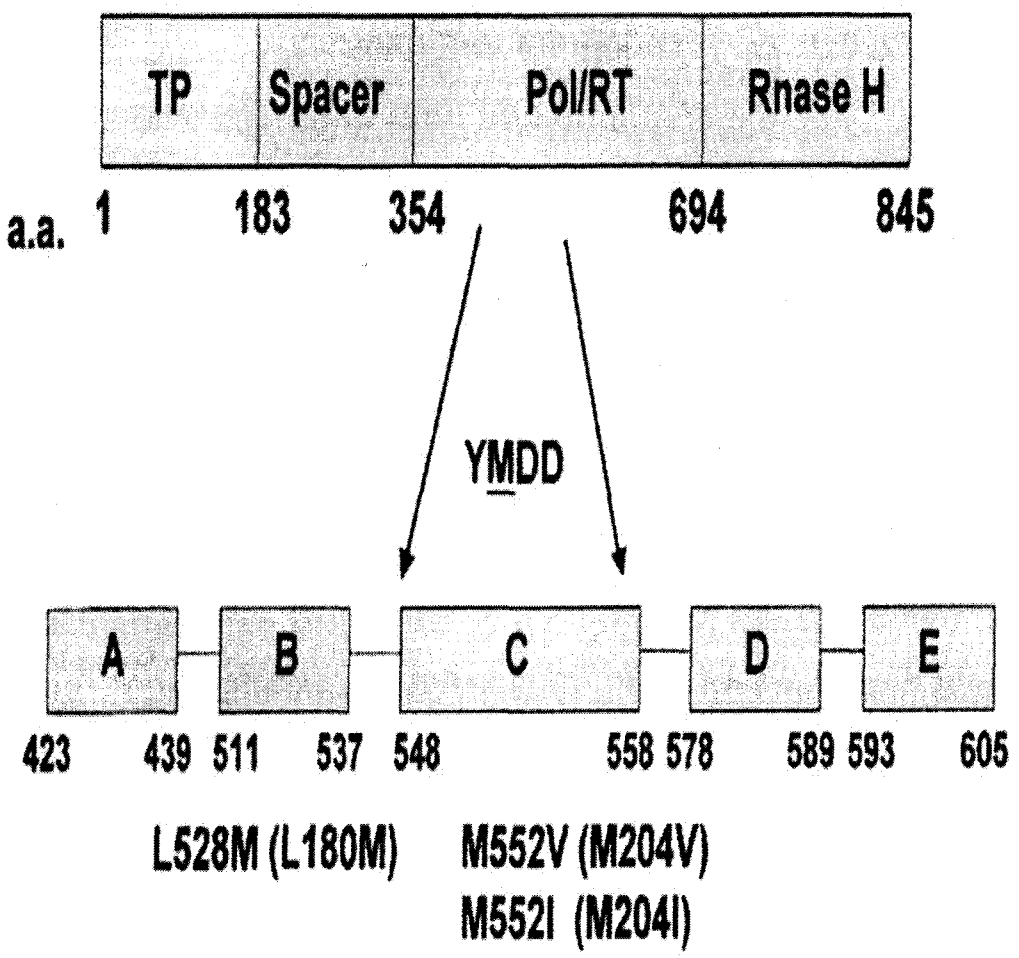

Figure 1.5 HBV polymerase and the YMDD motif mutations.

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mutation is situated in the B-domain, which has a role in template positioning (109). The mutations in the C domain reduce the replicative capacity leading to the replication disadvantage of the variant virus (109,115). Interestingly, in a study involving woodchucks, mutations were not found in the YMDD motif, which may be due to the negative effect of the mutations on WHV replication efficiency, but are instead generally found in the B region (102,116). It has been displayed that the HBV B domain mutation, which is generally coupled with a C domain mutation, aids in the compensatory restoration of the replicative ability of the mutant virus, and may strengthen the drug resistance (109,113,115). To account for this role, it is speculated that the B domain mutation could counter the conformational unevenness resulting from the C domain mutations (109). Precore mutations combined with lamivudine resistance also result in the restoration of viral replicative ability. Additionally, basal core promoter mutations in conjunction with the drug-resistant phenotype has lead to an increase in mutant viral replication, and at levels even greater than the wildtype version (86). On another note, neither of the drug-resistant mutations appears to have detrimental effects on the packaging of pregenomic RNA (114,117).

There has also been a third mutation that has been detected, rtV173L, which can be found along with the double mutation, and may also be compensatory (110). It may aid in increasing the replicative ability of the variant virus by two means. First, since residue 173 is located beneath the viral nucleic acid template strand, it may have an advantageous role in template strand positioning. Or since it is close to residue F88, which is involved in the polymerization process and interacts with the sugar ring of the nucleoside analog, residue 173 may have a role in facilitating the polymerization reaction

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by improving the position of the phenylalanine side chain (110). Overall, this mutation leads to increased drug resistance, and may be a result of long term lamivudine treatment (110,118). This mutation occurs at a site where the polymerase and surface antigen open reading frames overlap, thus causing surface antigen mutations, sE164D and I195M (110,112,118). The levels of HBsAg were not affected by the I195M mutation in one study, but surface antigen mutations could have an effect on the HBsAg and antibody binding (110,114,116,118). On the other hand, a mutation that substitutes alanine to threonine at position 566 of the WHV polymerase also gives rise to a stop codon in the S gene, which can lead to the truncation of the envelope proteins at the carboxy end (102,116). Regardless of the drug-resistant mutation, when the resistant virus emerges it becomes the dominant population, as opposed to the wildtype virus. Since the mutant virus can efficiently replicate and hepatocyte turnover is low, this would slow the evolution back to wildtype virus (116).

The polymerase binding pocket of HBV has a similar structure to that of other retroviruses, such as human immunodeficiency virus, HIV (117). There are even analogous mutations; the HIV reverse transcriptase may have a M184I mutation due to lamivudine resistance, which can then give rise to the M184V variant (113,119-121). However, the murine leukemia virus reverse transcriptase binding site may be too structurally different, since the wildtype active site YVDD is not only resistant to 3-TC but the mutant that switches the active site to the normally 3-TC sensitive YMDD is still somewhat resistant (122,123). Not only can the same drug invoke resistance amongst similar viruses, but there can be cross resistance with nucleoside analogs which have alike sugar moieties (124,125). Such as there is cross resistance between the 3-TC and

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FTC-resistant virus and 5-F-β-L-ddC and β-L-ddC which are structurally similar when used as nucleoside analogs against HIV (124,126).

There have been a number of models proposed to account for the mechanism of drug resistance. One theory is that drug-resistant variants may actually exist as a quasi species, and when there is the greatest suppression of wild type virus by lamivudine, the mutated virus may emerge (98,102). An explanation for the mechanism of drug resistance is that there is steric hindrance between the antiviral nucleoside analog and polymerase binding pocket. In the HBV mutation, M552I or M552V, there is steric hindrance between the β branched side chains of isoleucine or valine and the oxathiolane ring of the triphosphate of 3-TC, thereby preventing the binding of lamivudine (110,119). These residues, which branch out and are rather large can behave as steric barriers (117). This same conflict can be seen with HIV, where lamivudine can no longer bind to the polymerase binding pocket due to the hindrance at the site of the mutation (position 184) between the oxathiolane ring of 3-TC and the side chains of Val, Ile, or Thr (β branched amino acids (119). The B domain mutations also have a potential role in disturbing the lamivudine binding to the polymerase active site (116). In the M184I (or the M184V or M184T) HIV mutant, the Cγ2 methyl of isoleucine (or methyl substituents of valine or threonine) is in close proximity to the oxathiolane ring, and the β branched isoleucine (or valine and threonine) side chain is restricted through its interactions with its side chains, Y115 and Y183, overall contributing to the steric conflict (119,123). There may also be repositioning of the template-primer, which may alter the dNTP (deoxyribonucleotide triphosphate) binding pocket, and could prevent the formation of a closed complex including the binding pocket, DNA, and the triphosphate of 3-TC (119,123). A

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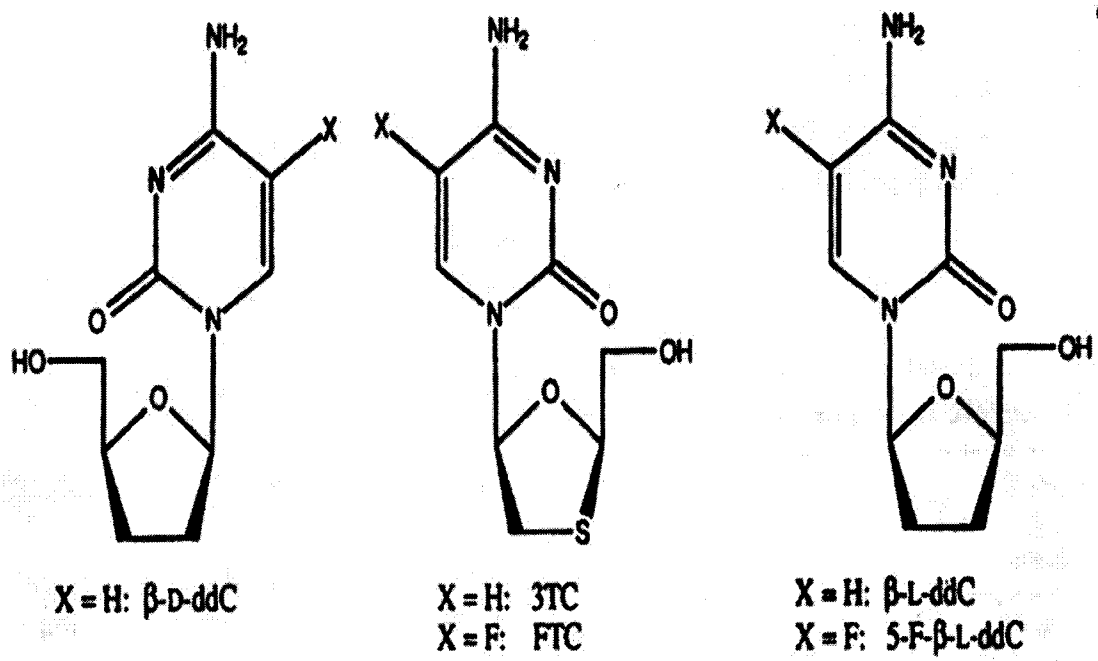

Figure 1.6 Cross-resistant nucleoside analogs. From ref. 124.

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template- primer shift may also result in the lamivudine triphosphate not being properly aligned in the complex. This could lead to an ineffectual transition state, since the correction of the primer shift could raise the free energy requirement, and incur too high of a cost for the transition state (119). Finally, any changes in the residues near the binding pocket could have potential detrimental effects leading to drug resistance (119).

1.11 Immune response to HBV.

The clearance of the virus depends on the innate and adaptive immune response. Generally, in acute hepatitis cases, the innate system releases cytokines and the humoral adaptive response clears most of the virions in the incubation stage. The viral infection is resolved when the hepatitis B antigens are eradicated, and there are memory T cells specific for HBV (8). However, at other times the infection is not cleared and a chronic infection develops. In some of these cases there is a correlation to the state of the patient's immune system. This can relate to age, with younger children more likely developing a chronic HBV infection due to an immature immune system (27,43). Or in some circumstances, patients who have developed a chronic infection do not have as strong of a cytotoxic T lymphocyte (CTL) response in comparison to patients who have cleared the virus, and there is a limited response to viral antigen (14,127-131).

The innate immune response involves cells such as natural killer (NK) cells, natural killer T (NKT) cells, and Kupffer cells (liver specific macrophages) to release cytokines. The cytokines are responsible for limiting viral presence in the first part of the

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infection by disrupting viral mRNA and removing nucleocapids, the site of viral replication (8,128). The infected cell first typically responds with release of interferon (IFN) α/β, which inhibits HBV replication (by using the 2', 5'-oligoadenylate synthetase or protein kinase R (PKR) pathway), prevents protein synthesis, and activates macrophages (8,132). The 2', 5'-oligoadenylate system causes the degradation of single stranded RNA, the intermediate in the viral life cycle (132). The PKR path is involved in the inhibition of translation, by sequestering eIF-2B, a translation initiation factor (132). The active macrophages are then able to recruit NK cells, which can target and kill the infected cell or produce interferon γ, tumour necrosis factor (TNF)α, and other cytokines used to limit HBV replication (8,128,129). A strong NK cell response can be detected quite early, during the acute HBV incubation stage, and as the viral load decreases so does the quantity of circulating NK cells (133). The HBx protein expressed by mammalian hepadnaviruses appears to prime cells for TNF α induced apoptosis, which may be responsible for some of the liver disease associated with an HBV infection (134). The macrophages (along with dendritic cells) are also responsible for producing chemokines, and the phagocytosis of viral associated proteins from which the antigens are used to prime the adaptive immune system. The NKT cells lyse the target cells, produce interleukin- 4 (IL-4) and IFN γ, and also assist in priming the adaptive immune response to specific antigens (8).

The adaptive immune response involves humoral and cell mediated immune responses. The humoral response focuses on the production of antibodies and establishing memory against HBV. The different antibodies that result from HBV are indicators of disease stages. Acute HBV cases are indicated by antibodies that neutralize

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HBcAg, meanwhile the resolve of the acute infection is represented with antibodies against HBeAg (4,8). When a patient has seroconverted to produce anti-HBe antibodies this often is coupled with a reduced viral load, normal ALT levels, and improved liver histology (18,63). In certain mutated strains of HBV, the patient is HBeAg negative but there remains actively replicating virus (4,8). Also during patient recovery from HBV, there is seroconversion to produce anti-HBs antibodies, against S, preS1, and preS2 glycoprotein antigens (8).

The cellular immune response involves the use of CTLs, specifically $CD4^+$ and $CD8^+$ cells. The $CD4^+$ and $CD8^+$ response is seen quite early in an HBV infection (such as in the incubation phase), and may precipitate infected cell lysis that can lead to liver damage or induce a noncytopathic mechanism (128,129,133,135). The CTL response against an HBV infection is polyclonal and multispecific with respect to HBV epitopes (124). $CD4^+$ cells use major histocompatibility complex (MHC) class II molecules, typically expressed by antigen presenting cells, to target the viral peptides. While on the other hand, $CD8^+$ cells identify viral peptides that are associated to MHC class I molecules, which are generally expressed by somatic cells (8,127-129). The soluble HBsAg can not only elicit an antibody response, but has been shown to also stimulate a $CD8^+$ CTL response to the antigen (136). By and large, the $CD8^+$ response involves destroying the infected cell by inducing apoptosis, while the $CD4^+$ cells act as effectors and immune system regulators as it releases various cytokines (128,129). Both the CTLs release TNF α and IFN γ, which are involved in target cell lysis, and may inhibit to some extent HBV replication (8,128,129). HBV nucleocapsids and replicative intermediates do not appear to be destroyed by CTL cytolytic events, but must rely on a noncytolytic

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means, such as through the use of inflammatory cytokines like IFN γ, to help clear the infection (128,137,138). Again, the CTL response is evident in peripheral blood early in infection (prior to ALT rise) and when the acute HBV infection is resolved the CTL presence is quickly diminished (8,127). Meanwhile, the memory T cells seem to persist for months after the resolution of an acute infection (127).

Liver damage caused by the immune response is mediated through the FAS ligand (FasL), which induces apoptosis, TNF α (produced by macrophages), and the perforin/granzyme paths. Hepatocarcinogenesis is associated to cases where the HBV DNA (especially the HBx protein, which activates transcription factors) is integrated into host cellular genomic DNA, but may also be due to long term effect of chronic hepatitis on facilitating chromosomal error (8,128).

In terms of immune function, there have been numerous proposals to account for chronic infections. One proposed reason for a persistent infection is due to an insufficient T cell response, such as a deficiency in activated $CD8^+$ T cells or the inability to release the suitable cytokines, thus facilitating the prevention of immune system from recognizing infected cells (8,128,133). Or helper T (Th) cells may be faulty, which could influence antibody production (139). Typically, chronically infected patients do not have as strong as a CTL response against various epitopes of the viral polymerase, nucleocapsid and envelope, as compared to the rigorous CTL response from those who have resolved an acute infection (127-129,140-142). Along the same line, there is the idea is that the magnitude of HBV replication can overwhelm the immune system, since the ratio of CTLs to infected hepatocytes has been estimated at 1:1000 (128,143). There is also the factor that activated CTLs may then undergo as programmed death (128).

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Furthermore, after spreading though out the liver, the virus may spread to areas outside the liver, and T cells specific to HBV may not be able to have an effect in those sites (8,14,127,128). An alternate theory is that there may be mutations on the T cell epitopes, such as amino acid substitutions on the human leukocyte antigen (HLA) binding motif. This could reduce the recognition of HBV peptides, and allow viral escape from a CTL response (8,127,144). Finally with respect to vertical transmission, chronic infection could be a result of neonatal immune system tolerance (128,145).

Regardless, of the mechanism of establishing chronic infection, defective T cell response is expected to restore, when the viral load is reduced. Lamivudine antiviral treatment has been exhibited to overcome the T cell hyporesponsiveness displayed in chronically infected patients (130,131). Perhaps over a long term schedule, drugs which inhibit HBV replication, may be able to provide a chance for the immune system to re-establish its effectiveness (8,135).

1.12 Pre-treatment considerations.

Liver disease remission is often associated with seroconversion to the anti-HBe antibody state along with the loss of HBV DNA. However, a complication lies with a mutation in the precore region of the HBV genome. In this case, there is no HBe antigen produced, so there is an anti-HBe antibody production associated with continued replication of HBV DNA, and acute liver disease. Thus, HBV DNA levels are used as a major predictor of liver injury. When HBV DNA levels are negative regardless of antigen levels, then minimal liver damage should result (3,146). Therefore, an important

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treatment target is viral replication. The overall goal of an antiviral treatment is the elimination of HBV DNA (and in some cases HBeAg), and the seroconversion to an anti-HBe antibody state. Consequently, there should be a reduction in hepatic inflammation and ALT levels should return to normal. Thus, the rate of morbidity associated with the disease should be decreased (3,103,146).

1.13 Treatments currently available.

1.13.1 Interferon.

Interferons belong to the pleiotropic cytokine family. These cytokines stimulate antiviral genes, have an antiproliferative effect, and acts as immunomodulating agents (132). The interferon used for treatment against HBV is the interferon α form. It has been used since 1991 and functions as an antiviral and an immunomodulating agent without being antigen specialized (103,132). The mechanism of action of interferon involves breaking down mRNA, through the use of an RNAse L path and the 2', 5'-oligoadenylate synthetase multienzyme. The presence of viral double stranded RNA activates the 2-5A synthetase, which responds by producing 2'-5'-oligoadenylates. The 2'-5'-oligoadenylates then bind and activate RNAse L, an endoribonuclease. RNAse L is responsible for degrading single stranded RNA, which is often the RNA intermediate in viral replication (132). The immunomodulatory effects of interferon α include stimulating B cell proliferation, which augments the antibody response as part of the humoral immune response. Interferon also helps upregulate MHC class 1 expression on

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infected cells, MHC class II expression on antigen presenting cells, and the presentation of viral antigens to $CD8^+$ CTLs and $CD4^+$ helper T cells. Interferon may also play a role in increasing CTL synthesis by aiding in the upregulation of T helper cells receptor components (132). Finally, in those patients who have responded to interferon treatment, there was an accompanying rise in monocyte production of other cytokines, TNF α and IL-1β, which can also contribute to the viral clearance activity (147).

The standard dosage of interferon is based on a four-month (sixteen week) course where five million units are delivered subcutaneous daily. In other trials ten million units were administered three times per week (3). An advantage of this therapy is that this treatment is administered in a finite schedule, and that the resulting response is persistent (66). In one trial, this regimen lead to the eradication of HBeAg in 30-40% patients, and 15-20% of patients seroconverted, developing anti-HBe antibodies (148,149). In a long term post treatment follow up (of up to nine years), 30% of patients lost serum HBeAg and HBV DNA (150). Even when HBeAg negative chronic carriers were treated for one year, 15-25% of those patients developed a sustained response (4). However, the effectiveness of this treatment is variable. In another study, after four months of therapy, 32% of patients became HBV DNA negative, compared to 7% in the control group (151). Furthermore, in a large study involving 419 Chinese patients, there was no significant difference in HBeAg seroconversion rates with patients treated with interferon as compared to the control group after twenty four months of follow up, and only 2.4% of interferon treated patients lost HBsAg (152). The lower rate of response amongst Chinese patients may be due to the probability that most were infected early on in age, in which case the immune system may be more tolerant to HBV (152).

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Interferon β, a similar cytokine produced by fibroblasts, has been tried as a treatment option for those who did not respond to interferon α . In response to interferon β, there did seem to be a modest effect on the normalization of ALT levels, reduction in serum HBV DNA, and a decrease in side effects (149). However, there was no loss of HBsAg, complete clearance of HBV DNA, or significant improvement in liver histology/necroinflammatory score (149).

Not only does the interferon α treatment not have a high rate of successful response rate, but there are numerous limitations associated with this therapy. First of all, patients have a limited response to interferon when their viral load is greater than 5.66 x 10^7 copies/mL (200 picograms/mL) (4). Other disadvantages associated with this treatment include the difficult administration (subcutaneous or parenteral route) and the numerous side effects. The side effects include neutropenia, leukopenia, thrombocytopenia, autoimmune disease (e.g. in the thyroid), hair loss, influenza like symptoms, rashes, fatigue, and depression (3,4,11,63,146,148,153,154). Due to these side effects, there is a limitation in its usage, especially in children (155,156). This treatment is also quite costly, in one report the cost of a sixteen week course of alpha interferon treatment was estimated at slightly over seven thousand dollars (€ 3264) (157). Additionally, there is the variable effectiveness depending on the specific patient profile (63,103). The type of patient that appears to benefit most from this treatment is a female, with low serum viral DNA levels, increased aminotransferase levels, pre-treatment cirrhosis, short infection time, and no other morbid illness (63,150-152,155). These characteristics of a patient, display a base line immune response against the hepatitis. For instance, the aminotransferase flare (when ALT levels rapidly rise) is a consequence of

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an active immune mediated clearance (63). Of those who respond to this therapy, ALT levels are at least five times greater than the upper limits of normal, while low responses to interferon occur when ALT levels are less than two times the upper limits of normal. It appears that interferon may just be speeding up the immune system clearance of HBV in those select patients (150,152,155,156,158). Interferon treatment also does not appear to have an effect on the survival rate or prevention of complications (such as the development of HCC) of chronic HBV carriers (150,152,158). Furthermore, interferon α therapy has been associated with allograft rejection in liver transplant recipients, due to its immunostimulatory effect (159). Finally, an ALT flare often occurs prior to seroconversion due to host T-cell activation in preparation of viral clearance, and reinstates an acute like phase. Therefore, if a patient is already suffering from compensated cirrhosis, the interferon could lead to liver failure (3,148).

1.13.2 Lamivudine.

Lamivudine is structurally known as (-)-2',3'-dideoxy-3'-thiacytidine, and is commonly referred to as 3-TC. It is a cytosine nucleoside analog that is intracellularly phosphorylated to its triphosphate form by host cellular kinases. In this form it can compete with the incorporation of host dCTP during DNA synthesis. As a result, viral DNA chain extension is terminated, thereby preventing the production of the negative strand of DNA (3,103,160-163).

Dosages are daily (approximately 100 mg), are administered orally, and are typically prescribed for one year or six months after HBeAg is lost and anti-HBe

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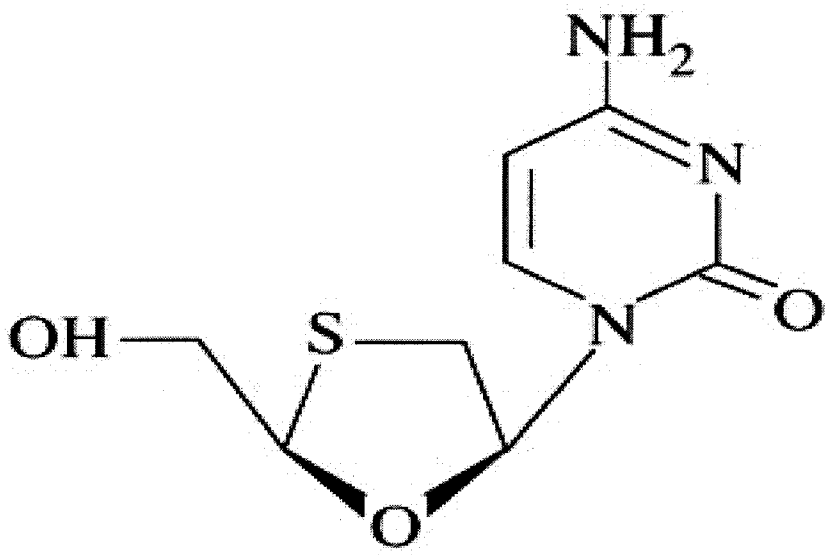

Lamivudine

Figure 1.7 Structure of lamivudine, (-)-2',3'-Dideoxy-3'-thiacytidine (3-TC). From ref. 164.

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antibodies are present (4,148,165, 166). The effective concentration (EC_{50}) of lamivudine used against HBV is between 0.01-5.6 mol/L (167). Lamivudine is quite capable of significantly decreasing HBV DNA levels, reducing transaminase levels, and improving liver histology (103,105,168,169). In one study, after six months of daily dosages of 100 mg of lamivudine, HBV DNA levels decreased by four to five logs, which was thereafter accompanied with a reduction in ALT levels (105). In another six month study using lamivudine, 80% of serum HBeAg and HBsAg was decreased in those receiving treatment, there was a normalization of ALT levels, an improvement in liver histology, and of the patients receiving dosages of 100 or 300 mg of treatment, 77% of them became negative for HBV DNA (168). Based on fifty two weeks of 100 mg daily 3-TC treatment of American patients, 52% of chronically infected patients had improved histology (a reduction of two points in the Histologic Activity Index score), 64% had reduced necroinflammatory activity, 32% lost serum HBeAg, 17% underwent HBeAg seroconversion, 44% sustained the loss of serum HBV DNA, 41% maintained normal ALT levels, and 2% lost serum HBsAg (170). The response profile appears to be consistent even within different regions, which have dissimilar modes of HBV transmission. Amongst Chinese patients, results were similar after one year of treatment with 56% of patients undergoing a decrease in necroinflammatory activity by two points, 16% underwent HBeAg seroconversion, there was a median decrease of 98% of HBV DNA during the course of the study, and 72% maintained normal ALT levels (171).

Similar to interferon treatment, when ALT levels are five times the upper limits of normal, there is a better chance of a seroconversion response (3,105,165). However, unlike interferon there are minimal side effects associated with this therapy, and this

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treatment is less expensive (3,103,105,153,169,171,172). As well, it can be used with patients who have a viral load greater than 200 pg/mL, when interferon has proven unsuccessful and with immunocompromised transplantation patients where interferon is not recommended (3,4,63). Lamivudine can also be used with children, a difficult population since they have high viral loads, immune tolerance to HBV, and a propensity to develop the chronic infection (156). Lamivudine is tolerable, and in one study involving patients aged two to seventeen, 23% lost HBeAg and HBV DNA (156). Lamivudine also impedes the progression of the hepatic injury, thus prolonging survival. In a study, more than 50% of patients undergoing therapy, experienced a hepatic inflammation reduction (3,148). Another advantage is that a liver biopsy does not need to be performed prior to prescribing a nucleoside analog, as in the case with interferon since there is typically no ALT flare. Furthermore, this drug is a viable option for patients with decompensated cirrhosis, improving the clinical outcome by suppressing viral replication (4,173).

One of the disadvantages of this therapy is that once treatment is ceased, virus levels rebound and hepatic inflammation ensues if seroconversion did not occur with respect to HBeAg (3,105). When lamivudine treatments are discontinued, an acute hepatitis like phase can occur, that can precipitate jaundice (174). The viral rebound may be due to the cccDNA reservoir in an HBV chronically infected patient (247). An additional limitation of this treatment option, is the emergence of drug-resistant mutants, which has already been discussed in an earlier section (63,105,175). Resistance can emerge as early as eight months into treatment, and as therapy continues there is an eventual complete changeover of wildtype HBV for the mutant species (105,165).

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Again, the mutation is a base pair substitution in the YMDD locus of the HBV DNA polymerase, either in the C domain (the catalytic site of the reverse transcriptase) or the B domain (63,103,105,112,165). A common mutation occurs at residue 552, where the methionine is replaced with a valine or isoleucine (3,112,165,176). A mutation at residue 528 has also been described, where the methionine has been replaced by a leucine amino acid (165,177). Some less common mutations with a less clear clinical relevance are the substitutions at position 501 involving phenylalanine to leucine, and at residue 515 leucine is substituted for methionine (3,178,179). The rate of occurrence of lamivudine resistance is 67% for patients who have undergone three years of therapy (3,148). As a result of the mutant virus, HBV DNA and ALT levels tend to rise, which typically precede an acute exacerbation (165). A further complication arises if the patient requires a transplantation, since it would be increasingly difficult to protect the graft if lamivudine resistance already developed (4,180,181).

When lamivudine resistance develops, the use of an alternate antiviral treatment, such as adefovir, is recommended, which is further discussed in a later section (111,182). The continuation of an antiviral therapy is important since the exacerbation induced by the emergence of the HBV mutant strain may induce a CTL response against the new variant and may facilitate HBeAg seroconversion (165). Furthermore, treatment cannot be stopped since the wild type virus would become the dominant population over the mutated version, and disease exacerbation could occur following an increase in viral titres (3,103,174,183,184). This is due to the fact that the mutated virus does not replicate as efficiently as the wild type virus (3,185). However, the resistant strain resurfaces and after a much shorter period when compared to original initial emergence.

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For instance, in the second round of treatment, mutated strains can emerge as early as three months (105). Regardless, the patient should be monitored closely after the surfacing of the resistant viral strain, since the acute hepatitis that can result may be severe (165).

1.13.3 Adefovir.

Adefovir (or hepsera) is an acyclic adenine nucleotide analog, and it is recognized structurally as 9-(2-phosphonyl- methoxyethyl)-adenine (132). It is administered in the form of a prodrug, adefovir dipivoxil, at 10 mg daily (101,148). Like lamivudine, it is based on oral administration (66). It also works in a similar fashion whereby the intracellularly diphosphorylated form of adefovir inhibits the HBV reverse transcription, and prevents chain elongation of HBV DNA. Adefovir is already monophosphorylated and only requires intracellular diphosphorylation (101). In addition, since the first nucleotide incorporated during the priming step is a purine, adefovir can also inhibit HBV DNA synthesis by blocking the priming step of reverse transcription (186).

The benefit of the use of adefovir is that it is effective against lamivudine-resistant strains (101,187). Westland et.al. stipulate that adefovir is active against the HBV drug-resistant strain since it possesses a flexible acyclic chain instead of a cyclic sugar moiety. This acyclic side chain can then avoid steric conflict in the HBV polymerase binding pocket. Adefovir is also very structurally similar to dATP, which may prevent the mutant virus from identifying the modified nucleoside, and the

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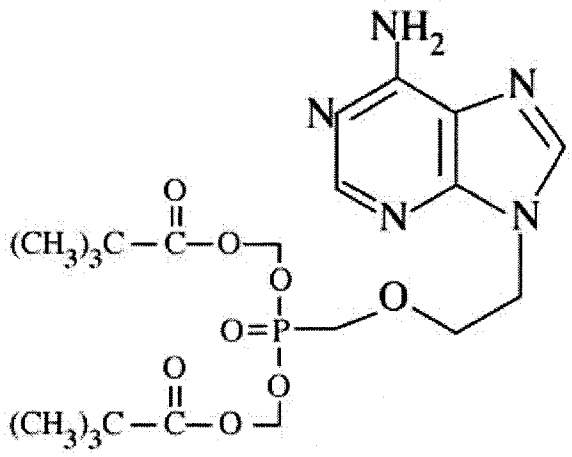
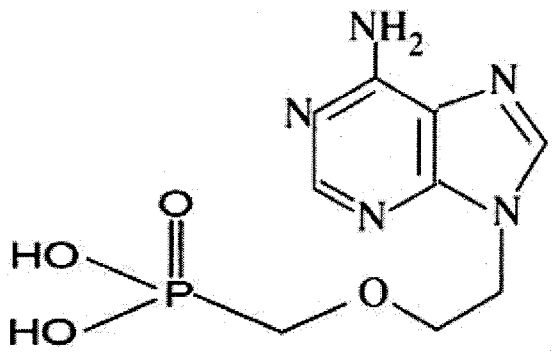


Adefovir dipivoxil (Hepsera)

Figure 1.8 Structure of adefovir dipivoxil, 9-[2-[bis[(pivaloyloxy) methoxy] phosphonyl] methoxy]- ethyl]adenine. From ref. 164.



Adefovir

Figure 1.9 Structure of adefovir, 9-(2-phosphonyl- methoxyethyl)-adenine. From ref. 188.

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phosphonate bond in adefovir (instead of a phosphate bond) aids in preventing its premature removal (101). In addition, this drug is even more effective than lamivudine in decreasing serum HBV DNA level, as the EC50 of adefovir against HBV is between 0.2-2.5 mol/L (167). After twenty four weeks of receiving the combination of lamivudine and adefovir, patients had the same resulting viral load decrease as the patients who just took adefovir (187). However, one of its main side effects is at dosages of 30 mg or higher, there is renal damage in the form of crystals forming in the proximal tubules (3,63,189,190). Furthermore, adefovir resistance has emerged, although at low frequencies. In one study involving fifty seven patients, after fifteen to sixteen months resistance developed in only two patients (191). This resistant species features a rtN236T mutation in the D domain of the HBV polymerase (191,192)

1.13.4 Other immunomodulatory treatments.

The distinguishing characteristic in patients who clear the hepatitis B virus is that they have a strong CTL and $CD4^+$ immune response, as compared to those who are persistently infected (127-129,133,140-142,193). Furthermore, viral clearance necessitates the loss of the cccDNA pool residing in the nucleus of infected hepatocytes, and the immune system may be involved with this process (193). Thus, the immune system appears to be a valid target for HBV therapy. However, immunomodulatory agents such as thymosin-α_1, interleukin-2, interleukin-12, and levamisole are non specific immunomodulatory modifiers that have been tested as treatment options, and have proven overall ineffective (3,106,141,194,195). For instance, thymosin- α_1, a synthetic

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twenty eight amino acid polypeptide derived from the thymus, has been investigated as a potential treatment for chronic HBV carriers (106,142,194,195). It presumably leads to increased production of cytokines (e.g. IFN-α , IFN-γ, IL-2 /IL-2 receptor, IL-3), 2',5'-oligoadenylate synthetase, NK cell activity, circulating peripheral blood lymphocytes and other CTLs, as well as facilitates T cell maturation (106,142,194,195). Although there are no side effects associated with this therapy, except for injection site irritation, in trials examining its efficacy there was no significant responsiveness in terms of ALT normalization and loss of HBV DNA and HBsAg (194,195). At its most successful regimen, it is just as effective as interferon therapy, with respect to the loss of HBV DNA, normalization of ALT levels, and liver histology (106).

1.13.5 Newly developed antiviral agents: FTC, LdT, and entecavir.

FTC, (also referred to as emtricitabine) is structurally known as 5-fluoro-1-(2R,5S)[2-(hydroxymethol)-1,3-oxathiolan-5-yl]cytosine. This deoxycytidine nucleoside analog is structurally similar to 3-TC except that FTC has a fluorine substituent at the C-5 position. This nucleoside analog is even more potent than 3-TC, and has been shown to reduce HBV DNA levels by three logs at a dosage of 200 mg daily, and is safe and tolerable (196-200). FTC is currently undergoing phase three clinical trials, but this antiviral is cross resistant to 3-TC resistant mutations (197, 201). Other nucleoside analogs that are currently being investigated in clinical trials are beta-L nucleosides such as LdT/telbuvidine(β-L-2'-deoxythymidine). This antiviral is potent, and selective, displaying no signs of toxicity (197,200-205). However, again this compound appears to

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be cross resistant to 3-TC resistant mutations (206).

Of the antivirals currently under development, one drug that holds strong potential as a future therapeutic is the nucleoside analog, entecavir. Entecavir, which is also known as BMS- 200475, is currently undergoing phase III clinical trials. This antiviral is a cyclopentyl deoxyguanosine nucleoside analog, 6-H-Purin-6-one-,2-amino-1,9-dihydro-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]. It has been proposed that it inhibits HBV replication through three different mechanisms of action. Similar to lamivudine and adefovir, in its triphosphorylated form entecavir acts like a substrate for the HBV polymerase, competing with the natural nucleotide substrate and leading to chain termination during DNA synthesis. This chain termination does not occur immediately since entecavir features two hydroxyl groups in the sugar moiety, so the chain termination occurs two to three nucleotides later due to the structural alterations of the sugar ring leading to inefficient DNA chain elongation. This nucleoside analog can also exert its effect by directly inhibiting the HBV polymerase. In addition, entecavir can block the priming step by competing with dGTP, which is the typical initial nucleotide incorporated during the priming step (207).

The drug is administered orally, and is tolerated quite well, eliciting no significant side effects. This antiviral is a potent inhibitor of HBV replication with a much lower EC_{50} than other antivirals, such as lamivudine. In HBV *in vitro* experiments, the EC_{50} of entecavir is as low as 3.75 nm, compared to lamivudine which has an EC_{50} of 116 nm (208). Dosages as low as 0.01 mg can reduce HBV DNA levels in chronically infected humans (209-213). Entecavir has displayed antiviral activity in lamivudine resistant strains and due to the potency of this nucleoside analog against wildtype HBV, it is

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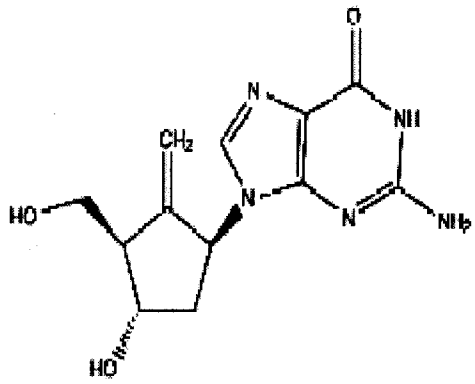
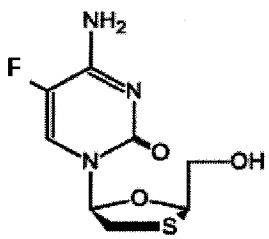
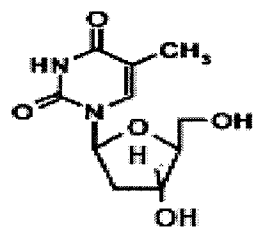


Structure of entecavir. From ref. 207.



Structure of FTC/ emtricitabine

From ref. 199.



Structure of β-L-dT

From ref. 205.

Figure 1.10 Structure of entecavir, FTC, and β-L-ddT.

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expected that the emergence of a resistance strain in response to entecavir monotherapy will take a longer time to develop (212). However, already studies have reported the surfacing of entecavir-resistant mutations, such as the mutation rtI169T and rtM250V, when this antiviral was used on lamivudine resistant strains of HBV (214).

1.13.6 Combination therapy.

Although the combination of lamivudine and interferon do not have a significant additive beneficial effect on liver histology or maintaining reasonable ALT levels, there did appear to be a prevention of the emergence of the YMDD mutant strains (103,169). Furthermore, in another study, 33% of the patients undergoing the combination therapy elicited a sustainable HBeAg seroconversion for forty eight weeks after the last treatment, as compared to a 15% rate in patients receiving solely lamivudine (162). In a study combining two to three nucleosides analogs, a synergistic effect was found, and there was even a delay of viral rebound after drug withdrawal when administered early to combat the cccDNA amplification process (215). Therefore, there may be hope in the future for combinatorial therapy that may be effective. This must especially be considered with respect to HBV when considering the relative success of combinatorial treatment against HIV. The use of multiple cocktail drugs, termed the highly active antiretroviral therapy (HAART), is far more effective against HIV as compared to a monotherapy (86). There is also the possibility that a synergistic antiviral effect will result when a combination of HBV reverse transcriptase inhibitors which have various mechanisms of action (chain termination, blocking priming steps etc.) are used in

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conjunction (103,193,215). The same idea could be applied with inhibitors of different targets in the HBV life cycle, such as nonpolymerase glucosidase inhibitors being used with reverse transcriptase inhibitors (216,217). The use of multiple inhibitory compounds should reduce the emergence of drug resistance that often results with monotherapy (103,175,193). Malik and Lee (3) suggest a future regimen of immunization to eliminate the carrier state, an antiviral agent or a combination of antiviral agents to reduce viral load, and to get rid of residual intracellular virus an immunomodulatory therapy must be used (142,194,195).

1.14 Future anti-HBV agents: rationale.

Nucleoside therapy is a promising route for treatment of chronic HBV infection. It has been proposed that by continually inhibiting HBV replication, it may allow for viral clearance through hepatic turnover and a CTL immune response against infected hepatocytes (103). One of the causes of T cell hyporesponsiveness has been credited to a high viral load or antigenemia (130). By using lamivudine to decrease the viremia, there was an augmentation of a CD4+ mediated responses to viral antigens and CD8+ CTL reactivity in chronically infected patients (130,131). Therefore, there may be a chance of restoring an effective T cell response by inhibiting HBV replication (142). Moreover, by using a combination therapy of nucleosides there may be a prevention or delay of the emergence of a mutant strain of HBV (215,218). As well, if allowed a longer treatment schedule there may be a chance to decrease the viral load, and decrease cccDNA amplification, while allowing the host immune response an opportunity to restore (215).

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Nucleoside analogs can exert an antiviral effect through various mechanisms of action. The first step involves the conversion of nucleoside analogs to nucleotides, followed by the triphosphorylation by host cellular kinases. The triphosphorylated form can then act either as substrates or inhibitors of the viral DNA polymerase (the HBV DNA polymerase). As substrates they are incorporated into the viral DNA chain during DNA chain elongation. Unlike cellular DNA polymerase, the viral reverse transcriptase does not possess a 3' to 5'exonucleolytic proofreading activity to remove any erroneous nucleotides already incorporated and the false nucleotide could lead to fatally mutated viral DNA. Another possible outcome is that for some analogs that do not have a 3'-OH for subsequent nucleotides to attach to, viral DNA elongation can be prevented. Thus, HBV viral replication is inhibited as the analogs act as chain terminators (103,122,161-162,219). Finally, the triphosphorylated nucleotide could act as direct inhibitors of viral DNA polymerase.

The rationale for the design of novel nucleosides as anti-HBV agents came from the following structural considerations.

1. It is hoped that since nucleoside analogs are structurally modified or unnatural, they cannot be recognized by cellular DNA polymerase and hence, will not have an inhibitory effect on the host polymerase. The overall effect should be that these nucleoside analogs are not cytotoxic. However, the nucleoside analogs should be similar enough as to compete with natural deoxyribonucleotide triphosphates (dNTPs) for phosphorylation and incorporation in the viral DNA elongation (220,221). In addition, the antiviral target, HBV polymerase, is distinct from the human DNA polymerase, which should minimize the threat of cytotoxicity (222). Furthermore, the

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stereospecificity can have an effect on the cytotoxicity of the nucleoside analogs. For instance, the L-enantiomer series of 2'3'-dideoxycytidine results in significantly reduced cytotoxicity (220). This same trend was seen with respect to another potent inhibitory nucleoside analog, 2',3'-dideoxy-5-fluorocytidine (220).

2. The presence of a hydroxyl (OH) group at the 3'-position may facilitate the incorporation of the nucleoside analog into the negative viral DNA strand being produced (by mimicking the 3'-OH in the 2'-deoxyribose ring of natural nucleosides) (115,223). The 3'-OH in the 2'-deoxyribose ring of natural occurring nucleosides is responsible for chain elongation, since it attacks the phosphorous (a nucleophile) of an already incorporated dNTP on the primer stand. Without this hydroxyl group, DNA strand elongation cannot proceed (224). Therefore, the lack of a 3'-OH in the ring structure is important for antiviral activity, since the incorporation of the nucleoside analog may lead to chain termination and increases the inhibitory activity of the analog (115,222,223). The hydroxyl group is also essential as the identifying group for nucleoside diphosphate (NDP) kinases, and increases the efficiency of the catalytic enzyme. The NDP kinase catalyzes the phosphorylation of a nucleoside diphosphate into its triphosphate form (225). Without this hydroxyl group, the nucleoside becomes a poor substrate for the phosphorylation reaction, since it is the hydroxyl group that donates a hydrogen bond to the oxygen connecting the β and γ phosphates in the triphosphorylated nucleoside (225). There is little specificity and involvement in the catalysis by the nucleoside base, since it does not have any polar interactions with the protein and the ribose 2' position (which is not involved in binding). Thus, there is a leniency in terms of these structures for modification (225).

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3. Another example of a structural factor involves the presence of a sulfur in the ribose ring of the nucleoside analog. The sulfur at the 3' position leads to increased inhibitory activity against HBV replication and less cytotoxicity with respect to 3-TC, in comparison to other 2'3'-dideoxy analogs (124). The sulfur in the ribose ring leads to increased binding affinity through its interaction with the non mutated methionine in the YMDD motif (125). The sulfur is bulky with a larger van der Waal radius as compared to carbon, and it may contribute to the steric hindrance that facilitates lamivudine resistance after the amino acid substitutions have occurred (119,123).

4. The stereospecificity of the nucleoside analog is also a consideration relating to its potency as an antiviral. For instance, in one series of 2',3'-dideoxycytidine analogs the β-L nucleosides were more potent than the β-D nucleoside enantiomers against WHV (226). The stereospecificity seems to affect various structures differently, and that relates to the specific structure binding to the HBV polymerase. As an example, the β and L-ring (such as with 3-TC) conformation can cause steric conflict with the mutated viral polymerase binding site (119,125).

5. The presence of an acyclic side chain may also be an important aspect in an anti-HBV agent. An acyclic chain allows flexibility in terms of the interaction of the nucleoside analog and the binding site, but it must mimic the glycosyl portion of a natural nucleoside in order to serve its function as a substrate (227). The open chain acyclic nature of the adefovir side chain may be responsible for its activity against lamivudine-resistant strains (101). It has been proposed by Das et al. that the presence of an acyclic side chain attached to a nucleoside analog would facilitate greater torsional flexibility between the nucleoside and the binding site, since it is the steric hindrance between the

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oxathiolane ring of lamivudine and the β branched amino acid residues in the binding site that may lead to resistance (125,228). Another similar consideration, is to avoid developing cross-resistant drugs for the HBV mutant strains, which could be anticipated with other β-L dideoxynucleosides with similar sugar moieties (124,125,190).

6. Furthermore, a nucleoside analog that may be more opposing to pyrophosphorolysis or ATP removal, may be more likely to contribute to anti-HBV activity (101,229,230). Pyrophosphorolysis is catalyzed by the viral polymerase and is facilitated by the presence of pyrophosphate (PPi), a byproduct of ATP or AMP hydrolysis (219). This reaction enables the removal of the incorporated nucleotides (dNTPs) from the elongating DNA chain. Lamivudine is more resistant to pyrophosphorolysis as opposed to another less effective related analog, ddC, which accounts for the disparity in antiviral activity (219). Nucleotides with sugar ring alterations or modifications appear to avoid pyrophosphorolysis, since they are inferior substrates (219). Moreover another factor to take into account is that nucleoside phosphorylase activities can be greater in the liver (along with the lung and colon), as compared to plasma and the drug can be degraded prior to its usage as an antiviral (231,232).

7. The type of base is also important. In a series of 2',3'-dideoxynucleoside analogs, the compounds with purine bases were found to be much more active as compared to the pyrimidine versions (233,234). However, there is a novel class of pyrimidine compounds that have displayed promising antiviral activity. These compounds are acyclic pyrimidine nucleosides with novel C-5 substituents, such as 1-[4-hydroxyl-3-(hydroxymethyl)-1-butyl]-5(1-azido-2-chloroethyl)uracil, or 1-[4-hydroxy-3-

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(hydroxy-methyl)-1-butyl]-5-(1-azidovinyl)uracil (222,223,227). Additionally, this series of compounds have not indicated any signs of cytotoxicity (223,227). In addition, pyrimidine nucleosides with acyclic modifications have proven to be more water soluble as compared to purine nucleoside analogs, which would augment their bioavailability, a consideration in drug formulation (227).

8. Finally, C-5 substituents can also contribute to the antiviral ability of certain nucleoside analogs. With respect to the C-5 substituent, an alkyl group (double bonds included) tends to enhance the inhibition against the HBV polymerase (222,227). A structure activity relationship has been established for the anti-retroviral C-5 and C-6 substituted dideoxy nucleoside analogs, such as the 5-halo (or methoxy)- 6-alkoxy (azido or hydroxy)-5,6-dihydro analogs of 2',3'-didehydro-2',3'-deoxythymidine, 3'-fluoro-2',3'-dideoxythymidine, and 3'-azido-3'-deoxythymidine, where the order of C-5 substituents which confer the strongest anti-HIV activity is I>Br>Cl (235-237). In some cases, by adding a C-6 substituent, such as methoxy or ethoxy, there is a resulting greater inhibitory activity (237). The type of C-6 substituent may not only determine the potency of the antiviral activity, but may also have an effect on the stability of the analog (238,239). Overall, the halogen substituent appears to be an important component in the structure of antiviral analogs (240).

1.15 Hypothesis and objectives.

There has been a tremendous amount of research done on the hepatitis B virus,

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and with due reason considering the vast implication in terms of the number of people affected worldwide and the potentially detrimental clinical outcome The treatment options currently available, interferon, lamivudine, and adefovir, have limitations with regards to their effectiveness, resistance and toxicity. Although there are newly developed antiviral agents (such as entecavir), which are promising future treatment options, there remains a need for the development of novel therapeutics that will be able to complement or supplement the already available drug treatments.

It is my hypothesis that modified C-5 and/or C-6 substituted pyrimidine nucleoside analogs, featuring an acyclic glycosyl moiety, would be selective inhibitors of HBV replication and this antiviral activity can be retained against the lamivudine-resistant viral strains. This series also incorporates other structural considerations for an antiviral compound such as it does not feature an oxathiolane ring, and has a modified acyclic sugar moiety (either with or without a 3'-OH like group). This acyclic side chain is either the one in common with the side chain seen in the purine antiviral acyclovir (ACV), a 1-[(2-hydroxyethoxy)methyl glycosyl moiety, or a 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl], which is featured in the purine nucleoside analog ganciclovir (GCV) . ACV, 9-[(2-hydroxyethoxy)methyl]guanine, is an effective herpes antiviral, but has a limited ability for HBV inhibition (223,241-244). GCV, 9-[(2-hydroxy-1-hydroxymethyl)ethoxy)methyl]guanine, can be used against Human Cytomegalovirus, and although it is a moderate HBV inhibitor, it has toxic effects (223,244). However, the only feature shared with ACV and GCV and this novel series of pyrimidine nucleoside analogs are the acyclic side chains located at the N-1 position, which are able to mimic the natural 2'-deoxyribose moiety.

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The aim of this project was to investigate the antiviral effect of newly designed 5- and/or 6- substituted acyclic pyrimidine nucleosides on the inhibition of HBV replication, and to determine the structure activity relationships of this new series of compounds. These novel nucleosides were synthesized and evaluated for anti-HBV ability against human and duck HBV using various *in vitro* assays. Their effects on cell proliferation were also tested to ensure that these compounds do not elicit any cytotoxicity. These compounds were also investigated for their antiviral capabilities against lamivudine-resistant strains of human HBV. Finally, selected compounds were tested *in vivo* using congenitally infected ducks. These studies were performed with the aim of providing an additional therapeutic option for chronic HBV carriers that may be effective against both the wildtype and lamivudine-resistant HBV strains.

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Chapter Two: Synthesis of two novel series of acyclic pyrimidine nucleoside analogues as anti-HBV agents.

2.1 Introduction.

As indicated in the introduction, anti-HBV nucleoside analogs exert their antiviral effect by acting as inhibitors of viral DNA polymerase and/or as chain terminators of viral DNA elongation. They are first converted to nucleotides and then triphosphorylated by host cellular kinases. The triphosphorylated nucleotides can then act as substrates or inhibitors of viral DNA polymerase. As substrates, they can get incorporated into the growing viral DNA chain and can lead to chain termination or fatally mutated viral DNA. Host DNA replication is not affected since cellular DNA polymerase has exonucleolytic proofreading ability to remove any flawed nucleotides, unlike the viral DNA polymerase. In addition, the host DNA polymerase shows high substrate specificity towards natural NTPs (nucleotide triphosphates) and does not recognize unnatural NTPs as substrates (1-7). Therefore, nucleoside analogs can serve as selective antivirals through the inhibition of viral replication.

Some of the structural features that were taken into account with respect to the novel series of acyclic pyrimidine nucleoside analogues were discussed in chapter one and are summarized here. In one of the series, a structural feature of the modified nucleosides is that they do not have a 3'-OH as in the 2'-deoxyribose ring, without which DNA elongation cannot occur (8). However, the presence of a 3'-OH is important in order for efficient phosphorylation by the NDP kinase and for incorporation in the

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growing DNA strand (9). The other series of acyclic pyrimidines synthesized, contains a 3'-OH like group. Another feature of these acyclic pyrimidine analogs is that there is no oxathiolane ring. Thus, there is no bulky sulfur group in a cyclic ring that may be the source of the steric hindrance between the oxathiolane ring and the β branched amino acid residues in the mutated 3-TC drug-resistant HBV polymerase binding pocket that prevents the binding of 3-TC (10-13). The acyclic side chain permits flexibility between the nucleoside and the HBV polymerase binding site to still be able to conform and fit into the mutated HBV binding pocket. The acyclic side chain structure may account for the ability of adefovir to retain activity with 3-TC-resistant HBV (14,15). The alterations to the sugar ring may also produce more effective nucleoside analogs since this makes them poorer substrates for pyrophosphorolysis, a process which removes the nucleotides from the chain, preventing its chain termination effect (4). Finally, the novel series of compounds will feature a modified uracil base, which may be structurally different enough to not confer cross resistance with 3-TC (13,16,17).

The new series of compounds will contain varying C-5 and in some cases C-6 substituents, such as halo, nitro, amino, alkyls, and substituted alkyls. Acyclic pyrimidine nucleosides with C-5 substituents, such as 1-[4-hydroxyl-3-(hydroxymethyl)-1-butyl]-5-(1-azido-2-chloroethyl)uracil, or 1-[4-hydroxy-3-(hydroxy-methyl)-1-butyl]-5-(1-azidovinyl)uracil have shown promising antiviral activity without being cytotoxic (15,18,19). As mentioned in chapter one, the C-5 substituents appear to contribute to anti-HBV activity and in some cases, the additional C-6 substituent may also increase the antiviral potential of the nucleosides (15,19,20). Specifically, the C-5 halogen substituent in various nucleoside series appears to contribute to inhibitory activity against

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different viruses, such as HIV and HSV, and may even be structure stabilizing (20-25).

In summary, the pyrimidine analogs that will be synthesized in this chapter possess a modified uracil base with an acyclic glycosyl moiety as in acyclovir, the 1-[(2-hydroxyethoxy)methyl] moiety, or in ganciclovir, 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl].

The new series of 5- and/or 6-substituted acyclic pyrimidine compounds with the 1-[(2-hydroxyethoxy)methyl] moiety [**15-28**] were prepared through a single step synthesis. The synthesis involved reacting respective silylated uracils possessing different substituents at the C-5 and/or C-6 positions, with 1,3-dioxolane in the presence of potassium iodide and chlorotrimethylsilane, as shown in figure 2.1. The compounds featuring the 1-[(2-hydroxy-1- (hydroxymethyl)ethoxy)methyl] chain [**42-54**] required a variety of reactions, as described in detail later. Compounds **42-54** were generally obtained by the reaction of silylated uracils with bromomethyl ether, as shown in figure 2.4, on page 87 (26-30,34,35).

The target compounds [**15-28 and 42-54**] will be synthesized and purified in this chapter, with the aim in later chapters of evaluating the effects of these compounds against HBV replication, and to determine the structure activity relationships for this novel series of compounds.

2.2 Materials and methods.

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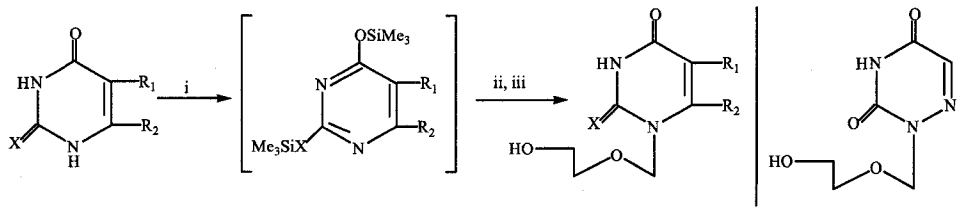
2.2.1 Synthesis of 5-and/or 6-substituted pyrimidines with a 1-[(2-hydroxyethoxy)methyl] glycosyl moiety [15-28].



Uracil [**1-14**] Product [**15-27**] Product [**28**]

Figure 2.1 Reaction scheme for the synthesis of modified 5-and/or 6-substituted pyrimidines with a 1-[(2-hydroxyethoxy)methyl] glycosyl moiety [**15-28**].

[Reagents: (i)Bis(trimethylsilyl)acetamide,dry acetonitrile ; (ii) 1,3-dioxolane, potassium iodide, trimethyl chlorosilane, 25 °C; (iii) quenched with MeOH and neutralized with $NaHCO_3$.]

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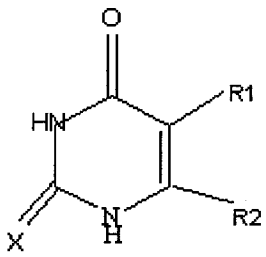
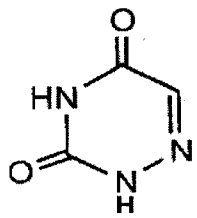


Uracil [**1-13**]



Uracil [**14**]

1 = R_1 = Br, R_2 = H, X = O
2 = R_1 = Cl, R_2 = H, X = O
3 = R_1 = NO_2, R_2 = H, X = O
4 = R_1 = NH_2, R_2 = H, X = O
5 = R_1 = C_2H_5, R_2 = H, X = S
6 = R_1 = H, R_2 = H, X = S
7 = R_1 = CF_3, R_2 = H, X = O
8 = R_1 = CH_2OH, R_2 = H, X = O
9 = R_1 = $COOC_2H_5$, R_2 = H, X = S
10 = R_1 = H, R_2 = CH_3, X = O
11 = R_1 = H, R_2 = $COOCH_3$, X = O
12 = R_1 = I, R_2 = CH_3, X = O
13 = R_1 = NO_2, R_2 = CH_3, X = O
14 = 6-Azauracil

Figure 2.2 Structures of uracil bases [**1-14**] used in the synthesis of target compounds [**15-28**].

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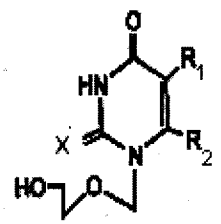
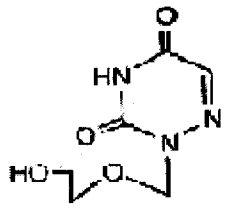


Compound [**15-27**]



Compound [**28**]

15 = R_1 = Br, R_2 = H, X = O

16 = R_1 = Cl, R_2 = H, X = O

17 = R_1 = NO_2, R_2 = H, X = O

18 = R_1 = NH_2, R_2 = H, X = O

19 = R_1 = C_2H_5, R_2 = H, X = S

20 = R_1 = H, R_2 = H, X = S

21 = R_1 = CF_3, R_2 = H, X = O

22 = R_1 = CH_2OH, R_2 = H, X = O

23 = R_1 = $COOC_2H_5$, R_2 = H, X = S

24 = R_1 = H, R_2 = CH_3, X = O

25 = R_1 = H, R_2 = $COOCH_3$, X = O

26 = R_1 = I, R_2 = CH_3, X = O

27 = R_1 = NO_2, R_2 = CH_3, X = O

28=1-[(2-hydroxyethoxy)methyl 5-Azauracil

Figure 2.3 Overall structures for target acyclic pyrimidine nucleosides, compounds **15-28**, possessing various substituents at the C-5 and/or C-6 positions of the uracil base with a 1-[(2-hydroxyethoxy)methyl] glycosyl moiety.

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Chemistry.

The target compounds 5-and/or 6-substituted 1-[(2-hydroxyethoxy)methyl]uracils (**15-28**) were achieved by a one step synthesis using iodomethyl [(trimethylsilyl)oxy]ethyl ether, prepared *in situ* from 1,3-dioxolane and trimethyl chlorosilane, instead of acetoxyethyl acetoxymethyl ether as alkylating reagent (31,32,33). By this method, steps involving synthesis of acyclic chain (2-acetoxyethyl acetoxymethyl ether) and deprotection of acetyl group were eliminated. Thus, uracils **1-14** silylated with bis (trimethylsilyl)acetamide in dry acetonitrile were reacted with trimethylchlorosilane, potassium iodide and 1,3-dioxolane at room temperature for 16-24 h to yield the desired compounds **15-28** (34).

1-[(2-hydroxyethoxy)methyl]-5-bromouracil (15). To a suspension of **1** (3 g, 15.7 mmol) in 40 mL dry acetonitrile, 9.0 mL (44.2 mmol) of bis(trimethylsilyl)acetamide was added and stirred at room temperature till a clear solution was obtained. To this solution, 1.2 mL (16.2 mmol) of 1,3-dioxolane, 2.6 g (15.6 mmol) of potassium iodide (KI) and 2.4 mL (22.09 mmol) of chlorotrimethylsilane were added. The reaction mixture was stirred at room temperature for 16 h at which time TLC (EtOAc: MeOH, 9.5: 0.5, v/v) indicated that the reaction was completed. The reaction medium was quenched with methanol and neutralized with 6.0 g of sodium bicarbonate. The solid obtained was purified by silica gel column chromatography using (EtOAc: MeOH 99: 1, v/v) to yield **15** as a solid (3.85 g, 92.5%): mp 150-152 °C (lit mp 147-148 °C)(31); ^{1}HNMR (DMSO-d_6) δ 3.55 (m, 4H, OCH_2CH_2O), 4.65 (br s, 1H, OH), 5.14 (s, 2H, NCH_2), 8.28 (s, 1H, H-6). Anal. ($C_7H_9BrN_2O_4$) C, H, N.

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The above procedure used for the synthesis of **15** was applied for the preparation of following compounds with variations as described.

1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (16). The reaction was carried out using **2** (0.5 g, 3.42 mmol), 40 mL of dry acetonitrile, 2 mL (9.8 mmol) of bis(trimethylsilyl) acetamide, 0.25 mL (3.37 mmol) of 1,3-dioxolane, 0.56 g (3.37 mmol) of KI and 0.5 mL (4.60 mmol) of chlorotrimethylsilane. Purification of the residue by silica gel column chromatography using EtOAc as eluent provided **16** as a solid (300 mg, 39.9%): mp 162 °C (dec.); ^{1}HNMR (DMSO-d_6)
δ 3.55 (m, 4H, OCH_2CH_2O), 4.64 (t, 1H, OH), 5.10 (s, 2H, NCH_2), 8.19 (s, 1H, H-6), 11.85 (s, 1H, NH). Anal. ($C_7H_9BrN_2O_4$) C, H, N.

1-[(2-hydroxyethoxy)methyl]-5-nitrouracil (17). The reaction was carried out **3** (1 g, 6.36 mmol), 40 mL of dry acetonitrile, 3.5 mL (17.2 mmol) of bis(trimethylsilyl)acetamide, 0.5 mL (6.75 mmol) of 1,3-dioxolane, 1.05 g (6.32 mmol) of KI and 0.95 mL (8.74 mmol) of chlorotrimethylsilane. Purification of the product by silica gel column chromatography using MeOH: EtoAc (1:9, v/v) as eluent yielded **17** as a light yellow solid (1.08 g, 73.8%): mp 200-202 °C; ^{1}HNMR (DMSO-d_6)
δ 3.48 (s, 2H, OCH_2), 3.65(s, 2H, CH_2O), 4.68 (t, 1H, OH), 5.28 (s, 2H, NCH_2), 9.26 (s, 1H, H-6), 12.03 (s, 1H, NH). Anal. ($C_7H_9N_3O_6$) C, H, N.

1-[(2-hydroxyethoxy)methyl]-5-aminouracil (18). A suspension of 5-aminouracil (**4**) (2.67 g, 21.0 mmol) in 40 mL of dry acetonitrile was stirred with 16.5 mL (81.1 mmol) of bis(trimethylsilyl)acetamide to get a clear solution. To this solution, 1.6 mL (21.5 mmol) of 1,3-dioxolane, 3.5g (21.0 mmol) of KI and 3.1 mL (28.5 mmol) of chlorotrimethylsilane were added and the reaction mixture was stirred at room

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temperature for 16 h. The reaction was then quenched by the addition of MeOH (20 mL) and neutralized with 4.27 g of sodium bicarbonate. The solid materials were removed by filtration. The filtrate was concentrated on rota-vacuo and the residue was purified from silica gel column using EtOAc: MeOH (8.5:1.5, v/v) as eluent to provide **18** as a creamy solid (1.41 g, 33.4%): mp 161-162 °C; ^{1}HNMR (DMSO-d_6)

δ 3.45 (m, 4H, OCH_2CH_2O), 4.18 (s, 2H, NH_2), 4.65 (t, 1H, OH), 5.02 (s, 2H, NCH_2), 6.8 0 (s,1H, H–6), 11.35 (s, 1H, NH). Anal. ($C_7H_{11}N_3O_4$) C, H, N.

1-[(2-hydroxyethoxy)methyl]-5-ethyl-2-thiouracil (19). The reaction was carried out using **5** (1.0 g, 6.4 mmol), 40 mL of dry acetonitrile, 3.5 mL (17.2 mmol) of bis(trimethylsilyl) acetamide, 0.95 mL (12.8 mmol) of 1,3-dioxolane, 2.12 g (12.7 mmol) of KI and 1.4 mL (12.8 mmol) of chlorotrimethylsilane. Purification of the product by silica gel column chromatography using MeOH:$CHCl_3$ (1:9, v/v) as eluent afforded **19** as a white solid (550 mg, 37.4%): mp 110-112 °C; ^{1}HNMR (DMSO-d_6) δ 1.15 (t, 3H, 5-CH_2CH_3), 2.40 (q, 2H, 5–CH_2CH_3), 3.60 (t, 2H, OCH_2), 3.68 (m, 2H, CH_2O), 4.81 (t, 1H, OH), 5.68 (s, 2H, NCH_2), 7.78 (s, 1H, H-6), 12.72 (s, 1H, NH). Anal. ($C_9H_{14}N_2O_3S$) C, H, N.

1-[(2-hydroxyethoxy)methyl]-2-thiouracil (20). Reaction of **6** (1 g, 7.8 mmol) in 40 mL of dry acetonitrile with bis(trimethylsilyl)acetamide (5 mL, 24.57 mmol), 1,3-dioxolane (1.15 mL, 15.5 mmol), KI (2.60 g, 15.6 mmol) and chlorotrimethylsilane (1.7 mL, 15.6 mmol), using the procedure described for the preparation of **20**, and purification of the product by silica gel column chromatography using MeOH:$CHCl_3$ (1:9, v/v) as eluent yielded **20** as a solid (140 mg, 9%): mp 148-150 °C; ^{1}HNMR (DMSO-d_6) δ 3.50 (m, 2H, OCH_2), 3.60 (m, 2H, CH_2O), 4.659 (t, 1H, OH), 5.58 (s, 2H, NCH_2), 5.98 (d, 1H,

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H-5), 7.82 (d, 1H, H-6). Anal. ($C_7H_{10}N_2O_3S$) C, H, N.

1-[(2-hydroxyethoxy)methyl]-5-trifluoromethyluracil (21). The reaction of 5-trifluoromethyluracil (**7**) (400 mg, 2.22 mmol) in 50 mL of dry acetonitrile with 1.25 mL (6.14 mmol) of bis(trimethylsilyl)acetamide, 0.25 mL (3.37 mmol) of 1,3-dioxolane, 0.55 g (3.33 mmol) of KI and 0.36 mL (3.32 mmol) of chlorotrimethylsilane provided **21** as a white solid (320 mg, 56.7%) after purification by silica gel column chromatography using MeOH:EtOAc (0.5:9.5, v/v to 0.3:9.7, v/v) as eluent: mp 142-145 °C (lit mp 142-143 °C) (31); ^{1}HNMR (DMSO-d_6) δ 3.50 (m, 2H, OCH_2), 3.58 (m, 2H, CH_2O), 4.65 (t, 1H, OH), 5.18 (s, 2H, NCH_2), 8.44 (s, 1H, H-6), 11.82 (s, 1H, NH). Anal. ($C_8H_9F_3N_2O_4$) C, H, N.

1-[(2-hydroxyethoxy)methyl]-5-hydroxymethyluracil (22). The reaction was carried out using **8** (0.5 g, 3.52 mmol) in 40 mL of dry acetonitrile, 2 mL (9.8 mmol) of bis(trimethylsilyl)acetamide, 0.26 mL (3.5 mmol) of 1,3-dioxolane, 0.58 g (3.49 mmol) of KI and 0.52 mL (4.78 mmol) of chlorotrimethylsilane and 1.5 g of $NaHCO_3$ to provide **22**. Purification of the residue by silica gel column chromatography using MeOH:EtoAc (2:8, v/v) as eluent afforded **22** as a white solid (500 mg, 65.8%): mp 170-172 °C; ^{1}HNMR (DMSO-d_6) δ 3.50 (m, 4H, OCH_2CH_2O), 4.15 (m, 2H, 5-CH_2OH), 4.62 (t, 1H, OH), 5.00 (t, 1H, CH_2OH), 5.10 (s, 2H, NCH_2), 7.58 (s, 1H, H-6), 11.35 (s, 1H, NH). Anal. ($C_8H_{12}N_2O_5$) C, H, N.

1-[(2-hydroxyethoxy)methyl]-5-carbethoxy-2-thiouracil (23). The reaction was carried out using **9** (0.5 g, 3.52 mmol) in 40 mL of dry acetonitrile, 2 mL (9.8 mmol) of bis(trimethylsilyl)acetamide, 0.26 mL (3.5 mmol) of 1,3-dioxolane, 0.58 g (3.49 mmol) of KI and 0.52 mL (4.78 mmol) of chlorotrimethylsilane and 1.5 g of $NaHCO_3$ to

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provide **23**. Purification of the residue by silica gel column chromatography using MeOH:EtoAc (2:8, v/v) as eluent afforded **23** as a white solid (500 mg, 65.8%): mp 170-172 °C; ^{1}HNMR (DMSO-d_6) δ 3.50 (m, 4H, OCH_2CH_2O), 4.15 (m, 2H, 5-CH_2OH), 4.62 (t, 1H, OH), 5.00 (t, 1H, CH_2OH), 5.10 (s, 2H, NCH_2), 7.58 (s, 1H, H-6), 11.35 (s, 1H, NH). Anal. ($C_8H_{12}N_2O_5$) C, H, N.

1-[(2-hydroxyethoxy)methyl]-6-methyluracil (24). The reaction was carried out using **10** (2 g, 15.8 mmol) in 50 mL of dry acetonitrile, 8.7 mL (42.7 mmol) of bis(trimethylsilyl)acetamide, 2.4 mL (32.3 mmol) of 1,3-dioxolane, 5.3 g (31.9 mmol) of KI and 3.5 mL (32.2 mmol) of chlorotrimethylsilane. Purification of the product obtained by silica gel column chromatography using MeOH:EtoAc (1.2:8.8, v/v) as eluent yielded **24** as a solid (660 mg, 20.82%): mp 186-188 °C; ^{1}HNMR (DMSO-d_6) δ 2.40 (s, 3H, CH_3), 3.58 (m, 4H, OCH_2CH_2O), 4.78 (t, 1H, OH), 5.34 (s, 2H, NCH_2), 5.64 (s, 1H, H-5), 11.34 (s, 1H, NH). Anal. ($C_8H_{12}N_2O_4$) C, H, N.

1-[(2-hydroxyethoxy)methyl]-6-methoxycarbonyluracil (25). The reaction of **11** (1 g, 5.88 mmol) in 40 mL of dry acetonitrile with 3.2 mL (15.7 mmol) of bis(trimethylsilyl)acetamide, 0.9 mL (12.14 mmol) of 1,3-dioxolane, 2.0 g (12.04 mmol) of KI and 1.3 mL (11.96 mmol) of chlorotrimethylsilane afforded **25** as a solid (270 mg, 19%), after purification by silica gel column chromatography using MeOH:$CHCl_3$ (0.6:9.4, v/v) as eluent: mp 150-151 °C; ^{1}HNMR (DMSO-d_6) δ 3.45 (m, 2H, OCH_2), 3.56 (m, 2H, CH_2O), 3.84 (s, 3H, CH_3), 4.62 (t, 1H, OH), 5.12 (s, 2H, NCH_2), 6.18 (s, 1H, H-5), 11.45 (s, 1H, NH). Anal. ($C_9H_{12}N_2O_6$) C, H, N.

1-[(2-hydroxyethoxy)methyl]-5-iodo-6-methyluracil (26). The reaction was carried out using **12** (0.5 g, 1.98 mmol) in 50 mL of dry acetonitrile, 1.08 mL (5.3 mmol)

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of bis(trimethylsilyl)acetamide, 0.3 mL (4.04 mmol) of 1,3-dioxolane, 0.660 g (3.97 mmol) of KI and 0.43 mL (3.95 mmol) of chlorotrimethylsilane and 1.58 g of $NaHCO_3$ to obtain **26**. Purification of the product by silica gel column chromatography using EtoAc as eluent and crystallization with MeOH yielded **26** as a solid (242 mg, 37.46%): mp 165-167 °C; ^{1}HNMR (DMSO-d_6) δ 2.64 (s, 3H, CH_3), 3.50 (m, 4H, OCH_2CH_2O), 4.60 (t, 1H, OH), 5.38 (s, 2H, NCH_2). Anal. ($C_8H_{11}IN_2O_4$) C, H, N.

1-[(2-hydroxyethoxy)methyl]-5-nitro-6-methyluracil (27). The reaction of **13** (1 g, 5.8 mmol) in 50mL of dry acetonitrile with 3.2 mL (15.7 mmol) of bis(trimethylsilyl)acetamide, 0.9 mL (12.1 mmol) of 1,3-dioxolane, 1.94 g (11.68 mmol) of KI and 1.3 mL (11.96 mmol) of chlorotrimethylsilane gave 27 as a solid (420 mg, 29.5%), after purification by column chromatography using MeOH:$CHCl_3$ (0.8:9.2, v/v) as eluent and crystallization using EtOAc: mp 107-109 °C; ^{1}HNMR (DMSO-d_6) δ 2.41 (s, 3H, CH_3), 3.48 (m, 2H, OCH_2), 3.55 (m, 2H, CH_2O), 4.68 (t, 1H, OH), 5.35 (s, 2H, NCH_2), 12.15 (s, 1H, NH). Anal. ($C_8H_{11}N_3O_6$) C, H, N.

1-[(2-hydroxyethoxy)methyl]-6-azauracil (28). The reaction was carried out using 6-azauracil (**14**) (1 g, 8.8 mmol) in 40 mL of dry acetonitrile with 5.0 mL (24.5 mmol) of bis(trimethylsilyl)acetamide, 0.65 mL (8.7 mmol) of 1,3-dioxolane, 1.47 g (8.85 mmol) of KI and 1.3 mL (11.9 mmol) of chlorotrimethylsilane. Purification of the product obtained by silica gel column chromatography using MeOH: EtOAc (1.5:8.5, v/v) as eluent provided the desired compound **28** as a white solid (842 mg, 51%): mp 144-146 °C; ^{1}HNMR (DMSO-d_6) δ 3.48 (m, 2H, OCH_2), 3.56 (m, 2H,CH_2O), 4.65 (t, 1H, OH), 5.20 (s, 2H, NCH_2), 7.51 (s, 1H, H-5), 12.22 (s, 1H, NH). Anal. ($C_6H_9N_3O_4$) C, H, N. (34).

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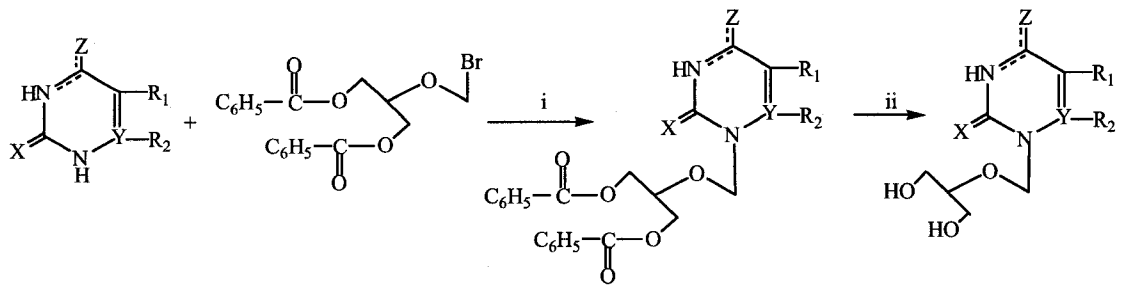
2.2.2 Synthesis of 5-and/or 6-substituted pyrimidines with a 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] glycosyl moiety [42-54].



Uracil [**29-41**] 2-(bromomethoxy)-1,3-propenediyl dibenzoate Product [**42-54**]

Figure 2.4 Reaction scheme for the synthesis of modified 5-and/or 6-substituted pyrimidines with a 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] glycosyl moiety [**42-54**].

[Reagents: (i) Hexamethyldisilazane, trimethylchlorosilane, potassium nonaflate, reflux with dry acetonitrile, (ii) 40% aqueous methylamine at 25 °C.]

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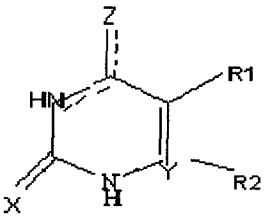

Uracil [**29-41**]

29 = R_1 = H, R_2 = H, X = O, Y = C, Z = O

30 = R_1 = F, R_2 = H, X = O, Y = C, Z = O

31 = R_1 = Br, R_2 = H, X = O, Y = C, Z = O

32 = R_1 = Cl, R_2 = H, X = O, Y = C, Z = O

33 = R_1 = CH_3, R_2 = H, X = S, Y = C, Z = O

34 = R_1 = CH=CHBr, R_2 = H, X = O, Y = C, Z = O

35 = R_1 = F, R_2 = H, X = O, Y = C, Z = NH_2

36 = R_1 = H, R_2 = CH_3, X = O, Y = C, Z = O

37 = R_1 = Br, R_2 = CH_3, X = O, Y = C, Z = O

38 = R_1 = I, R_2 = CH_3, X = O, Y = C, Z = O

39 = R_1 = H, X = O, Y = N, Z = O

40 = R_1 = CH_3, X = O, Y = N, Z = O

41= R_1 = CH_3, X = S, Y = N, Z = O

Figure 2.5 Structures of uracil bases [**29-41**] used in the synthesis of target compounds [**42-54**].

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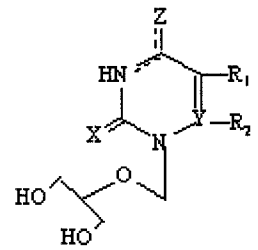

Compound [**42-54**]

42 = R_1 = H, R_2 = H, X = O, Y = C, Z = O

43 = R_1 = F, R_2 = H, X = O, Y = C, Z = O

44 = R_1 = Br, R_2 = H, X = O, Y = C, Z = O

45 = R_1 = Cl, R_2 = H, X = O, Y = C, Z = O

46 = R_1 = CH_3, R_2 = H, X = S, Y = C, Z = O

47 = R_1 = CH=CHBr, R_2 = H, X = O, Y = C, Z = O

48 = R_1 = F, R_2 = H, X = O, Y = C, Z = NH_2

49 = R_1 = H, R_2 = CH_3, X = O, Y = C, Z = O

50 = R_1 = Br, R_2 = CH_3, X = O, Y = C, Z = O

51 = R_1 = I, R_2 = CH_3, X = O, Y = C, Z = O

52 = R_1 = H, X = O, Y = N, Z = O

53 = R_1 = CH_3, X = O, Y = N, Z = O

54 = R_1 = CH_3, X = S, Y = N, Z = O

Figure 2.6 Overall structures for target acyclic pyrimidine nucleosides, compounds **42-54**, possessing various substituents at the C-5 and/or C-6 positions of the uracil base with a 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] glycosyl moiety.

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Chemistry.

For the synthesis of desired compounds, generally the 5- and/or 6- substituted uracils [**29-41**] were refluxed with hexamethyldisilazane (HMDS) and chlorotrimethylsilane (in a catalytic quantity) for approximately 3 h. The resultant clear solutions were then evaporated in vacuo to remove the excess of HMDS. The silylated intermediates produced were coupled with 2-(bromomethoxy)-1,3-propenediyl dibenzoate in dry acetonitrile for 6-12 hours at reflux temperature to yield the precursor ester derivatives (26). These ester derivatives were subsequently deprotected at 25 °C using 40% aq. methylamine to produce the desired 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]5-substituted uracils (35).

1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]uracil (42). A mixture of **29** (200 mg, 1.78 mmol), 0.77 g (1.96 mmol) of 2-(bromomethoxy)-1,3-propanediyl-dibenzoate, 0.36 mL (2.23 mmol) of hexamethyldisilane, 1.34 mL (12.33 mmol) of trimethylchlorosilane, 1.56 g (4.61 mmol) of potassium nonaflate in 100 mL dry acetonitrile was refluxed for 7 h at 80 °C. The reaction was brought to room temperature. The solid material was filtered off and washed with dichloromethane (20 mL). The mother liquor and washings were combined and concentrated on rota-vacuo. The residue obtained was redissolved in dichloromethane (300 mL) and washed with water. The organic phase was dried over anhydrous sodium sulfate, filtered, concentrated and purified on a silica gel column chromatography to yield the intermediate 1-[(2-benzoyloxy-(1-benzoyloxymethyl)ethoxy)methyl]uracil that was deprotected using 40% aq methylamine (60 mL) at room temperature for 2 h. The product was purified by silica gel column

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chromatography using EtOAc: MeOH (9.6:0.4) as eluent to yield **42** (140 mg, 36.3%). ^{1}HNMR (DMSO-d_6): δ 3.35-3.42 (m, 4H, OCH_2CH_2O) , 3.55 (m,1H, CH), 4.60 (m, 2H, OH), 5.18 (s, 2H, NCH_2), 5.60 (d, 1H, H-5), 7.64 (d, 1H, H-6), 11.25 (s,1H, NH). Anal. ($C_8H_{12}N_2O_5$) C, H, N.

1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-fluorouracil (43). The reaction of **30** (500 mg, 3.85 mmol) with 2-(bromomethoxy)-1,3-propanediyldibenzoate (1.66 g, 4.22 mmol), hexamethyldisilane (0.64 mL, 3.97 mmol), trimethylchlorosilane (2.6 mL, 23.93 mmol), and potassium nonaflate (3.38 g, 9.99 mmol) in 100 mL dry acetonitrile at 85 °C for 7 h, followed by debenzolyation using 34 mL of 40% aq. methylamine at room temperature, using the procedure outlines for the preparation of **42** and purification of the product by silica gel column chromatography using EtoAc: MeOH (8.6:1.4) as eluent, yielded **43** (600 mg, 66.66%). ^{1}HNMR (DMSO-d_6): δ 3.45-3.50 (m, 4H, OCH_2CH_2O), 3.54(m, 1H, CH), 4.65 (m, 2H, OH), 5.15 (s, 2H, NCH_2), 8.08 (d, 1H, H–6), 11.75 (s, 1H, NH). Anal. ($C_8H_{11}FN_2O_5$) C, H, N.

1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-bromouracil (44). The reaction of **31** (200 mg, 1.05 mmol) with 2-(bromomethoxy)-1,3-propanediyldibenzoate (0.45 g, 1.15 mmol), hexamethyldisilane (0.21 mL, 1.3 mmol), trimethylchlorosilane (0.78 mL, 7.72 mmol), and potassium nonaflate (0.920 g, 2.72 mmol) in 100 mL dry acetonitrile at 80 °C for 6 h, followed by debenzolyation using 40 mL of 40% aq. methylamine at room temperature, using the procedure outlines for the preparation of **42** and purification of the product by silica gel column chromatography using MeOH:EtoAc (0.5:9.5, v/v) as eluent, yielded **44** (150 mg, 48.56%). ^{1}HNMR (DMSO-d_6): δ 3.35-3.45 (m, 4H, OCH_2CH_2O), 3.58 (m, 1H, CH), 4.62 (m, 2H, OH), 5.20 (s, 2H, NCH_2), 8.22 (s, 1H, H-6), 11.78 (s,1H, NH).

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Anal. ($C_8H_{11}BrN_2O_5$) C, H, N.

1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-chlorouracil (45). The reaction of **32** (250 mg, 1.71 mmol) with 2-(bromomethoxy)-1,3-propanediyldibenzoate (0.74 g, 1.88 mmol), hexamethyldisilane (0.29 mL, 1.79 mmol), trimethylchlorosilane (1.2 mL, 11.05 mmol), and potassium nonaflate (1.5 g, 4.44 mmol) in 100 mL dry acetonitrile at 85 °C for 5 h, followed by debenzolyation using 20 mL of 40% aq. methylamine at room temperature, using the procedure outlines for the preparation of **42** and purification of the product by silica gel column chromatography using MeOH:$CHCl_3$ (1.2:8.8, v/v) as eluent, yielded **45** as a solid (200 mg, 46.72%): m.p. 142-145 °C; ^{1}HNMR (DMSO-d_6): δ 3.40-3.50 (m, 4H, OCH_2CH_2O), 3.55 (m, 1H, CH), 4.62 (m, 2H, OH), 5.20 (s, 2H, NCH_2), 8.18 (s,1H, H–6), 11.80 (s, 1H, NH). Anal. ($C_8H_{11}ClN_2O_5$) C, H, N.

1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-2-thio-5-methyluracil (46). The reaction of **33** (500 mg, 3.97 mmol) with 2-(bromomethoxy)-1,3-propanediyldibenzoate (1.72 g, 4.38 mmol), hexamethyldisilane (0.64 mL, 3.97 mmol), trimethylchlorosilane (2.67 mL, 24.58 mmol), and potassium nonaflate (3.49 g, 10.32 mmol) in 100 mL dry acetonitrile at 80 °C for 6 h, followed by debenzolyation using 35 mL of 40% aq. methylamine at room temperature, using the procedure outlines for the preparation of **42** and purification of the product by silica gel column chromatography using MeOH:EtOAc (0.4:9.6, v/v) as eluent, yielded **46** (600 mg, 65.74%). ^{1}HNMR (DMSO-d_6): δ 1.79 (s, 3H, CH_3), 3.37–3.42 (m, 4H, OCH_2CH_2O), 3.48 (m, 1H, CH), 4.60 (m, 2H, OH), 5.18 (s, 2H, NCH_2), 7.58 (s,1H, H–6), 11.22 (s, 1H, NH). Anal. ($C_9H_{14}N_2O_5$) C, H, N.

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1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-(2-bromovinyl)uracil (47). The reaction of **34** (150 mg, 0.691 mmol) with 2-(bromomethoxy)-1,3-propanediyldibenzoate (0.3 g, 0.763 mmol), hexamethyldisilane (0.11 mL, 0.682 mmol), trimethylchlorosilane (0.47 mL, 4.32 mmol), and potassium nonaflate (0.608 g, 1.78 mmol) in 50 mL dry acetonitrile at 80 °C for 2 h, followed by debenzolyation using 10 mL of 40% aq. methylamine at room temperature, using the procedure outlines for the preparation of **42** and purification of the product by silica gel column chromatography using MeOH:$CHCl_3$ (1.2:8.8, v/v) as eluent, yielded **47** (100 mg, 45.06%). ^{1}HNMR (DMSO-d_6): δ 3.35 (m, 2H, OCH_2), 3.42 (m, 2H, CH_2O), 3.58 (m, 1H, CH), 4.62 (m, 2H, OH), 5.20 (s, 2H, NCH_2), 6.84 ((d, 1H, CH (of vinyl)), 7.25 (d, 1H, CH (of vinyl)), 8.0 (s,1H, H–6), 7.58 (s,1H, NH). Anal. ($C_{10}H_{13}BrN_2O_5$) C, H, N.

1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-fluorocytosine (48). The reaction of **35** (200 mg, 1.55 mmol) with 2-(bromomethoxy)-1,3-propanediyldibenzoate (0.67 g, 1.70 mmol), hexamethyldisilane (0.26 mL, 1.61 mmol), trimethylchlorosilane (1.04 mL, 9.57 mmol), and potassium nonaflate (1.36 g, 4.02 mmol) in 100 mL dry acetonitrile at 80 °C for 6 h, followed by debenzolyation using 35 mL of 40% aq. methylamine at room temperature, using the procedure outlines for the preparation of **42** and purification of the product by silica gel column chromatography using MeOH:EtOAc (1:9, v/v) as eluent, yielded **48** (160 mg, 44.31%). ^{1}HNMR (DMSO-d_6): δ 3.38-3.42 (m, 4H, OCH_2CH_2O), 3.54 (m, 1H, CH), 4.58 (m, 2H, OH), 5.12 (s, 2H, NCH_2), 7.51-7.74 (2s, 2H, NH_2), 7.95 (d,1H, H–6). Anal. ($C_8H_{12}FN_3O_4$) C, H, N.

1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-6-methyluracil (49). The reaction of **36** (200 mg, 0.98 mmol) with 2-(bromomethoxy)-1,3-propanediyldibenzoate

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(0.57 g, 1.46 mmol), hexamethyldisilane (0.2 mL, 1.24 mmol), trimethylchlorosilane (0.73 mL, 6.72 mmol), and potassium nonaflate (0.86 g, 2.53 mmol) in 100 mL dry acetonitrile at 80 °C for 9 h, followed by debenzolyation using 40 mL of 40% aq. methylamine at room temperature, using the procedure outlines for the preparation of **42** and purification of the product by silica gel column chromatography using MeOH:EtOAc (0.8:9.2, v/v) as eluent yielded **49** as a white solid (80 mg, 26.54%): m.p. 145-147 °C; ^{1}HNMR (DMSO-d_6): δ 2.58 (s, 3H, CH_3), 3.30-3.46 (m, 4H, OCH_2CH_2O), 3.56 (m, 1H, CH), 4.38 (m, 1H, OH), 4.60 (m, 1H, OH), 5.42 (s, 2H, NCH_2), 11.75 (s,1H, NH). Anal. ($C_9H_{13}BrN_2O_5$) C, H, N.

1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-bromo-6-methyluracil (50). The reaction of **37** (200 mg, 0.98 mmol) with 2-(bromomethoxy)-1,3-propanediyldibenzoate (0.57 g, 1.46 mmol), hexamethyldisilane (0.2 mL, 1.24 mmol), trimethylchlorosilane (0.73 mL, 6.72 mmol), and potassium nonaflate (0.86 g, 2.53 mmol) in 100 mL dry acetonitrile at 80 °C for 9 h, followed by debenzolyation using 40 mL of 40% aq. methylamine at room temperature, using the procedure outlines for the preparation of **42** and purification of the product by silica gel column chromatography using MeOH:EtOAc (0.8:9.2, v/v) as eluent yielded **50** as a white solid (80 mg, 26.54%): m.p. 145-147 °C; ^{1}HNMR (DMSO-d_6): δ 2.58 (s, 3H, CH_3), 3.30-3.46 (m, 4H, OCH_2CH_2O), 3.56 (m, 1H, CH), 4.38 (m, 1H, OH), 4.60 (m, 1H, OH), 5.42 (s, 2H, NCH_2), 11.75 (s,1H, NH). Anal. ($C_9H_{13}BrN_2O_5$) C, H, N.

1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-iodo-6-methyluracil (51). The reaction of **38** (200 mg, 0.79 mmol) with 2-(bromomethoxy)-1,3-propanediyldibenzoate (0.47 g, 1.2 mmol), hexamethyldisilane (0.16 mL, 1.0 mmol), trimethylchlorosilane (0.53 mL, 4.88 mmol), and potassium nonaflate (0.7 g, 2.07 mmol) in

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100 mL dry acetonitrile at 80 °C for 9 h, followed by debenzolyation using 35 mL of 40% aq. methylamine at room temperature, using the procedure outlines for the preparation of **42** and purification of the product by silica gel column chromatography using MeOH:EtOAc (1.5:8.5, v/v) as eluent yielded **51** as a solid (80 mg, 28.31%): m.p. 142-143 °C; ^{1}HNMR (DMSO-d_6): δ 2.68 (s, 3H, CH_3), 3.25-3.42 (m, 4H, OCH_2CH_2O), 3.50 (m, 1H, CH), 4.38 (m, 1H, OH), 4.60 (m, 1H, OH), 5.40 (s, 2H, NCH_2), 11.60 (s,1H, NH). Anal. ($C_9H_{13}IN_2O_5$) C, H, N.

1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-6-azauracil (52). The reaction of **39** (200 mg, 1.77 mmol) with 2-(bromomethoxy)-1,3-propanediyldibenzoate (0.76 g, 1.95 mmol), hexamethyldisilane (0.3 mL, 1.86 mmol), trimethylchlorosilane (1.2 mL, 11.05 mmol), and potassium nonaflate (1.55 g, 4.58 mmol) in 100 mL dry acetonitrile at 80 °C for 5 h, followed by debenzolyation using 40 mL of 40% aq. methylamine at room temperature, using the procedure outlines for the preparation of **42** and purification of the product by silica gel column chromatography using MeOH:CH_2Cl_2 (1.5:8.5, v/v) as eluent, yielded **52** (80 mg, 20.80%). ^{1}HNMR (DMSO-d_6): δ 3.35- 3.45 (m, 4H, OCH_2CH_2O), 3.62 (m, 1H, CH), 4.57 (m, 2 H, OH), 5.27 (s, 2H, NCH_2), 7.48 (s,1H, H–5). Anal. ($C_7H_{11}N_3O_5$) C, H, N.

1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-methyl-6-azauracil (53) and 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-methyl-6-aza--2-thiouracil (54). Reaction of **41** (200 mg, 1.39 mmol), 603 mg (1.53 mmol) of 2-(bromomethoxy)-1,3-propanediyldibenzoate, 0.28 mL (1.73 mmol) of hexamethyldisilane, 1.04 mL (9.57 mmol) of trimethylchlorosilane, 1.23 g (3.34 mmol) of potassium nonaflate in 100 mL dry acetonitrile was refluxed for 10 h at 80 °C and stirred overnight at room temperature.

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The solid material obtained was filtered off, and washed dichloromethane (20 mL). The mother liquor and washings were mixed and concentrated on rota-vacuo. The residue obtained was redissolved in dichloromethane (300 mL). The organic phase was dried over anhydrous sodium sulfate, filtered, concentrated and purified on a silica gel column using MeOH:$CHCl_3$ (0.1:9.9, v/v) as an eluent to yield two products that were deprotected using 40% aq. methylamine for half an hour at room temperature and purified by silica gel column chromatography.

Product A eluted with EtOAc: MeOH (8.4:1.6, v/v) to provide compound **53** as a sticky solid (110 mg, 34.06%). ^{1}HNMR (DMSO-d_6): δ 1.99 (s, 3H, CH_3), 3.39-3.48 (m, 4H, OCH_2CH_2O), 3.55 (m, 1H, CH), 5.25 (s, 2H, NCH_2), 4.2- 4.8 (br, 2H, OH). Anal. ($C_8H_{13}N_3O_5$) C, H, N.

Product B eluted with EtoAc: MeOH (8.5:1.5, v/v) to yield **54** as a solid (50 mg, 14.48%): m.p. 150-152 °C; ^{1}HNMR (DMSO-d_6): δ 2.12 (s, 3H, CH_3), 3.35-3.49 (m, 4H, OCH_2CH_2O), 3.75 (m, 1H, CH), 4.60 (m, 2H, OH), 5.71 (s, 2H, NCH_2), 13.22 (s, 1H, NH). Anal. ($C_8H_{13}N_3O_4S$) C, H, N. (35).

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2.2.3 Purification and structural confirmation of compounds synthesized.

Melting points were determined with a Buchi capillary apparatus and are uncorrected. ^{1}H NMR spectra were determined for solutions in Me_2SO-d_6 on a Bruker AM 300 spectrometer using Me_4Si as an internal standard (34,35). The assignment of all exchangeable protons (OH, NH) was confirmed by the addition of the D_2O. Micro-analyses were within $\pm$ 0.4% of theoretical values for all elements listed, unless otherwise indicated. Silica gel column chromatography was carried out using Merck 7734 silica gel (100-200 μM particle size). Thin layer chromatography was performed with Machery-Nagel Alugam SiL G/UV silica gel slides (20 μM thickness). Substituted uracils were purchased from Sigma-Aldrich Chemical Co (34,35).

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Chapter Three: Anti-HBV evaluation of novel acyclic pyrimidine nucleoside analogues using a 2.2.15 HBV cell line and a DHBV infected primary duck hepatocyte assay. And determining cytotoxicity of the compounds in HepG2 and Vero cells.

3.1 Introduction.

The 5-and/or 6-substituted acyclic pyrimidine nucleosides synthesized in chapter two are investigated in this chapter for their *in vitro* antiviral activities. There are many *in vitro* models that have been developed for the purpose of studying HBV. Some of these include, the HepaRG cell line, which is based on human hepatoma cells infected with HBV. However a limitation with this cell line, is that in order for it to maintain HBV infection and hepatocyte function, it requires constant dimethyl sulfoxide or corticoid administration, agents which may obscure drug evaluation and toxicity testing results (1). Nonhepatic cells, such as murine fibroblasts, have been transfected with HBV, but the resulting cell line produced a decreased HBc antigenicity, a lesser quantity of large surface protein, and unstable virions (2). There is also the option of using a primary culture of woodchuck hepatocytes, which exhibits long term stability and survival. However, the WHV infection appears to be a more drawn out process, with slower replication within hepatocytes and a delayed emergence of cccDNA (3-5). Instead, two common *in vitro* systems used for hepatitis B investigations and for anti-HBV drug evaluation are assays based on the primary culture of DHBV infected duck hepatocytes and the 2.2.15 cell line. Both models are available, inexpensive, commonly

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used for assays, and when combined serve as predictors for the antiviral effect of potential drugs *in vitro* (6).

One of the systems that was used to evaluate the anti-HBV effectiveness of this series of compounds was the primary hepatocyte culture using ducks congenitally infected with DHBV. This system does rely on a DHBV infection rather than an HBV infection. However, the DHBV infected duck is often used for studying compounds that may be effective against HBV, even though the DHBV has a few distinguishing features. One such characteristic is that DHBV core particles have surface spikes and are considered fragile since many core particles are slightly disrupted (7,8). DHBV surface antigen particles are also morphologically different from HBV surface antigens. They have a lesser buoyant density and are spherically shaped, with a 40-60 nm diameter, as opposed to the 22 nm spherical and filamentous HBsAg (8-10). The DHBsAg is smaller in size; the molecular weight of this polypeptide is 17 500 daltons, while the HBV counterpart is 25 000 to 29 000 daltons (with the upper weight representing the glycosylated version of the lighter polypeptide), but the DHBsAg polypeptide tryptic map is similar to that of the non glycosylated version of the HBsAg adw type polypeptide (10). The DHBV also encodes for two envelope protein (L and S), and does not encode the third envelope protein (M) common in mammalian viruses. They also do not have much sequence homology (less than 10%) with HBV (7,11). In addition, their viral genomes are slightly lesser in size; the DHBV genome is slightly smaller with approximately 3000 base pairs, as compared to the HBV genome of roughly 3200 base pairs (7,11). Finally, the DHBV lacks the open reading frame X (ORF X), which typically encodes for regulatory proteins (11-13). However on this point, there have been

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studies, specifically one done by Chang at el. (14), which discusses the presence of a hidden open reading frame. This open reading frame can encode for DHBx, a transcription regulatory protein with similar functions to the mammalian counterpart. However, it has been demonstrated *in vitro* that the mammalian HBx protein is not essential for viral replication, transcription, and structural protein production (12). Nonetheless, the DHBV is not the only viral species to have differences with the HBV. For instance, the WHV has minimal nucleic acid homology with the HBV (3-5%), and morphologically different surface antigen particles (8,15). Meanwhile, there is limited evidence of liver disease being linked to GSHV, and limited liver histological changes result from the infection (16,17). The woodchuck may serve as a good model for the course of hepatocellular carcinoma (HCC), since the WHV is clearly associated with a high frequency of liver disease and has been shown to have integrated viral DNA in liver tumours (8,9). However as a disadvantage, the woodchuck is not domesticated and breeds at a reduced rate, as compared to the duck, which reduces their availability (9). The ground squirrels also generally breed solely in the middle of spring (16).

Despite the differences between DHBV and HBV, they share the important features of genome structure and relative size (all are partially double stranded circular genomes maintained by its cohesive ends, of approximately the same size in the range of 3-3.3kb), virion structure, replication mode and use of a DNA polymerase, limited host range, significant hepatatropism, sub viral particles, and general antigenic composition (7,8,11,13,16-19). Thus, the DHBV infected Pekin duck can be utilized both as an animal model and for *in vitro* testing (7,8,11,18,20). There is even similarity in the DHBV polymerase dNTP binding pocket, compared to its HBV counterpart, and the

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same chain terminating mechanism of action for nucleoside analogs, such as 3-TC, is considered applicable (21,22). As well, the common liver disease linked to HBV, HCC, has been displayed in DHBV infected ducks (9). Thus, overall these viruses are quite similar. For *in vitro* assays, a duck primary hepatocyte culture system has been widely used. This tissue culture assay has exhibited the standard presence of DHBV replicative intermediates, proteins, and surface antigens, and supports full cycles of *de novo* viral replication (20,23). By using congenitally infected ducks, it can be assumed that each of the hepatocytes in culture can support replication (20). Therefore, a primary culture system based on duck hepatocytes congenitally infected with DHBV is a valid assay that can be used for antiviral assessment.

The second system that was used for *in vitro* evaluation of antiviral activity was an assay based on the 2.2.15 cell line. The 2.2.15 cell line was originally developed in the George Acs laboratory (24). This is a human hepatoblastoma cell line (HepG2 cells) transfected with the HBV genome, thus the DNA is integrated. Associated with this cell line are the correct sizes and patterns of viral RNA transcripts, replicative intermediates, surface antigen, and proteins. This cell line is useful in that there is high rates of viral replication associated with these cells, and HBV virions (with infectivity capability) are constantly released (24,25). This system is also advantageous in that it allows for longer term studies of replication (24). Moreover, as with carcinoma cell lines, the hepatocytes are more likely to maintain their functional capabilities (such as plasma protein synthesis) and differentiation unlike other immortal cell lines (26,27). This assay has been used under numerous applications for studying HBV replication and as a screening assay for compounds that may have an inhibitory effect on HBV replication (24,28-30).

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The standard MTT cell proliferation assay was used to determine if any toxic effects could be attributed to these novel compounds. This colorimetric assay is based on viable cells being able to cleave the yellow MTT tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into purple formazan crystals (Roche kit). The MTT assay is sensitive, a good indicator of mitochondrial and other forms of toxicity, and allows for a quantification of cell viability (26,31). This assay was performed using HepG2 and Vero cells. The HepG2 cell line is derived from a human hepatocellular carcinoma by Knowles et. al., and forms the basis for the 2.2.15 cell line (27). This cell line features continuous replication and the cells generally retain hepatocyte function (26,32,33). The HepG2 cells have been used previously for toxicity testing, especially since these cells have a significant mitochondria DNA presence (26,32). The other cell type used for the MTT assay were Vero cells, which are derived from the African green monkey kidney tissue, by Y. Yasumura and Y. Kawakita (34). Vero cells are also a standard cell type used for toxicity testing (31).

The series of nucleoside analogs synthesized in chapter two were investigated to determine if they were able to have an inhibitory effect on DHBV replication in the primary DHBV infected duck hepatocyte assay and on human HBV in the 2.2.15 cell assay. The compounds were tested at various concentrations in triplicate wells every alternate day for a two week period. Generally, the dot blot method was used to display the activity of these compounds and to give an indication of their respective level of antiviral activity. In the case, with compound **16**, 1-[(2-hydroxyethoxy)methyl]5-chlorouracil, where it exhibited the strongest activity in the primary culture assay of DHBV congenitally infected hepatocytes, real-time PCR (RT-PCR) was used to obtain a

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more sensitive quantification of its antiviral ability (35-37). The acyclic pyrimidine nucleoside analogs were also tested to determine if there was any cytotoxicity resulting from a four day incubation with the compounds, by using the MTT cell proliferation assay. HepG2 and Vero cells were used, and the compounds were again tested in triplicates, at concentrations of 100, 10, and 1 μg/mL.

3.2 Materials and Methods.

3.2.1 DHBV congenitally infected duck liver perfusion.

Primary duck hepatocytes were obtained by performing liver perfusions (adapted Tuttleman (20) method) on DHBV congenitally infected ducks from the University of Alberta farms. When born, the ducklings were confirmed to be positive for DHBV by a dot blot hybridization using their sera. The ducks used for liver perfusions were approximately two weeks old.

The duck was euthenized through an injection in the duck leg vein with euthenal and washed. The abdomen was cut open and a catheter was attached to the right ventricle of the heart, and the other end was attached to the perfusion pump. After cutting the hepatic portal vein, a warmed (37 °C) perfusion solution [500 mL of MEM (Sigma) supplemented with 5 mL of 100x penicillin G (Sigma)/streptomycin sulfate (Gibco), and 2.5 mL of 100 mM EGTA] was pumped through the duck, until the liver changed color to a light brown and all the blood was drained. A warmed (37 °C) collagenase solution [200 mg of collagenase (Sigma, type IV), 200 μl of 1 mg/mL insulin, and 2 mL of 100x penicillin G (Sigma)/streptomycin sulfate (Gibco) supplemented in Hank's salt solution

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with Ca^{2+}] was then infused more slowly through the duck. The liver was then washed with 50 mL of cold (4 °C) wash solution [50 mL EGTA base (Hank's solution without Ca^{2+} and Mg^{2+}) with 5mL of 50 mM EGTA , 50 µl of 1 mg/ml insulin, 0.5 mL of 100x penicillin G (Sigma)/streptomycin sulfate (Gibco), then a second cold (4 °C) wash solution [50mL of EGTA base (Hank's solution without Ca^{2+} and Mg^{2+}) with 0.26 mL of heat inactivated FBS, 2.5 mL of 50 mM EGTA, and 0.5 mL of 100x penicillin G (Sigma)/streptomycin sulfate (Gibco)]. The liver was then removed and teased in petri dishes filled with warm L-15 medium with L-glutamine [supplemented with 5% heat inactivated FBS (Gibco), 5 mL of 100x penicillin G (Sigma)/streptomycin sulfate (Gibco)]. The liver suspension was filtered with sterile gauze, and the resultant suspension was spun at <1000 rpm in a Sorval centrifuge. The cells were washed at least three times, using fresh L-15 media, allowing the hepatocytes to settle and removing the overlying media, before a final spin at <1000 rpm in a Sorval centrifuge, and then the cells were diluted in L-15 media for counting and plating.

3.2.2 Primary duck hepatocytes.

The hepatocytes obtained from the liver perfusion were counted using a hemacytometer counter and cell viability was taken into account by using the trypan blue dye. The cell suspension was diluted to a concentration of $1x10^6$ million cells/mL in supplemented L-15 media. Each well of treated 6-well plates, received 3 mL of the cell suspension to result in $3x10^6$ hepatocytes/well, which created a confluent monolayer. The cells were kept in a humidified 37 °C incubator with 0 CO_2 % level. The primary

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duck hepatocytes medium consisted of 500 mL of L-15 Leibovitz medium (Sigma), with L-glutamine, supplemented with 5% heat inactivated FBS (Gibco), 5 mL of 100x penicillin G (Sigma)/streptomycin sulfate (Gibco), and was sterilized with a 0.22 μm filter. The cells were left overnight and then the media was changed. The following day the schedule of drug treatments began.

3.2.3 2.2.15 cells.

The cells were kept in a humidified 37 °C incubator with a 5% CO_2 level. The 2.2.15 cell culture medium consisted of 500 mL of MEM (Sigma), supplemented with 10% heat inactivated FBS (Gibco), geneticin/G148 sulfate (final concentration 380 μg/mL), 5-7 mL of 7.5% sodium bicarbonate, and L-glutamine (final concentration of 2 mM), and was sterilized with a 0.22 μm filter. The cells were grown from a liquid nitrogen stocks, until they were seeded from a confluent T150 flask into treated 6-well plates using a trypsin solution (pH 7.4-7.5). When the wells exhibited a confluent cell layer, the drug treatments were initiated.

3.2.4 Drug solutions and treatments.

The target acyclic pyrimidine nucleoside analogs [**15-28** and **42-54**] were initially dissolved in DMSO to make a concentration of 10 mg/mL The compounds were then diluted into a sterile phosphate buffer solution, PBS (pH 7.3), and then further diluted into individual aliquots of cell culture medium; a variety of concentrations were used.

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The drug solutions were mixed and left covered with aluminum foil, to avoid potential decomposition of the compounds. The solutions were kept in a 4 °C cold room. Prior to a drug treatment, the solutions were warmed in the 37 °C incubator.

The compounds were tested in triplicate for each concentration. The drug solutions were added at a volume of 3 mL per well, replacing the prior media. The drug treatments began when the wells reached confluence and continued for two weeks, with drug treatments occurring every second day, for seven treatments in total. On the day subsequent to the last treatments, the cells were harvested.

3.2.5 Harvesting the primary duck hepatocytes and 2.2.15 cells, and isolating intracellular viral nucleic acids.

The cell layer in each well was rinsed with 3 mL of PBS, pH 7.3, warmed to 37 °C. Then 1 mL of room temperature lysis solution #1 (10 mM tris.HCl pH 7.5, 1 mM EDTA pH 8.0, 50 mM NaCl, 8% sucrose, 0.25% nonidet P-40) was added. The cell layer/suspension was transferred to sterile/autoclaved eppendorf tubes. The samples were microfuged for 5 minutes at 12 000 rpm, and the supernatant was transferred to new autoclaved eppendorf tubes, and the pellets were discarded. After adding 6mM $MgCl_2$ and DNAse/RNAse solution (a final volume of 20 µg was required for both the DNAse and RNAse per sample), the samples were vortexed and left in a 37 °C water bath for 45 minutes-1hour. 330 µl of 26% PEG in 1.4 M NaCl and 25 mM EDTA was added, and the samples were left in a 4 °C ice bath for at least 30 minutes. The supernatant was then removed after microfuging the samples at 4 °C for 4 minutes at 12000 rpm. To the

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visible pellet remaining, 200 µl of lysis solution #2 (10 mM tris.HCl pH 7.5, 5 mM EDTA, 1% SDS) was added. For protein digestion, 10 µl of 20 mg/mL fungal proteinase k (Invitrogen) was added. Samples were vortexed and left in a 42-55 °C water bath overnight, until there was no visible pellet remaining. For the phenol/chloroform extraction, one volume (200 µl) of tris saturated phenol was added, then the samples were vortexed. Followed by the addition of two volumes of chloroform (400 µl), and again the samples were vortexed. The samples were microfuged for 5 minutes at 12000 rpm, and 200 µl of the aqueous phase was transferred to new autoclaved tubes. To ethanol precipitate out the DNA, 0.1 M NaCl, 1 µl of 20 mg/mL yeast tRNA, and 2 volumes (400 µl) of ice cold 95% ethanol were added. The samples were vortexed, then left in –20 °C, for longer than 30 minutes or in –70 °C for 30 minutes. The samples were then microfuged for 10-15 minutes at 4 °C, and the salt was rinsed out with the addition of 3 volumes (600 µl) of ice cold 70% ethanol, and again the samples were microfuged for 2 minutes at 4 °C. The remaining pellet was left to air dry overnight, after the supernatant was removed. The dry DNA pellet was then resuspended in 50 µL of TE buffer (10 mM tris.HCl, 1 mM EDTA), in preparation for its use in the dot blot assay.

3.2.6 Dot blot hybridization and semiquntitative detection of viral DNA (for both the primary duck hepatocyte and 2.2.15 cell assays).

Using the dot blot Bio-Dot apparatus (Bio-Rad), 10 or 15 µL of each sample was spotted on the nylon membrane (Hybond-N+, Amersham Biosciences). The nylon membrane was then denatured by placing it face up on top of filter paper soaked in

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denature solution (0.5 M NaOH, 1.5 M NaCl) for 30 minutes. This process was then repeated with neutralization solution (0.5 M tris.HCl pH 8.0, 1.5 M NaCl) for 30 minutes or longer. To fix the nucleic acids to the membrane, it was then UV cross linked for 3 minutes using a UV transilluminator (UVP Inc.). Membranes were then prehybridized with a buffer (30% of 20xSSC, 70% of 10% SDS) for at least two hours at 65 °C. Either α-^{32}P 2.2.15 HBV plasmid or α-^{32}P DHBV core sequence DNA radiolabeled probe (using plasmid, pALTer) was used. The probe was denatured either by heat or by alkaline lysis. Radiolabeled probe was then added (10^6 CPM/mL), and left overnight for hybridization. To remove excess probe, two sets of washes (of 1xSSC/0.1% SDS solution) were performed for 15-30 minutes each, followed by one wash (of 0.1SSC/0.1% SDS) for 15-30 minutes. The membrane was then visualized using autoradiography, and the results were quantified using the NIH Image 1.62b7 program. The percent replication was calculated for each set of triplicate wells for each compound at a particular concentration. This calculation involved taking the average of the triplicate wells (in terms of their intensity measurement in pixels) and subtracting from it the average of the background of the membrane, which was all divided by the average of the triplicates of the wells treated with only media and DMSO (the negative control). This value was then multiplied by one hundred to convert it to a percentage. The percent inhibition of the compound was derived from the percent replication.

Percent replication:

[(average intensity of triplicate wells in presence of compound) – (background average)]/ (DMSO+media treated average) *100

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Percent inhibition:

100-percent replication

3.2.7 Real-time PCR quantification for the inhibition of viral replication by the compound, 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (16) in the duck primary culture assay.

Real-time PCR (RT-PCR) reactions were performed using the LightCycler machine (Roche Diagnostics), and a Lightcycler Faststart DNA Master Sybr Green 1 kit (Roche Applied Science, Germany). DHBV standards were based on the plasmid of DHBV, using pALTer, and ranged from the 1ng to 1 ft, separated in increments of one log. These DHBV standards were dissolved in TE buffer, since the DNA samples to be studied were also reconstituted in this buffer. The primers used were JS-5, 5'-TGAAGCAATCACTAGACC-3' and JS-28, 5'-ATGGTGGCTGCTCGAACT-3'. Prior to performing studies with experimental samples, $MgCl_2$ titrations ranging from final concentrations of 2.25 to 4 mM were carried out with the DHBV standards, as well different primer concentrations were tested. A final concentration of 3.34 mM of $MgCl_2$, 0.5 μM of each primer, and 1 μL of each DNA template was used for subsequent trials. The reactions were performed as per the manufacturer's instructions. Copy numbers were calculated for the DHBV standards and inputted into the program. Noise was removed form the readings, and minimal error applied, and standard curves were plotted.

In the first study with experimental samples, one well from each of the 1-[2-hydroxyethoxy]methyl]-5-chlorouracil (**16**) concentrations (20, 10, and 1 μg/mL) were

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tested against an untreated well. These samples were the intracellular viral DNA extractions from a previous experiment where the results were displayed using the dot blot. A repeat RT-PCR experiment was performed using all three wells of the 1-[2-hydroxyethoxy]methyl]-5-chlorouracil (**16**) from the same experiment, with 3-TC and untreated samples. The mean viral copy number for the samples/wells of each compound at each concentration was plotted in a standard curve.

3.2.8 MTT cell proliferation assay using HepG2 and Vero cells.

Cell viability was studied using the cell proliferation 1 (Roche, Cell Proliferation Kit 1/MTT) kit, as per the manufacturer's instructions. For this assay, both HepG2 cells and Vero cells (obtained from Holly Saffron, University of Alberta) were used. HepG2 cells were maintained in a cell culture media consisting of 500 mL of MEM supplemented with 10% heat inactivated FBS (Gibco), 5-7 mL of 7.5% sodium bicarbonate, 5mL of 100x penicillin G (Sigma)/streptomycin sulfate (Gibco), and L-glutamine (final concentration of 2 mM). The Vero cells were grown using 500 mL of D-MEM media (Gibco), supplemented with 5% heat inactivated FBS (Gibco), 5mL of 100x penicillin G (Sigma)/streptomycin sulfate (Gibco), and L-glutamine (final concentration of 2 mM). The cell culture medium were filtered with 0.22μm filters. Drug solutions (featuring compounds **15-28** and **42-54**) were originally in a concentration of 10 mg/mL in DMSO, and were made into 100, 10, and 1 μg/mL, concentrations by diluting the compounds into cell medium. Both HepG2 cells and Vero cells were seeded into separate 96 well plates at a concentration of 5 x 10^5 cells per well (100 μl of 5000

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cells/µl cell suspension). 50 µl of each drug solution were added to each well. Each drug concentration was tested in triplicates. The cells were left to incubate for 4 days in a 37 °C incubator with a 5% CO_2 level with the drug solutions. Then 15 µl of MTT reagent was added to each well. The cells were then further incubated for an additional 4 hours in a 37 °C incubator with a 5% CO_2 level. 100 µl of solubilization solution (10% SDS in 0.01 M HCl) was added to each well, and the plates were then left in the 37 °C incubator with a 5% CO_2 level overnight. Each well was then pipetted to break any formazan crystals that remained, and the plates were swirled on a plate shaker, and mixed using the microplate reader function. The plates were read on a microplate spectrophotometer reader (Spectra Max Plus[384], Molecular Devices Corporation) using 570 nm as the first reference wavelength, and 650 nm as the second reference wavelength. To quantify the results, the wells that were left blank represented the background readings, the average of which was subtracted from each individual well reading. An average was taken of the triplicate wells for each drug at each concentration, and this value was compared to the wells treated with just cell culture media for each plate, to account for the percent absorbance. The toxicity percentage was derived from the percent absorbance value.

Percent absorbance:

[(average absorbance of each drug concentration in triplicate)-(background average)]/average of triplicate wells treated with cell culture media)*100

Percent toxicity:

100-percent absorbance

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3.3 Results.

3.3.1 The inhibitory effect of the compounds against DHBV replication using a primary DHBV congenitally infected duck hepatocyte culture.

The summaries of compounds that were evaluated for antiviral activity against DHBV are included as tables 3.1 and 3.2. The data is expressed as the percent inhibition of viral DNA replication, in the presence of 10 μg/mL of the particular compound, when compared to the untreated DHBV infected controls. The EC_{50} represents the drug concentration (μg/mL) required to reduce the viral DNA in infected cells to that of 50% of the untreated controls; basically, the concentration that can reduce 50% of the viral DNA levels. At 10 μg/mL, the inhibition of DHBV was calculated and standard deviations were within 10% of the average values. After the preliminary testing at 10 μg/mL, the compounds were serially diluted to obtain more precise EC_{50} values. Tests were repeated two to three times. In the case where the EC_{50} obtained from three experiments was within a 10% standard deviation, average values are given, otherwise a range of EC_{50} values is shown.

Specifically, the summary of the anti-DHBV activity of the 5-and/or 6-substituted 1-[(2-hydroxyethoxy)methyl] uracils [**15-28**] is included as table 3.1. In this series, the 5-bromo-(**15**), 5-chloro-(**16**), 5-amino-(**18**), and 5-triflouromethyl-(**21**) analogs were amongst the most potent inhibitors of DHBV, with respective EC_{50} values of 1-5, 0.1-0.5, 1 and 1-10 μg/mL. The 5-nitro (**17**), 5-hydroxymethyl (**22**), and 5-carboxyethyl (**23**) analogs also exhibited effective inhibition of DHBV with EC_{50}s of 10 μg/mL. With respect to the series featuring the C-6 substituents, either alone or coupled with a C-5

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substituent, the 6-methyl (**24**), 6-carbomethoxy (**25**), 5-iodo-6-methyl (**26**), and 5-nitro-6-methyl (**27**) analogs all shared EC_{50} values of 5-10 μg/mL, also demonstrating effective anti-DHBV activity. However, the 1-[(2-hydroxyethoxy)methyl]-6-azauracil (**28**) displayed quite reduced antiviral activity, with only 25% inhibition of viral replication at 10 μg/mL. This suggests that the substitution at the 6-position of a carbon atom with a nitrogen is unfavorable to antiviral activity. Overall, in this series the 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) displayed the greatest anti-DHBV activity. An example of a dot blot featuring the effect of this derivative can be seen in figure 3.1. The 5-bromo analog (**15**) displayed an EC_{50} of 1-5 μg/mL, which is approximately ten fold less potent than the 5-chloro derivative (**16**). In a previous study, a derivative with a 5-iodo substituent was also investigated and found to have an EC_{50} of 10 μg/mL. Therefore it seems that in this series, a C-5 halogen substituent has a direct effect on the anti-DHBV activity. It should be noted that in comparison to 3-TC (the reference drug and positive control), the 5-chloro derivative (**16**) was ten times less active.

Table 3.2 outlines the anti-DHBV activity of the 5-and/or 6- substituted 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] uracils [**42-54**]. From this series, the 5-chloro (**45**), 5-(2-bromovinyl) (**47**), and 5-bromo-6-methyl (**50**) analogs emerged as the most potent DHBV antivirals with EC_{50} values of 1-5, 1-5, and 5-10 μg/mL, respectively. The anti-DHBV activity of compounds **45** and **47** was found to be similar to that of corresponding potent acyclic purine nucleoside analogs, which was reported to have an EC_{50} of 1.5 μg/mL in the DHBV assay (55). At 10 μg/mL, modest to no inhibition was found with compounds **43**, **44**, **46**, **48**, **49,** and **51-54.** In addition, the 5-unsubstituted uracil (**42**) had absolutely no anti-DHBV activity, confirming the importance of the C-5

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substituent in this series of pyrimidine analogs. Amongst the halogen C-5 substituents, there were differential antiviral effects, with the 5-bromo (**44**) and 5-chloro (**45**) analog exerting a greater inhibitory effect than the 5-flouro (**43**) derivative. This may be due to the impact of the different sizes and/or electronegativities of the various halogens. Another structure activity relationship noted is that the change of the thymine analog (**46**) to the 6-azathymine derivative (**53**) had no effect on resulting antiviral activity. However, the presence of a double bond at the 5-position tends to increase the inhibitory activity of the analog as seen with compound **47**, which agrees with other reported data (38-41). Again, in this series, the compound with the most potent activity featured the 5-chloro (**45**) substituent. However, the compound **45** possessing 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] side chain exhibited reduced antiviral activity as compared to the analogous compound **16** in the 1-[(2-hydroxyethoxy)methyl] uracil series (table 3.1). The same trend was apparent with compounds **49** and **51** and their 1-[(2-hydroxyethoxy)methyl] counterparts, compounds **24** and **26**. Nevertheless, in both series, the major determinant of the difference in activities of the acyclic pyrimidine nucleoside analogs was the nature of the C-5 substituent.

To confirm the potency of the 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**), the intracellular viral DNA extracts from an experiment, was used for RT-PCR. In the first run of RT-PCR, one well each of the concentrations of 1-[(2-hydroxyethoxy)methyl] 5-chlorouracil (**16**) at 20, 10, and 1 μg/mL was tested against one untreated well. At 20 μg/mL, 1-[(2-hydroxyethoxy)methyl] 5-chlorouracil (**16**), was able to completely inhibit DHBV viral replication, as no virion copy numbers were detected and it paralleled the negative control (TE buffer). At 10 and 1 μg/mL, there was an approximately 1.7 and 1.2

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log reduction in viral DNA, (respectively), compared to the untreated control. A second run was also performed including samples of wells treated with 1-[(2-hydroxyethoxy)methyl] 5-chlorouracil (**16**), 3-TC (at 20, 10, and 1 μg/mL), and the untreated samples (wells treated solely with media). At 20 μg/mL, 1-[(2-hydroxyethoxy)methyl] 5-chlorouracil (**16**), was associated with a 3.5 log reduction, and even at 1 μg/mL there was approximately a 1.3 log reduction in viral DNA. These results can be seen in figure 3.2. The RT-PCR was used to confirm the findings observed in the dot blot and allowed for a more precise quantification of results. Overall, these results displayed the potent antiviral activity of this particular compound (**16**), using a primary culture of DHBV infected hepatocytes.

3.3.2 The inhibitory effect of the compounds against HBV replication using the 2.2.15 assay.

The summary of compounds that were examined for antiviral activity against HBV in the 2.2.15 cell line assay is displayed in tables 3.1 and 3.2. The EC_{50} was calculated in the same manner as described for the primary duck hepatocyte assay.

The activity of the compounds featuring the 1-[(2-hydroxyethoxy)methyl] glycosyl moiety is summarized in table 3.1. Most of the compounds which were active against DHBV in the primary culture assay retained anti-HBV activity in this assay. Of this series, the 5-chloro analog (**16**), again emerged as the most potent compound. This derivative had an EC_{50} value of 1-5 μg/mL, which can be compared to the reference drug, 3-TC, which had an EC_{50} of 0.5-1 μg/mL. For compounds **15**, **16**, and **18** there was a

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reduction in anti-HBV activity as compared to anti-DHBV activity by two to five fold and ten fold, respectively. Although for compounds **22-23,** the activity of these compounds increased by approximately two fold in this assay. In this assay, compound **28**, the 1-[(2-hydroxyethoxy)methyl]-6-azauracil, again exhibited minimal antiviral activity similar to that seen in the DHBV assay, which confirms that the substitution of the carbon at the 6-position for a nitrogen atom hinders antiviral activity.

The anti-HBV activity of the series of nucleoside analogs featuring the 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] side chain is outlined in table 3.2. Again, most of the compounds that did exhibit anti-DHBV activity retained activity against HBV in the 2.2.15 assay. From this series, the most potent analogs were compounds **45**, **47**, and **50**, with EC_{50} values of 5-10 μg/mL, which is only ten to twenty fold less active than 3-TC. Again, the 5-unsubstituted analog (**42**) did not exhibit any antiviral activity, which is the same effect observed in the DHBV assay. This confirms the importance of the C-5 substituent for antiviral activity. In addition, like in the DHBV assay, the compounds featuring the 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] side chain, generally displayed decreased inhibitory activity in comparison to their 1-[(2-hydroxyethoxy)methyl] counterparts.

The possible reasons for the differences in activity observed in the DHBV versus the HBV assay will be discussed in the discussion, but briefly these results could be attributed to the differences in metabolism, genomic organization, and inherit differences between the two viruses.

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3.3.3 Toxicity assays.

Both the Vero cells and the HepG2 cells were used in a standard MTT assay at concentrations of 100, 10, and 1 μg/mL. After a four day incubation of the cellular layer with the compounds, none of the compounds exhibited substantial inhibitory effects on cellular proliferation. The summary of the MTT assay results can be viewed in table 3.3 and 3.4. All the compounds had a CC_{50} greater that 100 μg/mL. In addition, during the two week of treatments of the primary culture of DHBV congenitally infected hepatocytes and 2.2.15 cells, there was no evidence of cell lysis or changes in morphology.

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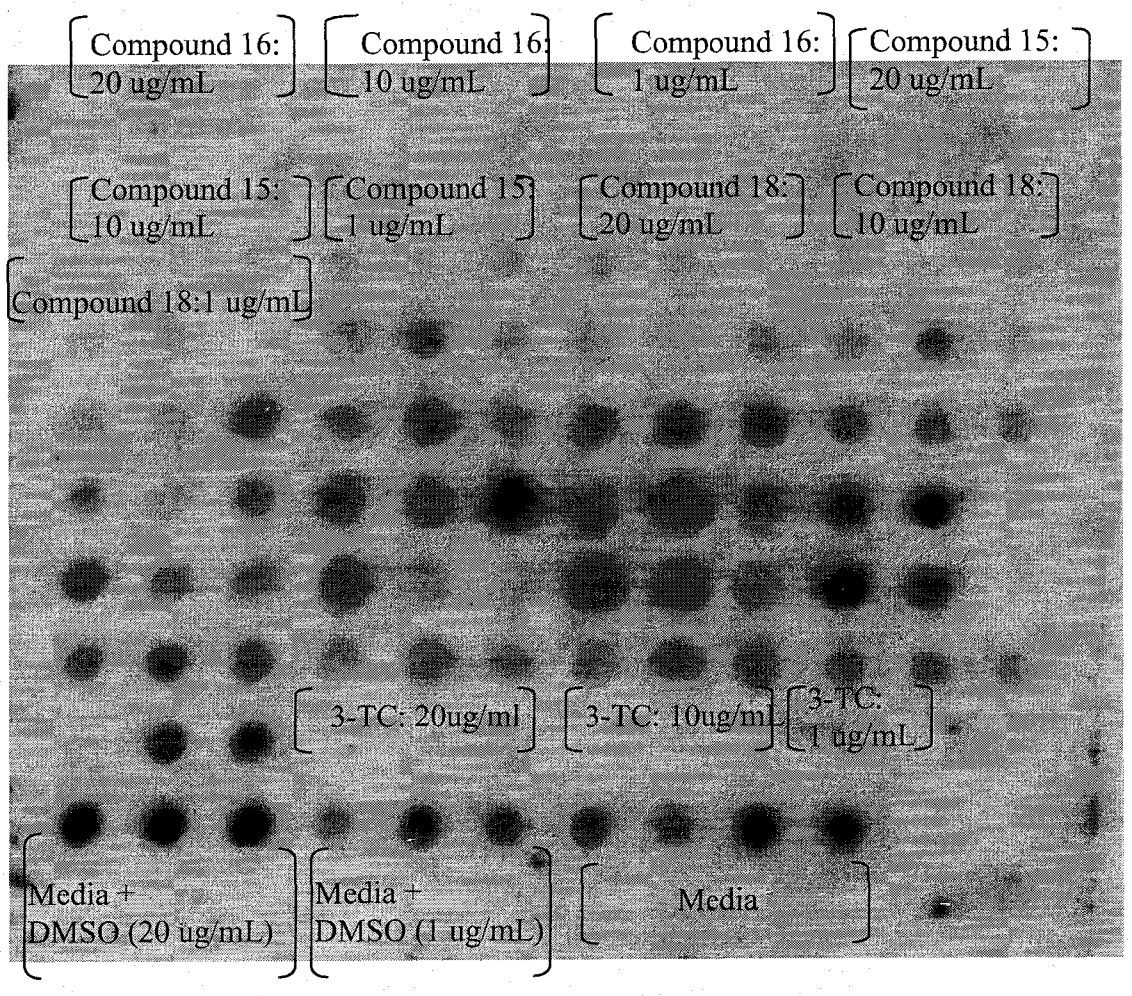

Figure 3.1 Example of a dot blot displaying the effect of selected compounds on DHBV replication using a primary hepatocyte culture assay.

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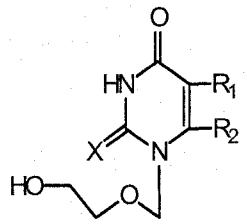
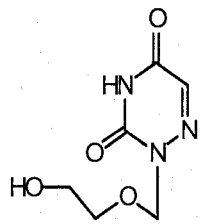


15-27



28

Compound	R_1	R_2	X	% inhibition @10 μg/ml [**EC_{50} (μg/ml)**]	
				DHBV Primary duck hepatocytes	2.2.15 Wildtype HBV
15	Br	H	O	89 [1-5]	60-73 [5-10]
16	Cl	H	O	94 [0.1-0.5]	82 [1-5]
17	NO_2	H	O	53 [10]	54 [10]
18	NH_2	H	O	65 [1]	50 [10]
19	C_2H_5	H	S	45 [>10]	40 [>10]
20	H	H	S	46 [>10]	42 [>10]
21	CF_3	H	O	76 [1-10]	55-65 [5-10]
22	CH_2OH	H	O	55 [10]	50-73 [5-10]
23	$COOC_2H_5$	H	S	53 [10]	50-64 [5-10]
24	H	CH_3	O	60 [5]	55-72 [5-10]
25	H	$COOCH_3$	O	56 [10]	60 [10]
26	I	CH_3	O	51 [10]	53 [10]
27	NO_2	CH_3	O	68 [5-10]	60-80 [5-10]
28	-	-	-	25 [>10]	25 [>10]
3-TC				96 [0.01-0.05]	88 [0.5-1]

Table 3.1 *In vitro* antiviral activity against wild-type (duck HBV and human HBV) of 5- and/or 6-substituted analogs of 1-[(2-hydroxyethoxy)methyl]uracils.

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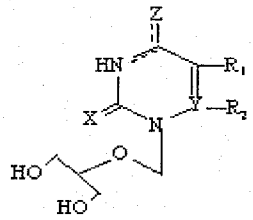

42-54

Compound	R_1	R_2	X	Z	% inhibition @10 μg/ml [**EC_{50} (μg/ml)**]	
					DHBV Primary duck hepatocytes	2.2.15 Wildtype HBV
42	H	H	O	O	0	0
43	F	H	O	O	24 [>10]	26 [>10]
44	Br	H	O	O	48 [>10]	40 [>10]
45	Cl	H	O	O	69 [1-5]	60 [5-10]
46	CH_3	H	O	O	45 [>10]	37 [>10]
47	CH=CHBr	H	O	O	75-90 [1-5]	53 [10]
48	F	-	-	O	20 [>10]	0
49	H	CH_3	O	O	27 [>10]	0
50	Br	CH_3	O	O	73 [5-10]	55 [10]
51	I	CH_3	O	O	44 [>10]	38 [>10]
52	H	-	O	O	41 [>10]	32 [>10]
53	CH_3	-	O	O	45 [>10]	37 [>10]
54	CH_3	-	S	O	25 [>10]	0
3-TC					96 [0.01-0.05]	88 [0.5-1]

Table 3.2 *In vitro* antiviral activity against wild-type (duck HBV and human HBV) of 5-and/or 6-substituted analogs of 1-[(2-hydroxy-1-hydroxymethyl)ethoxy)methyl]uracils.

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Treatment	Dose (ug/mL)	Percent Toxicity	Treatment	Dose (ug/mL)	Percent Toxicity	Treatment	Dose (ug/mL)	Percent Toxicity
	100	0.00		100	17.26		100	0.00
	10	0.00		10	13.53		10	21.63
15	1	9.85	**25**	1	15.75	**48**	1	19.27
	100	0.00		100	6.72		100	9.05
	10	15.37		10	1.20		10	0.00
16	1	11.87	**26**	1	12.03	**49**	1	0.00
	100	7.65		100	1.22		100	0.00
	10	0.00		10	1.22		10	4.92
17	1	6.43	**27**	1	14.28	**50**	1	0.25
	100	21.19		100	0.00		100	16.90
	10	4.10		10	0.00		10	26.82
18	1	0.00	**28**	1	0.00	**51**	1	15.90
	100	0.00		100	18.67		100	14.62
	10	19.79		10	12.20		10	0.07
19	1	18.55	**42**	1	0.00	**52**	1	0.00
	100	0.00		100	4.86		100	0.00
	10	0.00		10	0.00		10	0.00
20	1	0.99	**43**	1	0.00	**53**	1	0.00
	100	0.60		100	0.00		100	0.00
	10	0.14		10	0.00		10	0.00
21	1	0.00	**44**	1	11.57	**54**	1	6.18
	100	0.00		100	3.71	**Media+DMSO (average for all plate)**	100	2.41
	10	0.00		10	9.44		10	0.00
22	1	0.00	**45**	1	10.45		1	1.07
	100	0.00		100	9.77	**Media**	N/A	0.00
	10	15.47		10	6.09			
23	1	9.87	**46**	1	2.58			
	100	5.11		100	0.48			
	10	10.55		10	7.80			
24	1	0.00	**47**	1	13.53			

Table 3.3 MTT cell proliferation assay results for acyclic pyrimidine nucleosides [**15-28**, **42-54**] at concentrations of 100, 10, and 1 μg/mL using Vero cells.

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Treatment	Dose (ug/mL)	Percent Toxicity	Treatment	Dose (ug/mL)	Percent Toxicity	Treatment	Dose (ug/mL)	Percent Toxicity
	100	38.70		100	37.30		100	31.75
	10	17.23		10	0.00		10	19.84
15	1	0.00	**25**	1	0.00	**48**	1	0.00
	100	0.00		100	19.84		100	0.00
	10	6.22		10	9.00		10	0.00
16	1	1.42	**26**	1	18.52	**49**	1	0.00
	100	40.48		100	24.58		100	16.83
	10	8.47		10	3.39		10	0.00
17	1	0.00	**27**	1	0.00	**50**	1	0.00
	100	18.65		100	25.14		100	37.32
	10	0.00		10	4.81		10	6.97
18	1	0.00	**28**	1	0.00	**51**	1	17.42
	100	41.53		100	0.00		100	26.03
	10	0.00		10	0.00		10	2.54
19	1	0.00	**42**	1	0.00	**52**	1	0.00
	100	20.06		100	45.77		100	11.11
	10	5.09		10	16.67		10	0.00
20	1	0.00	**43**	1	11.38	**53**	1	0.00
	100	27.40		100	19.69		100	13.34
	10	4.52		10	0.00		10	0.00
21		0.00	**44**	1	0.00	**54**	1	0.00
	100	19.58		100	31.75	**UT+DMSO** (average for all plates)	100	31.74
	10	0.00		10	3.18		10	9.85
22	1	0.00	**45**	1	2.38		1	5.13
	100	26.46		100	22.54	**UT**	N/A	0.00
	10	22.75		10	0.00			
23	1	6.62	**46**	1	0.00			
	100	21.75		100	48.94			
	10	0.00		10	16.14			
24	1	5.94	**47**	1	0.00			

Table 3.4 MTT cell proliferation assay results for acyclic pyrimidine nucleosides [**15-28, 42-54**] at concentrations of 100, 10, and 1 μg/mL using HepG2 cells.

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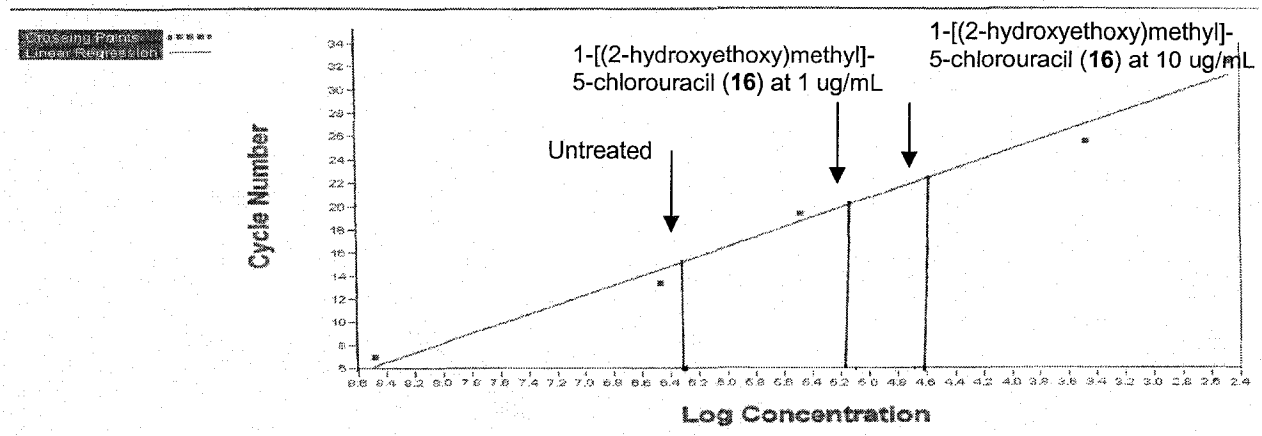
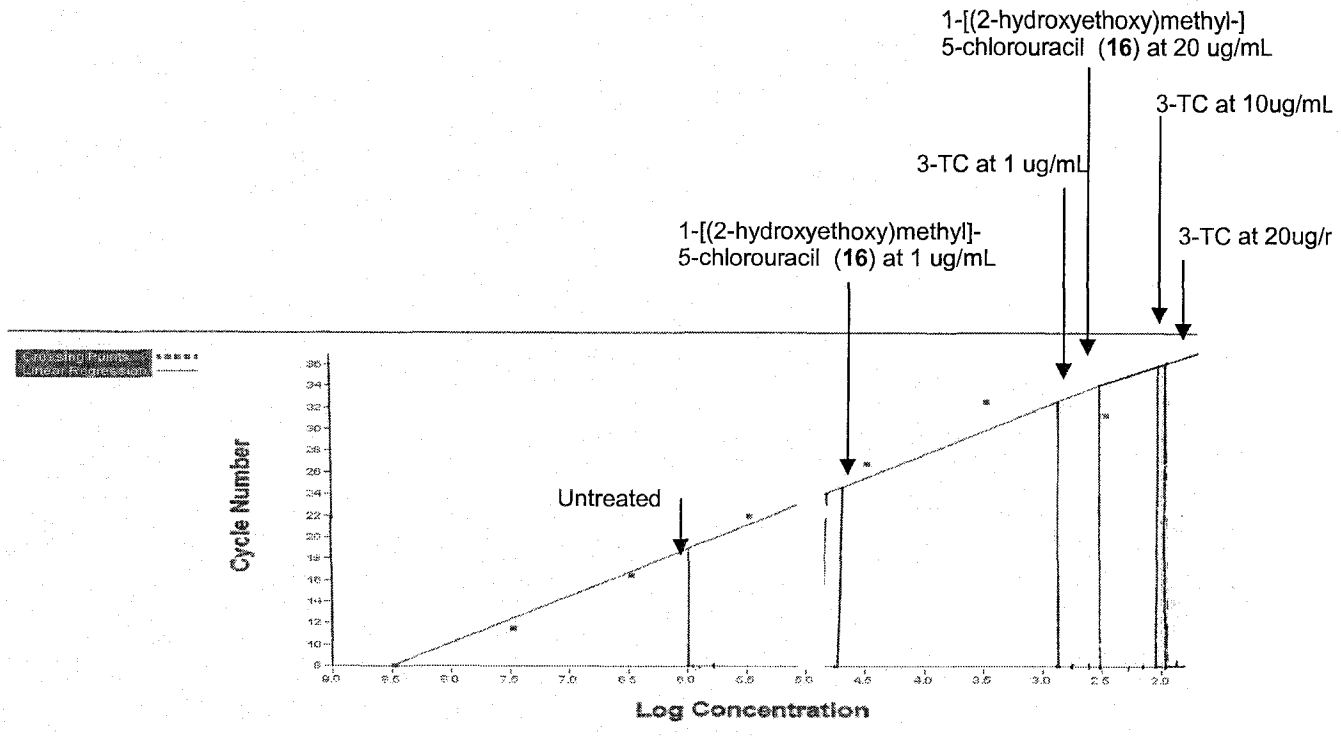


(a)



(b)

Figure 3.2 Real-time PCR standard curves exhibiting DHBV copy number reduction resulting from treatment with compound 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) and 3-TC at 20, 10, and 1 μg/mL.

[Graph (a) represents an experiment solely with the compound **16** and graph (b) represents a second experiment displaying the effect of the compound **16** and 3-TC].

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3.4 Discussion.

These novel 5-substittued acyclic pyrimidine nucleoside analogs have demonstrated effective and selective antiviral activity against DHBV and HBV, and show promise as a new subclass of analogs. Although there are a number of compounds that displayed strong activity, the compounds 1-[(2-hydroxyethoxy)methyl]-5-bromouracil (**15**), and 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) emerged as the most active in both the DHBV and HBV *in vitro* assays.

Generally, it would seem that the 1-[(2-hydroxyethoxy)methyl] side chain is associated with greater antiviral effects as compared to analogous compounds with the 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] glycosyl moiety. A possibility for this difference in activity may be that the1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] side chain possesses an extra hydroxyl group as compared to the 1-[(2-hydroxyethoxy)methyl] side chain. This extra hydroxyl group may act similar to a 3'-OH in natural dNTP and may not facilitate the premature chain termination required to inhibit DNA synthesis.

Overall it appeared that a halogen was an important C-5 substituent for conferring antiviral activity. The C-6 substituent did not seem to contribute to the antiviral nature of these compounds, and the azauracil where the C6 is substituted with a nitrogen (compound **28**), did not exhibit an antiviral effect. However, a double bond at the C-5 position may be important, as was seen with 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-(2-bromovinyl) uracil (**47**). In general a halogen C-5 substituent is an important determinant of antiviral activity, which could be due to size

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and/or electronegativity. This is not an isolated pattern. As mentioned previously, with respect to the 5-(1-azido-2-haloethyl)-2'-deoxyuridines series, the chloro C-5 substituent rendered the strongest inhibition against HSV-1, and the bromo group against HSV-2 (51). The iodo group at the C-5 position resulted in the greatest antiviral activity against HIV-1 with respect to the 5-halo (or methoxy)- 6-alkoxy (azido or hydroxy)-5,6-dihydro analogs of 2',3'-didehydro-3'-deoxythymidine (52-54).

In the 2.2.15 cell line assay, most compounds that exhibited activity against DHBV were able to maintain activity against HBV replication. Although, the level of inhibitory activity tended to be reduced in this assay. There could be a number of possibilities for this trend. One potential reason could be due to the continuous high level of replication inherit in a cell line, that may not be truly representative of the viral replication rates. Moreover, in this assay confluent cultures were used to maintain stable metabolism of the compounds, but at this stage HBV replication is quite high (42,43). In addition, these cells have undergone many passages, which may have also had an effect on the cell line characteristics (42). As well, in this assay total intracellular DNA was measured as an indicator of HBV replication, which includes integrated and episomal DNA (44). Finally, there could differential metabolism and phosphorylation efficiency between the duck hepatocytes and 2.2.15 cells. For instance, in the primary culture of duck hepatocytes the compounds may be more efficiently triphosphorylated by host cellular kinases which could explain their higher activity in this assay, as compared to in the 2.2.15 cell line where the compounds may not be as successfully phosphorylated. Regardless of the reason, the inhibitory effect of the compounds using the 2.2.15 cell line

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assay was reduced as compared to the effect observed in the duck primary hepatocyte culture.

For the purposes of determining which compound did indeed have an antiviral effect, activity in both systems was evaluated, but more significance was attributed to the results from the duck *in vitro* system since it is an assay based on a primary culture. Even though there are some differences between DHBV and HBV, which were discussed earlier, the primary culture may be a more accurate representation of the effect of these novel compounds on normal functioning hepatocytes. The DHBV primary culture system also incorporates more of the overall elements of *in vivo* hepadnaviral replication, and pathogenesis, such as after one week in culture, there is cccDNA amplification, which at first occurs at a linear rate but becomes exponential by ten to fourteen days post plating (30). The 2.2.15 cell line is quite useful in that it offers infinite cellular replication, whilst the cells in a primary culture system do not proliferate and can only maintain the cellular differentiation for a limited time in the range of weeks (45,46). However, when dealing with cell lines since they are based on transformed tumor cells, there can be quite a few deviations in characteristics from regular cells from that tissue (47). There are also considerable cellular phenotypic alterations and increases in proliferation rates that can occur after a low amount of passages (48). Overall, although HBV transfected human hepatoma cell lines appears to be the closest human based *in vitro* model, the hepatoma derived cell lines do differ from primary human liver cells. For instance, with respect to HepG2 cells, (the hepatoma cell line from which 2.2.1 5 cells are based), they express substantially less oxidative, reductive, and hydrolytic enzymes involved in drug metabolism, and may not be the most accurate predictor

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system for drug efficacy (13,46). There is also the presence of heterologous promoters in addition to the endogenous ones in the HBV plasmid used for transfection into human hepatoblastoma cells, which may have an effect on HBV replication in the 2.2.15 cells (44). A primary culture of human hepatocytes infected with HBV, would seem to be the most effective *in vitro* system for assaying potential antiviral compounds, but there is a limitation on the availability of human liver material (1). Furthermore, hepatocytes obtained *ex vivo* undergo spontaneous apoptosis and do not survive long after extraction (45,49).

The dot blot method of quantification has been criticized for its lack of sensitivity, but it is an effective technique to be used as a method for evaluating a large volume of compounds at different concentrations to give an approximate indication of the potency of the compounds. Moreover, the results obtained from the RT-PCR experiments corroborated the conclusions made from the dot blot results, but were just more sensitive quantitatively. In the future, to obtain more precise quantifications the method of RT-PCR can be more widely used.

Overall, the nucleoside analog in the acyclic pyrimidine series that displayed the strongest antiviral effect was compound **16,** featuring the C-5 chloro substituent coupled with the acyclic 1-[(2-hydroxyethoxy)methyl] side chain. In the DHBV assay this nucleoside analog exhibited an EC_{50} of 0.1-0.5 μg/mL and in the HBV assay it was able to retain its activity with an EC_{50} value of 1-5 μg/mL, which is quite comparable to 3-TC potency. RT-PCR quantification was used to confirm this finding, and at 20, 10, and 1 μg/mL this compound was associated with at least a 3.5, 1.7, and 1.2 log reduction compared to the untreated sample, respectively.

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These series of compounds did not exhibit any toxicity as can be seen by the MTT assay results in HepG2 and Vero cells, and by the lack of morphological alterations and cell lysis during the drug treatment in the duck primary culture and 2.2.15 assay. With respect to the MTT assay, the cell layers were incubated with the compound for four days, a reasonable time to allow for significant cellular toxicity to become apparent. This is important since host cellular mitochondrial DNA toxicity accrues, and becomes evident with exposure to the compound over time (11). Also, the cells were seeded onto the plates at a specific density, but were not left to reach confluence for this study. By not having the cells at a confluent state, there were still active proliferating cell undergoing cellular division and, macromolecular production. Thus, there was an increased potential for a compound to incur an effect on an integral stage, such as DNA replication, and display signs of toxicity (43). The levels of toxicity that were exhibited by these compounds in HepG2 cells, were dose related and paralleled the effects on cellular proliferation seen with media supplemented with DMSO controls. Therefore, the toxicity that did result could have been due to the DMSO concentration and not due to the actual nucleoside analog. Using the Vero cells with the MTT assay, the media plus DMSO control did not exhibit any toxicity, and the compounds did not generally exhibit the same dose related toxicity, but regardless, there was no substantial anti-proliferative effects induced by the compounds. Furthermore, the duck primary culture and 2.2.15 assay both involved a two week period of treatments on alternate days, which again provided adequate time for any evidence of toxicity to surface. The lack of toxicity suggests that these compounds exert their effect specific to HBV or DHBV viral polymerase and not host cellular DNA polymerases. The exact mechanism has not yet

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been elucidated. If these compounds are similar to other antiviral nucleoside analogs, then the specificity of their activity may be due to the phosphorylation by host cellular kinases, which allows these triphosphorylated derivatives to selectively inhibit the HBV polymerase. To determine if there is a delayed lethal toxicity, longer term studies can be performed in the future.

In the next chapter, compounds that exhibited effective antiviral activity against the DHBV and HBV were selected for further investigation. These acyclic pyrimidine nucleoside analogs were evaluated for their activity against two lamivuidne (3-TC)-resistant cell lines, in order to determine if the structure of these analogs would be beneficial with respect to their activity in the clinically relevant HBV mutated strains.

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Chapter Four: Assessing the antiviral activity of selected novel acyclic pyrimidine nucleoside analogs in lamivudine (3-TC)-resistant strains of HBV cell lines.

4.1 Introduction.

The HBV mutations and resulting drug-resistant variants have been previously discussed in the introduction (chapter one), so this topic will only be briefly summarized here. As indicated in the introduction, the long term usage of lamivudine (3-TC) leads to the development of HBV drug-resistant strains featuring either a rtM204I/V single mutation or a double mutation of rtM204V/rtL180M in the HBV polymerase (1-6). The emergence of lamivudine resistance can possibly lead to liver failure, hepatocellular carcinoma (HCC), and death (7). The amino acid substitution of methionine to isoleucine or valine (rtM204I/V) occurs in the HBV polymerase C domain in the YMDD motif, while the leucine to methionine mutation (rtL180M) is located in the B domain (2,3,8). The C domain mutations lead to a decrease in the replicative ability of the virus, while the B domain mutation results in a compensatory increase in viral replication (2,8,9). This helps explain why the double mutation typically confers stronger resistance to lamivudine as compared to the single mutation (4). One of the reasons proposed to explain the limited effect of lamivudine on these HBV mutants, is due to the steric conflict between the oxathiolane ring of the triphosphate of 3-TC and the β branched side chains of isoleucine or valine of the mutant HBV polymerase. This steric hindrance prevents lamivudine from binding to the constrained HBV polymerase binding pocket (3,10,11). Consequently, it has been speculated that the structural feature of an acyclic side chain linked to the nucleoside analog would allow for increased torsional flexibility,

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and a greater chance for binding to the HBV polymerase pocket (12,13). It is our aim to determine if the novel class of pyrimidine nucleoside analogs featuring an acyclic side chain will be able to retain antiviral activity against the lamivudine-resistant strains.

There are a couple of options for *in vitro* assays that can be used to determine the antiviral capability of compounds in HBV lamivudine strains. There are the tetracycline responsive hepatoma cell lines transfected with the cDNA of the mutant HBV pregenomic RNA, which can be utilized to study the effect of novel compounds on lamivudine-resistant HBV replication (14,15). However, this assay solely involves a M550V (rtM204V) or M539V mutation, and does not include other applicable and common clinical drug-resistant variants (14,16). Moreover, this system only produces low levels of viral replication (15). An alternate *in vitro* system that is applicable for studying drug-resistant strains, is an assay based on the use of a recombinant baculovirus to transduce HepG2 cells with HBV genomes (17). This system is able to induce high levels of viral replication, and efficiently detect cccDNA accumulation (17). However, as Fu and Cheng point out, it is a difficult system to use, the transfection method can be inefficient, there is variation between experiments, and the long term stability of the infected cells is still in question (18). Furthermore, it still confirms the antiviral capability of certain compounds already established with other easier to use transfection systems (17).

To determine the effect of selected compounds on lamivudine-resistant HBV replication, two cell lines produced by Kathi-Anne Walters in the Tyrrell laboratory were used. These cell lines feature HBV constructs with either the mutations rtM204I or rtL180M/M204V stably transfected into HepG2 cells (16). The mutations rtM204I and

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rtL180M/M204V are common mutations associated with lamivudine resistance in clinical isolates (19,20). Both intracellular and extracellular HBV production has been exhibited from these cells. Furthermore, these HepG2 cells transfected with mutant HBV genomes, have been confirmed to be stable even after seventy two passages, producing consistent levels of virions and viral antigens (18). Common antiviral compounds (such as lamivudine and penciclovir) were tested using these resistant cell lines, and the results demonstrating their decrease in effectiveness agreed with other published data (16). Overall, this assay is a viable option for determining if compounds active against HBV replication can also retain activity against lamivudine-resistant strains.

As mentioned previously, the pyrimidine nucleoside analogs being investigated possess the acyclic side chains in common with the ones featured in either acyclovir, the 1-[(2-hydroxyethoxy)methyl] glycosyl moiety, or the side chain on ganciclovir, 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]. Although data on the effect of acyclovir (ACV) on lamivudine-resistant strains of HBV could not be found, there was some limited and conflicting reports on the effect of ganciclovir (GCV). In one study, six patients who became resistant to 3-TC were administered ganciclovir for six months. As a result of this therapy, there were no significant improvements in terms of loss of serum HBV DNA or normalization of ALT levels, and two patients died from liver failure (21). Although in another experiment, a patient who developed lamivudine resistance and a related overlapping surface antigen mutation, was treated successfully with ganciclovir in addition to lamivudine. This patient became HBV DNA and HBeAg negative, anti-HBe antibodies were detected, and his ALT levels normalized (22). Due to the inadequate and inconsistent data it is difficult to make any conclusions about the effect of ACV or GCV

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on resistant strains. The lack of studies with ACV or GCV on the effect in 3-TC-resistant strains may be due to the limited efficacy of these nucleoside analogs on wildtype HBV (23-27). However, the only feature that is in common between the series of novel nucleoside analogs being currently investigated and ACV and GCV are the acyclic side chains.

The acyclic pyrimidine nucleoside analogs (**15-27**, **45-47**, **50**) with specific C-5 substituents that led to antiviral activity against DHBV in the primary culture and HBV in the 2.2.15 assay were selected for investigation against the resistant cell lines.

4.2 Materials and methods.

4.2.1 Drug solutions for resistant cell lines.

The compounds were initially dissolved in DMSO to a concentration of 10 mg/mL. The selected compounds were then diluted into a sterile phosphate buffer solution, PBS (pH 7.3) and then further diluted into individual aliquots of cell culture medium to produce a variety of concentrations. The cell culture medium consisted of 500 mL of MEM (Sigma), supplemented with 10% heat inactivated FBS (Gibco), geneticin/G148 sulfate (final concentration of 380 μg/mL), 5-7 mL of 7.5% sodium bicarbonate, and L-glutamine (final concentration of 2 mM). The media was filter sterilized with a 0.22 μm filter. The drug solutions were mixed and left covered with aluminium foil, to avoid potential decomposition of the compounds. The solutions were kept in a 4 °C cold room. Prior to a drug treatment, the solutions were warmed in the 37 °C incubator.

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4.2.2 Resistant cell lines (D88 and B1) drug treatments.

The 3-TC-resistant cell lines were grown out of liquid nitrogen frozen stocks, and were previously constructed by Walters, K.A. from the Tyrrell laboratory (16). The D88 cell line contains a double mutation of the HBV genome (rtL180M/M204V), and the B1 cell line features a single mutation (rtM204I). The cells were maintained in a humidified 37 °C incubator with a 5% CO_2 level. The cells were trypsinized and seeded from a confluent T150 flask into treated 6-well plates. When the cells exhibited confluence within each well, the first drug treatment was applied. The compounds were tested in triplicate for each concentration. The drug solutions were added at a volume of 3 mL per well, replacing the prior media. Treatments were administered every second day for seven treatments, two weeks in total. On the day subsequent to the last treatment, the cells were harvested.

4.2.3 Harvesting the cells and isolating intracellular viral nucleic acids.

The cell layer in each well was rinsed with 3 mL of PBS, pH 7.3, warmed to 37 °C. Then 1 mL of room temperature lysis solution #1 (10 mM tris.HCl pH 7.5, 1 mM EDTA pH 8.0, 50 mM NaCl, 8% sucrose, 0.25% nonidet P-40) was added. The cell layer/suspension was transferred to sterile/autoclaved eppendorf tubes. The samples were microfuged for 5 minutes at 12 000 rpm, and the supernatant was transferred to new autoclaved eppendorf tubes, and the pellets were discarded. After adding 6mM $MgCl_2$ and DNAse/RNAse solution (a final volume of 20 µg was required for both the DNAse

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and RNAse per sample), the samples were vortexed and left in a 37 °C water bath for 45 minutes-1hour. 330 µl of 26% PEG in 1.4 M NaCl and 25 mM EDTA was added, and the samples were left in a 4 °C ice bath for at least 30 minutes. The supernatant was then removed after microfuging the samples at 4 °C for 4 minutes at 12000 rpm. To the visible pellet remaining, 200 µl of lysis solution #2 (10 mM tris.HCl pH 7.5, 5 mM EDTA, 1% SDS) was added. For protein digestion, 10 µl of 20 mg/mL fungal proteinase k (Invitrogen) was added. Samples were vortexed and left in a 42-55 °C water bath overnight, until there was no visible pellet remaining. For the phenol/chloroform extraction, one volume (200 µl) of tris saturated phenol was added, then the samples were vortexed. Followed by the addition of two volumes of chloroform (400 µl), and again the samples were vortexed. The samples were microfuged for 5 minutes at 12000 rpm, and 200 µl of the aqueous phase was transferred to new autoclaved tubes. To ethanol precipitate out the DNA, 0.1 M NaCl, 1 µl of 20 mg/mL yeast tRNA, and 2 volumes (400 µl) of ice cold 95% ethanol was added. The samples were vortexed, then left in –20 °C, for longer than 30 minutes or in –70 °C for 30 minutes. The samples were then microfuged for 10-15 minutes at 4 °C, and the salt was rinsed out with the addition of 3 volumes (600 µl) of ice cold 70% ethanol, and again the samples were microfuged for 2 minutes at 4 °C. The remaining pellet was left to air dry overnight, after the supernatant was removed. The dry DNA pellet was then resuspended in 50 µL of TE buffer (10 mM tris.HCl, 1 mM EDTA), in preparation for its use in the dot blot assay.

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4.2.4 Dot blot hybridization and semiquantitative detection of viral DNA.

Using the dot blot Bio-Dot apparatus (Bio-Rad), 10 μL of each sample was spotted on the nylon membrane (Hybond-N+, Amersham Biosciences). The nylon membrane was then denatured by placing it face up on top of filter paper soaked in denature solution (0.5 M NaOH, 1.5 M NaCl) for 30 minutes. This process was then repeated with neutralization solution (0.5 M tris.HCl pH 8.0, 1.5 M NaCl) for 30 minutes or longer. To fix the nucleic acids to the membrane, the membrane was UV cross linked for 3 minutes using a UV transilluminator (UVP Inc.). Membranes were then prehybridized with a buffer (30% of 20xSSC, 70% of 10% SDS) for at least two hours at 65 °C. α-^{32}P HBV plasmid DNA radiolabeled probe was then denatured by heat or by alkaline lysis. Radiolabeled probe was then added (10^6 CPM/mL), and left overnight for hybridization. To remove excess probe, two sets of washes (of 1xSSC/0.1% SDS solution) were performed for 15-30 minutes each, followed by one wash (of 0.1SSC/0.1% SDS) for 15-30 minutes. The membrane was then visualized using autoradiography, and the results were quantified using the NIH Image 1.62b7 program . The percent replication was calculated for each set of triplicate wells for each compound at a particular concentration. This calculation involved taking the average of the triplicate wells (in terms of their intensity measurement in pixels) and subtracting from it the average of the background of the membrane, which was all divided by the average of the triplicates of the wells treated with only media and DMSO (the negative control). This value was then multiplied by one hundred to convert it to a percentage. The percent inhibition of the compound was derived from the percent replication.

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Percent replication:

[(average intensity of triplicate wells in presence of compound) – (background average)]/ (DMSO+media treated average) *100

Percent inhibition:

100-percent replication

4.3 Results.

In these studies, abacavir, [1S,4R]-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol, was used as a positive control since it has been reported to be active against the 3-TC-resistant HBV strains (14). 3-TC was also used as a negative control, to ensure that the cells displayed lamivudine resistance. Finally, DMSO was supplemented into media at equivalent concentrations as the drug solutions. Wells treated with this DMSO and media mixture were used as negative controls, to function as comparative determinants of activity. There were also wells treated with just media to serve as an additional control. No appreciable difference was found amongst the different DMSO concentrations in media and the cells solely treated with media.

Table 4.1 summarizes the activity of the 5-and/or 6-substituted 1-[(2-hydroxyethoxy)methyl]uracils selected for evaluation against the lamivudine-resistant strains. Table 4.2 represents the antiviral activity of specific 5-and/or 6-substituted 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]uracils. The data is expressed as the percent inhibition of viral DNA replication in the presence of 10 μg/mL of the particular compound, when compared to the untreated HBV infected controls. The EC_{50} represents

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the drug concentration (μg/ml) required to reduce the viral DNA in infected cells to that of 50% of the untreated infected controls; the concentration that can reduce 50% of the HBV DNA levels. At the 10 μg/mL concentration, the inhibitions of HBV were calculated and standard deviations were within 10% of the average values. After the preliminary testing at 10 μg/mL, the compounds were serially diluted to obtain more precise EC_{50} values and tests were repeated two to three times. In the cases where the EC_{50} obtained from three experiments was within a 10% standard deviation, average values are given, otherwise a range of EC_{50} values is shown.

One of the assays that was used to evaluate antiviral activities of the selected compounds against lamivudine-resistant HBV was the B1 cell line, featuring a single rtM204I mutation. The compounds 1-[(2-hydroxyethoxy)methyl]-5-bromouracil (**15**), 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**), and 1-[(2-hydroxyethoxy)methyl]-5-triflouromethyluracil (**21**) exhibited strong antiviral activity with EC_{50} values of 5-10, 1-10, and 10 μg/mL, respectively (table 4.1). Other analogs with the 1-[(2-hydroxyethoxy)methyl] side chain that exhibited moderate inhibition include compounds **17-20**, **23**,**24**,**26**,and **27**, where at 10 μg/mL these compounds were generally able to inhibit 20-30% of viral replication. From the series featuring the 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] side chain, the compounds containing the 5-chloro (**45**) and the 5-bromo-6-methyl (**50**) substituents maintained activity against the single mutant HBV, similar to that observed against wildtype HBV (table 4.2). Generally, the activity of the investigated acyclic pyrimidine nucleoside analogs correlated well with the antiviral effects displayed in wildtype HBV described in chapter three.

The compounds (**15-27**,**45-47, 50**) were also evaluated for antiviral activity in the

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D88 cell line, which is transfected with a double mutated (rtL180M/M204V) HBV DNA and confers stronger resistance to 3-TC as compared to a mutant HBV with only a single amino acid substitution (4,16). In this assay, the compounds, 1-[(2-hydroxyethoxy)methyl]-5-bromouracil (**15**), and 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**), again displayed the strongest antiviral activity (table 4.1). Both compounds had EC_{50} values of 1-10 μg/mL, which are comparable to the EC_{50} of abacavir (1-5 μg/mL), the positive control. The 5-triflouromethyl (**21**) analog maintained the same level of activity that was exhibited in the B1 cell line, with an EC_{50} of 10 μg/mL. It can be inferred that these structures are not cross-resistant to these specific HBV drug mutations. Other analogs that possessed the 1-[(2-hydroxyethoxy)methyl] glycosyl moiety, compounds **18**, **19**, **22**, **26**, and **27,** were moderately inhibitory. At 10 μg/mL these compounds were able to inhibit 20-40% of viral replication. From the series featuring the 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] side chain (table 4.2), the compounds containing the 5-chloro (**45**) and the 5-bromo-6-methyl (**50**) substituents still retained inhibitory activity. However, this antiviral effect was slightly less against the double mutant HBV as compared to the single mutant HBV.

In both of these assays, the lamivudine-resistant strains were much less sensitive towards 3-TC, as compared to wildtype HBV. The introduction of a single HBV mutation resulted in a 45% inhibition of viral replication at 10 μg/mL and in the double mutant there was a drop to a 30% inhibition at the same concentration. There is a marked decrease in lamivudine sensitivity when compared to the results obtained in the 2.2.15 assay featuring wildtype HBV, where 3-TC was able to inhibit 88% of viral replication at 10 μg/mL. This general reduction of sensitivity to 3-TC in the resistant strains and the

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specific ability of the double mutant to confer stronger resistance to 3-TC as compared to the single mutant, agrees with other published *in vitro* studies (16).

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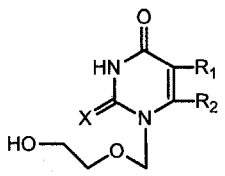

15-27

Compound	R_1	R_2	X	% inhibition @10 μg/ml [**EC_{50} (μg/ml)**]	
				B1 cell line M204I mutant HBV	D88 cell line L180M/M204V mutant HBV
15	Br	H	O	67 [5-10]	73 [1-10]
16	Cl	H	O	69 [1-10]	70 [1-10]
17	NO_2	H	O	30 [>10]	0
18	NH_2	H	O	30 [>10]	30 [>10]
19	C_2H_5	H	S	20 [>10]	40 [>10]
20	H	H	S	30 [>10]	0
21	CF_3	H	O	53 [10]	50 [10]
22	CH_2OH	H	O	0	30 [>10]
23	$COOC_2H_5$	H	S	20 [>10]	0
24	H	CH_3	O	30 [>10]	0
25	H	$COOCH_3$	O	0	0
26	I	CH_3	O	30 [>10]	25 [>10]
27	NO_2	CH_3	O	25 [>10]	20 [>10]
3-TC	-	-	-	45 [>10]	30 [>10]
Abacavir	-	-	-	80-90[1-5]	72[1-5]

Table 4.1 *In vitro* antiviral activity against 3-TC drug-resistant HBV of 5- and/or 6-substituted analogs of 1-[(2-hydroxyethoxy)methyl]uracils.

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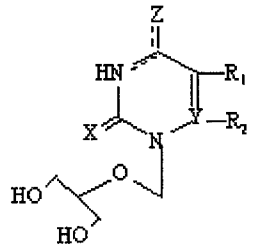

42-54

Compound	R_1	R_2	X	Z	% inhibition @10 μg/ml [**EC_{50} (μg/ml)**]	
					B1 cell line M204I mutant HBV	D88 cell line L180M/M204V mutant HBV
45	Cl	H	O	O	56 [5-10]	40 [>10]
46	CH_3	H	O	O	0	0
47	CH=CHBr	H	O	O	ND	ND
50	Br	CH_3	O	O	51 [10]	30 [>10]
3-TC					45 [>10]	30 [>10]
Abacavir					80-92 [1-5]	72 [1-5]

Table 4.2 *In vitro* antiviral activity against 3-TC drug-resistant HBV of 5- and/or 6-substituted analogs of 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]uracils. [ND: Not determined].

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4.4 Discussion.

In summary, the compounds that exhibited the strongest inhibitory activity against the lamivudine-resistant cell lines in the acyclic pyrimidine nucleoside analog series, involved the 5-halouracil analogs possessing the 1-[2-hydroxyethoxy) methyl] acyclic side chain. Specifically, the 5-bromo (**15**) and 5-chloro (**16**) analogs exhibited the strongest antiviral activity. These compounds exhibited the greatest antiviral activity in assays involving both the single and double mutants. Compound **21**, the 5-triflouromethyl analog also displayed effective antiviral activity. These compounds were also amongst the most active in the DHBV infected primary culture and the 2.2.15 HBV assay discussed in chapter three, which again supports the notion that a halogen C-5 substituent is important for anti-HBV activity. However, it is the acyclic side chain that can be credited with the ability to have any activity against 3TC-resistant variants, since it is this feature that is in common with adefovir, a very active compound against lamivudine-resistant HBV (28). The flexibility of the acyclic side chain in place of a glycosyl ring may not create a steric barrier between the nucleoside analog and the binding site, accounting for its activity (3,10,11,28).

The type of activity exhibited by the acyclic pyrimidine nucleoside analogs, **15**, **16**, and **21,** in these mutant HBV assays was similar to the activity displayed in wildtype HBV as seen with the 2.2.15 assay in chapter three. These acyclic nucleoside analogs were not cross-resistant to lamivudine. Compounds that were inhibitory against the wildtype HBV, retained analogous activity against the single and double HBV mutants. This is the same pattern of activity exhibited by adefovir, which has an EC_{50} of 0.2, 0.15-

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1.5, and 0.7-3.0 μg/mL in wildtype HBV, singly mutated HBV and double mutant HBV, respectively (2). The lack of cross-resistance may also be due to the absence of an oxathiolane ring structure in these nucleoside analogs, which is an integral structural component of 3-TC (1).

On the other hand, there was a substantial decrease in sensitivity demonstrated by 3-TC. This reduction in sensitivity to lamivudine was most strongly displayed with the HBV featuring the double mutation, and then with the HBV polymerase featuring a single mutation, which agrees with previous studies (16). Moreover, the single mutant HBV was more susceptible to abacavir as compared to the double mutation HBV (16). This corresponds well with the theory that the double mutant can replicate at high levels, since the B domain mutation that accompanies the replicative debilitating C domain mutation, helps in restoring the replicative ability of the variant virus (2,8,9). Thus the single mutant virus with only the C domain mutation would replicate less efficiently and be less sensitive to abacavir. With the acyclic pyrimidine nucleoside analogs investigated, there were generally similar levels of inhibitory activity for each compound between the two mutant cell line assays. However, in some cases selected compounds (**15** and **16**) displayed slightly more activity in the D88 cell line, featuring the double mutation, as compared to the B1 single mutation cell line. A possible explanation is that perhaps the B domain mutation may be aiding the ability of the nucleoside analog to exert its antiviral effect, due to the increased replication associated with this mutation. If the D88 cell line features greater replication rates, there may be a greater opportunity for the compound to exhibit an effect on the replication rates, as compared to the B1 cell line which may feature a lower rate of viral production to begin with. Another possibility is

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that the D88 cells have a B domain mutation along with a common C domain mutation. It has been proposed that the B domain mutation is involved with template primer positioning which changes the dNTP binding pocket and may even offset the conformational disproportion of a C domain mutation (2,8,29). While this may have a detrimental effect for 3-TC and other similar analogs, perhaps with these compounds the template repositioning may improve the binding of the nucleoside analog to the HBV polymerase. Again, since these compounds were not cross-resistant, they did not exhibit the same pattern of sensitivity as 3-TC in the two cell lines and may not share the same binding mechanism.

The use of *in vitro* lamivudine-resistant assays is the most convenient and effective model to determine the antiviral capability of compounds with respect to 3-TC-resistant HBV. Although an *in vivo* model would be more informative, there is a limited availability of lamivudine-resistant animal models for drug evaluation, since infecting animals with the mutant strain is not a viable option due to the reversion to the more replication fit wildtype strain. As well, for lamivudine resistance to occur naturally it would require a long term of administration of 3-TC to the chronic carriers. For instance, in the lamivudine treated chronically infected woodchucks it took ten to twelve months of therapy for drug resistance to emerge (30). The woodchuck can provide somewhat of a model, but the lamivudine-resistant point mutations differ from those seen in humans infected with the mutant strain (17,30-32). The WHV drug-resistant mutations are not in the YMDD motif but are upstream in the B domain region (30,31).

From the chapters three and four, the 1-[2-hydroxyethoxy) methyl]-5-bromouracil (**15**), 1-[2-hydroxyethoxy) methyl]-5-chlorouracil (**16**), 1-[2-hydroxyethoxy) methyl]-5-

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aminouracil (**18**) and 1-[2-hydroxyethoxy) methyl-5-triflouromethyluracil (**21**), showed the greatest overall activity in the primary culture of hepatocytes infected with DHBV assay, the HBV cell line assay, and/or the lamivudine-resistant cell lines. In this next chapter, these compounds were examined *in vivo* using DHBV congenitally infected ducks to assess their antiviral capabilities *in vivo*.

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Chapter Five: *In vivo* studies using the DHBV congenitally infected duck model.

5.1 Introduction.

In this chapter, the objective was to investigate selected compounds that displayed antiviral activity *in vitro* against DHBV, HBV, and lamivudine-resistant HBV, *in vivo* in an animal model of HBV infection. The nucleoside analogs that were selected for *in vivo* investigation include: 1-[(2-hydroxyethoxy)methyl]-5-bromouracil (**15**), 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**), 1-[(2-hydroxyethoxy)methyl]-5-aminouracil (**18**), and 1-[(2-hydroxyethoxy)methyl]-5-trifluoromethyl uracil (**21**). These compounds were tested over a time course of treatments in an *in vivo* model, using DHBV congenitally infected Pekin ducks. The purpose was to assess if the *in vitro* antiviral activity of these compounds translated into an effective *in vivo* response.

The use of DHBV infected ducks for these *in vivo* studies is a practical choice when considering the constraints on the other available HBV animal models. The only other non-human host of HBV is the chimpanzee, and these animals are limited in supply (1-3). Chimpanzees are excellent models for HBV, but are endangered and are quite costly to adequately house, maintain and retire, which restricts their usage (4-10). Lower primates, such as the rhesus monkey, have also been investigated as potential HBV animal models. However, they are less susceptible to an HBV infection and when they are infected they do not acquire the typical liver injury and the enzyme elevation characteristic of HBV (8). Wooly monkeys are susceptible to the WMHBV, but are also considered endangered (9). Woodchucks can serve as good experimental models for the related WHV, but are more difficult to handle and obtain than ducks (1,2,11-13).

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Transgenic mice have been developed which can also serve as animal models. These mice feature integrated HBV genomes that produces HBV DNA, replicative intermediates, viral antigens, and Dane type particles (14,15). The main limitation associated with this model is that the course/pathogenesis of a chronic HBV infection (including not being able to produce cccDNA) is not actually simulated, since the mouse is not a natural susceptible host (14-16). As well, these mice produce low quantities of sera viral particles and there is a lack of *de nova* infection in other cells (14,15). BALB/c nude mice have been injected with a clone of HBV DNA, which has resulted in a chronic HBV like state (17). Thus, these mice could serve as *in vivo* HBV models, but a constraint with this system is that HBV replication appears to cease months after the injection (17).

Ducks are useful *in vivo* models since they are affordable, have high viral titers, are available, and can maintain the viral supply through congenital infections (18-21). The ability of DHBV to be transmitted vertically, by means of the embryonated egg, allows for a stock of DHBV and animal models (13,20,22-25). In fact, DHBV viral replication begins as early as the twelfth day of the life stage of an embryo (25). The resulting infection from congenital transmission is reliable and leads to a persistent infection without severe hepatitis (1,22,26). Non-congenitally infected Pekin ducks can also be injected with the virus, either in the embryo or after hatching, to result in a chronic DHBV infection (1,13,18,22). Moreover, when non-congenitally infected Pekin ducks are inoculated with virus, there is a good correlation between the severity of the hepatitis and the generated viremia (1). There is also a correlation with the significance of an infection (indicated by the presence of viral particles and enzyme activity) and the

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severity of liver disease (21,27). Furthermore, the injected virus does not induce a significant amount of mortality and liver damage associated with the infection (1,18,20,22). Finally, it has also been found that DHBV, like HBV, can be integrated in duck hepatocellular carcinomas (21,28). Therefore, the duck model can also be used to study hepatocarcinogenesis. Overall, the duck model has been used for a wide array of experimentation in the hepatitis B field to investigate HBV pathogenesis and molecular biology (24,27).

The specific compounds were selected based on their ability to induce an antiviral effect *in vitro*, with most of the emphasis on the effect in the *in vitro* duck primary culture assay. With respect to *in vivo* testing, an additional beneficial feature of these compounds is that they are pyrimidine nucleosides with acyclic modifications. They are considered to be more water soluble as compared to purine nucleoside analogs, thus augmenting their bioavailability (29). This may lead to lower dosages, lesser chance of toxicity issues, and allows for the possibility of oral administration (30). The compounds were administered through the intraperitoneal (i.p.) route. This is an effective route for delivery to the liver target, since the concentrated compound has a greater opportunity to interact with the liver (30,31). The objective of this chapter was to determine if these compounds that exhibited *in vitro* antiviral activity, were able to demonstrate activity in the *in vivo* model or even have an augmented effect with the assistance of the immune system.

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5.2 Materials and methods.

5.2.1 Pekin ducks (*Anas domesticus*).

The ducks used for this study were bred and hatched at the University of Alberta Ellerslie Farms. On the first day of hatching the ducks were confirmed to be DHBV congenitally infected by Gerald Lachance (Tyrrell laboratory). The ducks were bled from a leg vein and the collected sera were used for dot blots. A α-^{32}P DNA radiolabeled probe featuring DHBV core sequence (using plasmid, pALTer) was used for viral detection. Only ducks that exhibited a strong viremic presence were used for the *in vivo* studies. The body weights of the ducks were recorded prior to the commencement of the study, on a weekly basis, and at the completion of the study. After the completion of each study (discussed below), the sera was dot blotted using 5 µl sera samples and again a α-^{32}P DNA radiolabeled probe featuring DHBV core sequence was used. The results were quantified using the NIH Image 1.62b7 program, and DHBV presence/intensity was measured in pixels. The first bleed (the pre bleed) was obtained prior to any drug treatment and was considered to represent 100% of DHBV replication for that particular subject. All subsequent sera samples (collected during drug treatments) were compared to the respective pre bleeds.

Percent replication of DHBV in sera for each duck at a specific time point:

$$\frac{\text{Presence of DHBV at that specific time point (after drug treatment)}}{\text{Presence of DHBV at the pre bleed (before drug treatment)}} \times 100$$

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5.2.2 *In vivo* studies of acyclic pyrimidine nucleosides.

The nucleoside analogs that were selected for testing include: 1-[(2-hydroxyethoxy)methyl]-5-bromouracil (**15**), 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**), 1-[(2-hydroxyethoxy)methyl]-5-aminouracil (**18**), and 1-[(2-hydroxyethoxy)methyl]-5-trifluoromethyl uracil (**21**). These compounds were dissolved in a 5-10% DMSO/PBS solution, and the control groups were injected with equivalent solutions. The compound solutions were kept in the refrigerator (4 °C) or kept frozen (-20 °C), and covered with aluminum foil until ready for use. They were warmed up to 37 °C prior to injecting and were administered through the intraperitoneal route. The dosages used for injections ranged from 25-40 mg/kg, and when 3-TC was used as a positive control it was administered at a dosage of 25 mg/kg.

Two to four ducklings were used in each drug treatment group and up to three ducklings were used as controls. For each study, the ducklings used were usually born on the same day, and were one week to sixteen days old when the treatments began. The injection schedules varied from once a day to bi daily, and the treatment program lasted from ten to twenty days. Blood samples were collected the day prior to the treatment (pre bleed), during the treatments, and in some cases for an extended period of time after the cessation of treatment (referred to as extended bleeds). After obtaining a blood sample, it was spun for 4 minutes at 12 000 rpm (Sorvall MC 12 V microfuge), and the sera was extracted. The sera samples were then stored in the refrigerator (4 °C) or the freezer (-20 °C), and kept covered until used for analysis.

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5.2.10 Statistics.

Statistical analyses for P values were performed using the statistical SAS program. The mixed method statistical procedure was used with respect to the NIH Image 1.62b7 program measurement values (in pixels) of each sera representation in the dot blot. The different covariance models including compound symmetry, heterogeneous compound symmetry, univariate, autoregressive, and heterogeneous autoregressive were applied and tested. A P value <0.05 was considered statistically significant.

5.3 Results.

The *in vivo* studies featuring the treatments of 1-[(2-hydroxyethoxy)methyl]-5-bromouracil (**15**), 1-[(2-hydroxyethoxy)methyl]-5-aminouracil (**18**), and 1-[(2-hydroxyethoxy)methyl]-5-triflouromethyluracil (**21**) did not exhibit considerable antiviral effects. Specifically, there was no statistically significant reduction in viremia in the treatment groups as compared to the control groups.

The compound, 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**), displayed a substantial antiviral effect when it was investigated *in vivo* at the dosage of 25 mg/kg (see figures 5.1-5.4). Based on the initial study, compound **16** had a significant effect that was also sustained even post treatment during the extended bleeding schedule. The results are represented graphically in figures 5.1 and 5.3, and the actual dot blots exhibiting these effects can be seen in figures 5.2 and 5.4. During the period of time when the ducks were receiving injections (see results displayed in figures 5.1 and 5.2),

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the interaction of the treatment over time was statistically significant with a P value of <0.0001. Moreover, during the period of twenty five days post treatment (results exhibited in figures 5.3 and 5.4), the effect of the treatment was still statistically significant with a P value of 0.0177. This experiment was repeated, but the results did not display the same level of antiviral activity. There could have been an issue with dosage. The ducks used in the second study were slightly older and were quite a bit larger in terms of body weight. For instance, in the initial study of this compound by week two the average of the duck weights were 0.29 kg, but the average of the duck weights in the repeat study for week two was 0.52 kg, almost double. The dosages and the allocation of the compound were based on duck weights recorded in previous studies. As a result, the dosage in the repeat study was almost 50% lower than the 25 mg/kg administered in the initial study. Therefore, the anti-HBV effect of compound **16** was not clear in the repeat experiment.

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Percent Replication of DHBV Detected in Sera over an Eighteen Day Schedule of Daily Injections of 1-[2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) in Congenitally Infected Ducks.

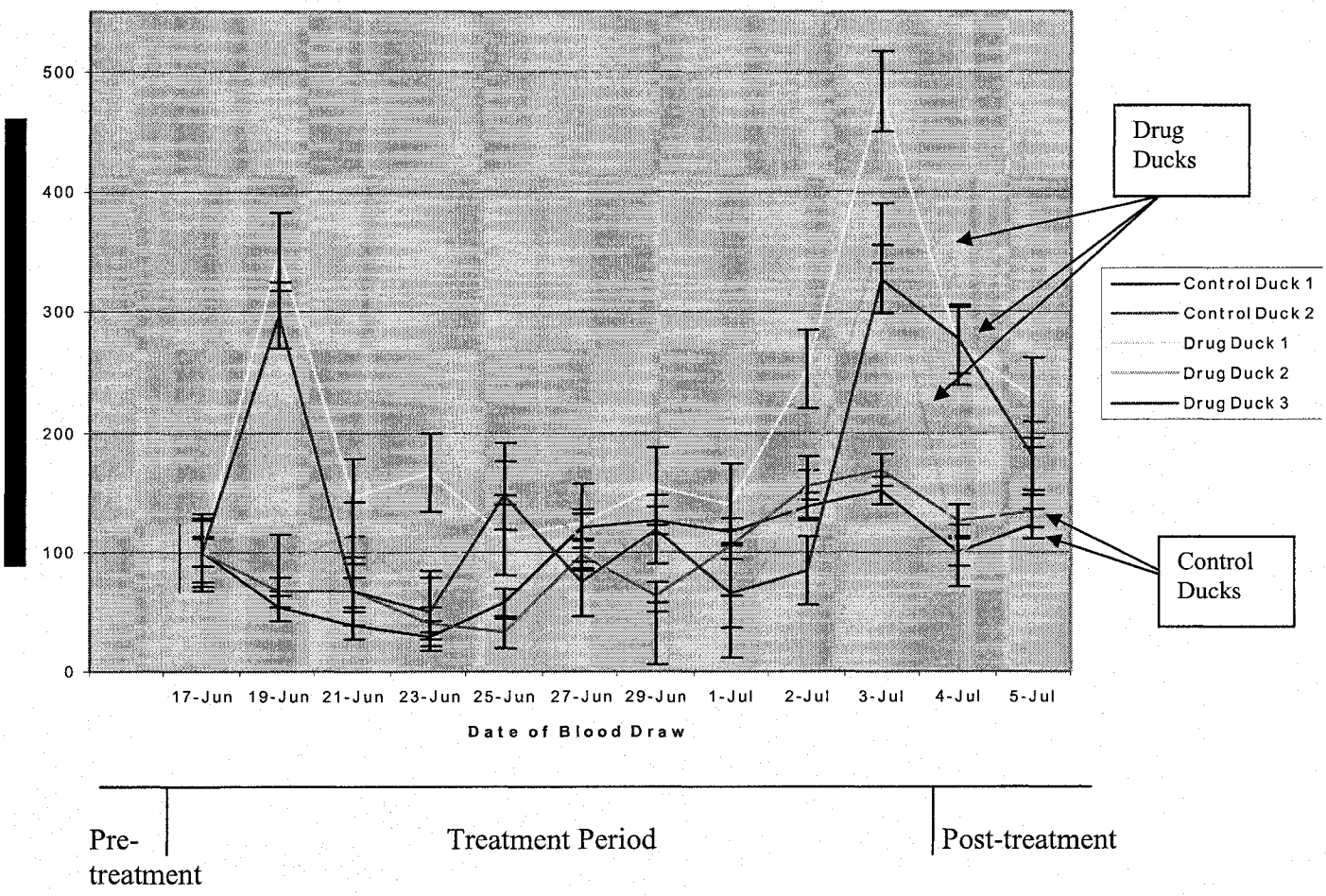

Figure 5.1 The effect of 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) on DHBV replication.

[Compound **16** was administered at a dosage of 25 mg/kg.]

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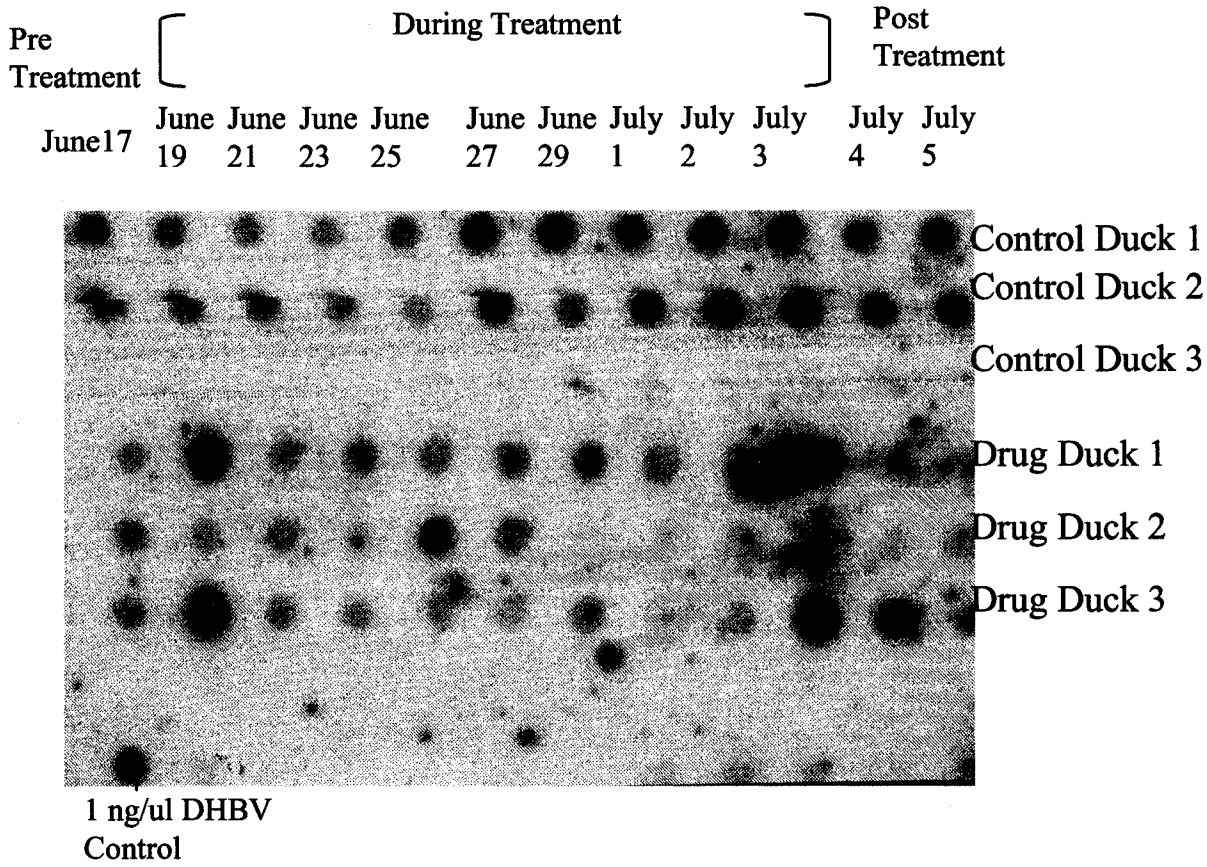

Figure 5.2 Dot blot of the *in vivo* study determining the effect of 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) on DHBV replication.

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Percent Replication of DHBV Detected in Sera over an Extended Bleeding Schedule.
[Drug: 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**)].

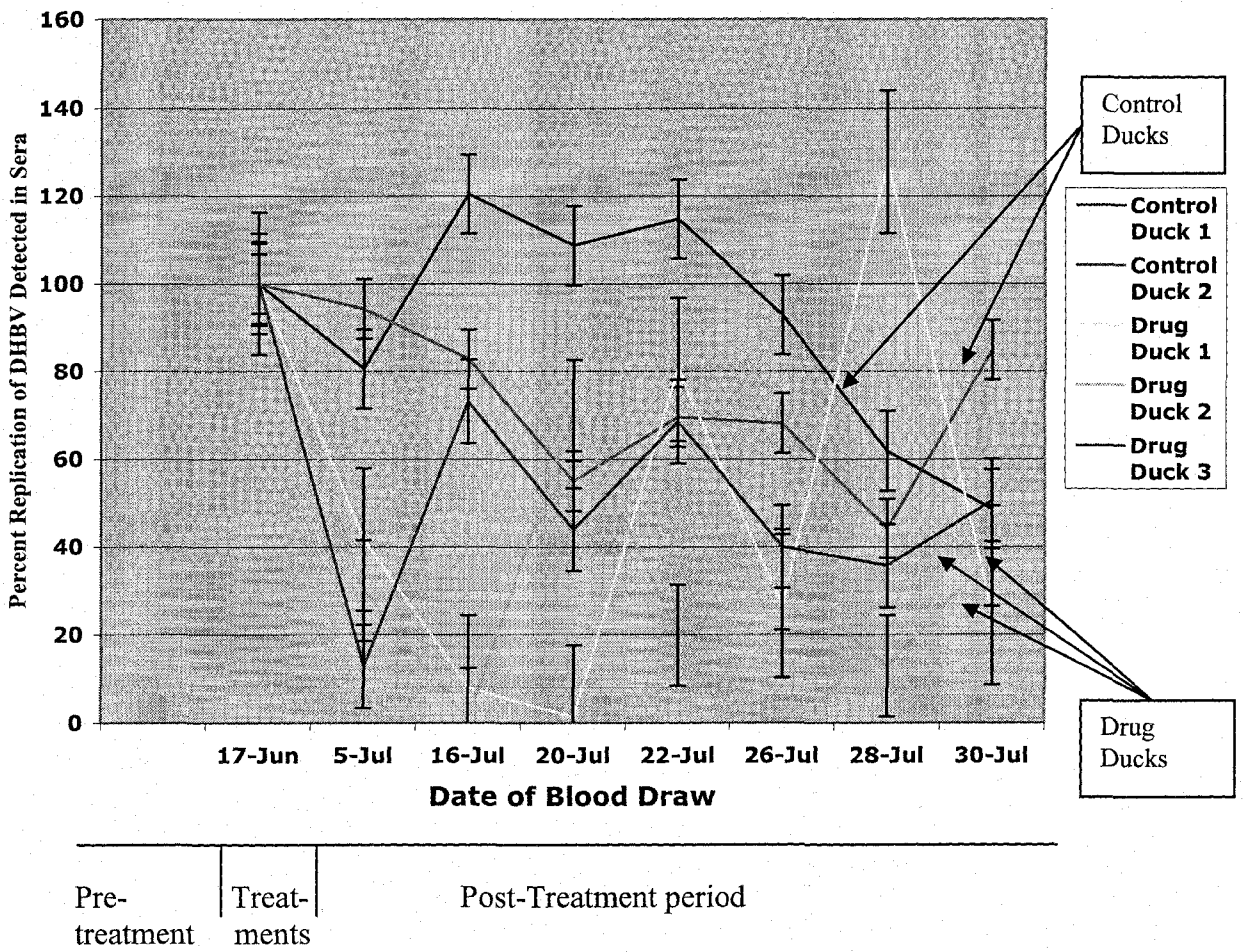

Figure 5.3 The effect of 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) on DHBV replication over an extended bleeding schedule.

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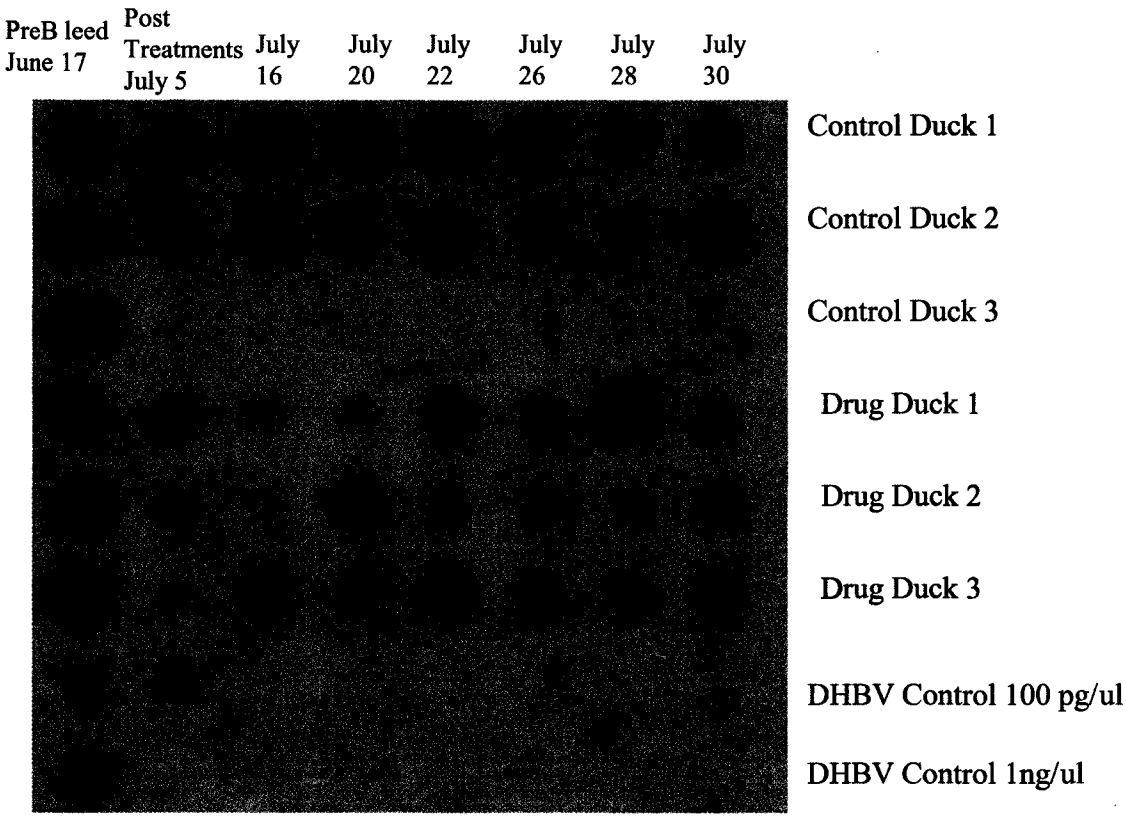

Figure 5.4 Dot blot of in the vivo study determining the effect of 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) on DHBV replication over an extended bleeding schedule.

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5.4 Discussion.

The compounds 1-[(2-hydroxyethoxy)methyl]-5-bromouracil (**15**), 1-[(2-hydroxyethoxy)methyl]-5-aminouracil (**18**), and 1-[(2-hydroxyethoxy)methyl]-5-triflouromethyluracil (**21**) did not exhibit a substantial antiviral effect *in vivo*. The lack of *in vivo* activity could be due to insufficient dosage and/or drug delivery to target site, or metabolic inactivation.

However, despite inherit viremic fluctuations seen in DHBV infections (23), the 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) was able to exert a statistically significant antiviral effect. It is assumed then that it was triphosphorylated by duck cellular enzymes and either inhibited the DHBV DNA polymerase and/or resulted in the chain termination of the elongating viral DNA strand it was incorporated into (32). The nucleoside analog, (**16**), not only exerted an effect during the scheduled treatments, but maintained the antiviral effect post treatment during a period of twenty five days after the last injection. In a repeat experiment the results were not as significant, because the ducks utilized were older and therefore, the body weights were larger (by approximately two times). The study had been planned assuming the duck weights would have been similar as in the first study, thus the dosages based on body weight were reduced by approximately half. As a result, the second study actually tested a lower dosage, which as expected exhibited a lesser effect. Since, it did not exhibit the same level of antiviral activity at a lower dosage, it almost serves as a pseudo dose response, which corroborates the potential antiviral effect of this compound. Overall, the 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) shows promise as a potential antiviral

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compound, and may display even better results in a future study using higher dosages and a longer treatment schedule.

Although ducks are not as sensitive to drug toxicity as compared to woodchucks and humans (5), there was no morbidity or other observable detrimental health conditions that resulted from the treatments. The body weights of the ducks in the treatment groups were typically on par with the control group ducks and in some cases were even greater. Overall, the tested compounds did not elicit any signs of toxicity. These results further support the MTT cytotoxicity assay results (discussed in chapter two), which implied that these compounds were not toxic since they had a CC_{50} greater than 100 μg/mL in both HepG2 and Vero cells.

The difficulty with *in vivo* data is that there is not always a direct correlation with results from *in vitro* experimentation, and there are many variables to consider in addition to the compound being tested (33). Although the 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) did exhibit a statistically significant antiviral effect, there are some factors that may have affected its ability to exhibit an even greater potential for activity. One such consideration is the drug delivery and/or route of administration. The intraperitoneal route was used, since the target organ for drug delivery was the liver and this route is commonly used in hepatitis B *in vivo* studies. Nevertheless, future studies could try alternative routes of administration, such as intramuscularly, intravenously or even orally, since these pyrimidine nucleosides are more water soluble and bioavailable (34).

There are also the different factors affecting the metabolism of the compound to consider (35). The avian class does tend to have higher metabolic rates as compared to

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mammalians, and may need dosages that are relevant to this issue (36). Furthermore, in a study by Dalvi et al., various members of the avian species (including the duck) were compared to the mammalian rat, with respect to presence of drug metabolic enzymes. It was found that the avian species generally possesses a lesser quantity of microsomal metabolic enzyme protein per gram of liver, and less cytochrome P-450 (containing oxidase abilities) when compared to the mammalian species. Cytochrome P-450 is an important determinant of the therapeutic ability of a drug, and can contribute to a difference between species with respect to drug metabolism (37). Moreover, the duck also has lower amounts of N-demethylase and analine hydroxylase metabolic activity when compared to the rat, which can reduce the rate of drug biotransformation in ducks (37). Although nucleoside analogs are generally treated as natural substrates and do not require to be metabolized as drugs, in the case of abacavir it has been shown it is metabolized by the drug metabolic enzymes, alcohol dehydrogenase and glucuronyl transferase. The exact mechanism of metabolism of these specific compounds have not yet been studied and should be investigated in the future.

Another factor to take into account is that each species has different phosphorylation abilities, which will affect the ability of a nucleoside analog to exert its effect (21). There are also differences to consider in light of the fact that avian red blood cells (RBCs) are nucleated as compared to the anucleated erythrocytes in humans (36,39-41). It is possible that the compounds could get internalized and metabolized by the duck RBCs, reducing target cell delivery and lowering plasma half lives. In addition, the presence of a nucleus in RBCs can affect the plasma membrane Ca^{2+} ATPase activity, which in turn can affect the phosphorylation by protein kinases (42). Therefore, there can

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be different levels of phosphorylation between a species that has nucleated erythrocytes versus a species that does not. Again, the nucleoside analogs require that they be triphosphorylated by host cellular kinases as a requisite for activity. Thus, despite the fact that the duck model is an excellent *in vivo* animal model, there are still some factors that can limit the direct correlation of activity between avians and mammals.

One other feature that may have contributed to the limited *in vivo* antiviral effect of some of these compounds is the age of the ducks used in this study. At the start of the studies, the age of the congenitally infected ducks receiving treatment ranged from one week to sixteen days old. In a study by Tsiquaye et al., it was found that young ducks of three weeks of age had much greater DNA polymerase activity, hence active viral replication, as compared to ducks which were even just weeks older (22). Hatchlings (less than one day old) had even greater viral polymerase activity (22). Thus, the compounds were tested in a period of extremely high levels of viral replication, and the ability of these compounds may not have been truly represented. Although older ducks may serve as a better model, an opposing factor to consider is that with increasing age there is a significant increase in body weights. As a result, substantially more compound is needed to apply the same dosage. In a future study, it may be more beneficial to study the inhibition of viral replication in these young ducks by measuring the levels of replicative intermediates, such as the single strands of viral DNA in tissue (23). Since congenital ducks are born with such high viral titres to begin with, it may not be fully indicative of the actual antiviral effect of the compound by looking at total DHBV detected in sera. Nevertheless, the compound 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) was able to exert an inhibitory effect on viral replication despite the fact

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that the ducks began treatments at one week of age. Thus, displaying the potent antiviral activity of this particular nucleoside analog.

In summary, the acyclic pyrimidine nucleoside analog, 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) is quite promising in terms of its potential antiviral ability. It was able to significantly reduce DHBV detected in sera during the treatment schedule, and maintained an effect during a twenty five day post treatment period. Accordingly, this compound warrants future study. To get a better understanding of the ability of this compound, studies investigating its metabolism, phosphorylation, and effect in older ducks may be beneficial in obtaining a better understanding of its effect. Furthermore, it may be worthwhile to prepare ester prodrugs, such as valeryl, to increase its bioavailability and activity.

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Conclusions and Future Studies

In this section I have briefly summarized the project and findings. The first phase of this project was to synthesize two novel series of acyclic pyrimidine nucleoside analogs. These analogs featured a variety of substituents at the C-5 and/or C-6 positions of the uracil base, and a 1-[(2-hydroxyethoxy)methyl] or a 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] flexible glycosyl moiety at the N-1 position that have abilities to mimic a natural 2'-deoxyribose moiety. These compounds were synthesized and purified in order to perform a number of *in vitro* and *in vivo* studies. The compounds were first evaluated for their anti-HBV activity using a primary culture of DHBV congenitally infected duck hepatocytes obtained through a liver perfusion and the 2.2.15 cell line (hepatoblastoma cells transfected with HBV). The compounds were tested at various concentrations and the level of inhibition of viral replication was used as a measure of activity. Of the compounds that exhibited antiviral activity, there appeared to be a trend. The compounds containing the 1-[(2-hydroxyethoxy)methyl] glycosyl moiety coupled with a halogenated C-5 substituent exhibited the strongest activity in the series. Specifically, the compound 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) had substantial inhibitory activity in the primary duck hepatocytes assay. In the 2.2.15 assay, this compound exhibited slightly lower activity, which could be due to the high levels of viral replication inherit in this cell line or due to differences in metabolism.

The activity of selected compounds was then tested against two clinically relevant lamivudine-resistant strains of HBV. Although 3-TC sensitivity decreased remarkably, there were approximately the same levels of activity displayed by the acyclic pyrimidine

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nucleosides analogs tested, in both the resistant strains as was seen in the wildtype HBV 2.2.15 assay. This led to the conclusion that there was no cross-resistance between this series of nucleoside analogs and 3-TC, and that the acyclic side chains were facilitating the compounds binding to the mutant HBV polymerase binding pocket.

Finally, selected acyclic pyrimidine nucleoside analogs were tested *in vivo* in young congenitally infected ducks, and again the compound 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) displayed potent activity. Not only was there a statistically significant reduction in DHBV detected in sera, but the inhibition on viral replication *in vivo* was sustained during the post treatment period. A repeat study with this compound was performed, but the duck ages and body weights were different and the dosages were reduced as compared to the initial study. The fact that the viral reduction was not as comparable to the first study can even indicate a pseudo dose response effect. In the future, this study could be repeated again with higher dosages and over a longer term treatment schedule, to see if greater viral clearance can be obtained. Alternate routes of administration and drug delivery could also be examined. In addition, older ducks could be used for a future study, since the younger ducks have exceptionally high states of viral replication.

In the future, it may be beneficial to perform a metabolic study. In order for the nucleoside analog to be active, the compound must be triphosphorylated by cellular kinases. Therefore, by looking at the level of phosphorylation it may explain why certain compounds were not active (due to lack of phosphorylation), and why some compounds could exert an effect (if phosphorylated). It may even explain why a compound can have

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a strong antiviral effect in the primary duck hepatocyte assay versus the 2.2.15 cell line, if there was differential phosphorylation efficiency.

Overall, the compound 1-[(2-hydroxyethoxy)methyl]-5-chlorouracil (**16**) has exhibited strong inhibitory activity against HBV replication in these studies. Based on the data presented here and with increasing future studies and investigations, this nucleoside analog may have potential as a future antiviral that can be used against the hepatitis B virus. This series of 5-substitued acyclic pyrimidine nucleoside analogs holds promise as a new generation of anti-HBV agents and should be explored further with various other C-5 substituents and acyclic side chains at the N-1 position in order to investigate novel chemotherapeutic agents for the treatment of an HBV infection. This new class of acyclic pyrimidine nucleoside analogs could be used either alone, sequentially, or in combination therapy to reduce the toxicity and avoid the emergence of drug resistance.

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