The Molecular and Structural Basis of HBV-resistance to Nucleos(t)ide Analogs

Nidhi Gupta¹, Milky Goyal², Catherine H. Wu¹ and George Y. Wu*¹

¹Department of Medicine, Division of Gastroenterology-Hepatology, University of Connecticut Health Center, Farmington, USA; ²Department of Microbiology, College of Basic Sciences and Humanities, Punjab Agriculture University, Ludhiana, Punjab, India

Abstract

Infection with hepatitis B virus (HBV) is a worldwide health problem. Chronic hepatitis B can lead to fibrosis, liver cirrhosis, and hepatocellular carcinoma (HCC). Management of the latter two conditions often requires liver transplantation. Treatment with conventional interferon or pegylated interferon alpha can clear the virus, but the rates are very low. The likelihood, however, of viral resistance to interferon is minimal. The main problems with this therapy are the frequency and severity of side effects. In contrast, nucleos(t)ide analogs (NAs) have significantly lower side effects, but require long term treatment as sustained virological response rates are extremely low. However, long term treatment with NAs increases the risk for the development of anti-viral drug resistance. Only by understanding the molecular basis of resistance and using agents with multiple sites of action can drugs be designed to optimally prevent the occurrence of HBV antiviral resistance.

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Introduction

The hepatitis B virus (HBV) is estimated to affect more than 400 million individuals worldwide,¹ and approximately two million people in United States. Chronic hepatitis B can lead to fibrosis, liver cirrhosis, and hepatocellular carcinoma (HCC). The geographical distribution of HBV infection varies widely (Fig. 1). High genomic variability of HBV has resulted in the evolution of eight distinct genotypes (A-H), some of which are further divided into subtypes (1, 2 etc.).²

HBV was discovered in 1967,³ but treatment was not available until the development of interferon, which was first used in 1976.⁴ This was the mainstay therapy until 1998 when the mechanistic similarity between human immunodeficiency virus (HIV) reverse transcriptase and HBV polymerase led to the study and approval of lamivudine, the first oral nucleos(t)ide analog (NA) approved for HBV treatment. Subsequently, several NAs were developed for HBV treatment, including adefovir in 2002, entecavir in 2005, telbivudine in 2006, and tenofovir in 2008.⁵

Current treatment for HBV infection consists of two major classes:⁶ immuno-modulatory agents (interferon-based therapies) and oral NAs. Advantages of treatment with conventional interferon or pegylated (long-acting) interferon alpha include low risk of developing viral resistance and slightly increased clearance of HBV with time. However, severe side effects such as anxiety, depression, and anemia have limited its benefits. Although NAs have significantly lower side effects, long term treatment with NAs is generally required because the rate of sustained virological response is extremely low.⁷ Since the replication rate of HBV is high and a proof reading mechanism of its polymerase is absent, the mutation rate is very high, 1.4–3.6 × 10⁻⁵ nucleotide substitutions per site per cycle. This results in a high risk for the development of drug resistance⁷ against NA therapy.

Genome organization and genotypes

HBV is one of smallest known animal viruses, 32–42 nm in diameter. The viral particle consists of an outer envelope and an inner nucleocapsid that enclose a viral DNA genome and other proteins (Fig. 2a). The outer lipoprotein envelope has hepatitis B surface antigens (HBsAg) embedded in it. These antigens are found in blood either free or bound to viral particles. Free HBsAg is non-infectious, but is involved in induction of long-term immunity against HBV.⁹–¹¹ The viral core consists of hepatitis B core antigen (HBcAg), hepatitis B e antigen (HBeAg), a partially double stranded DNA molecule, and DNA polymerase. HBeAg is a non-secretary marker of infectious viral material found inside HBV-infected cells. HBeAg is a form of HBcAg secreted in blood, and is usually an accurate index of viral load.¹²–¹⁴ The polymerase, a DNA-dependent DNA polymerase, is involved in reverse transcription for viral replication¹⁵–¹⁷ and is research target for the development of drugs against HBV.⁵,¹⁸–²⁰

The compact organization and multiple overlapping open reading frames (ORFs) has required the HBV to utilize its genome efficiently (Fig. 2b).¹⁶,²¹ The viral genome consists of a 3.2 kb partially double-stranded DNA molecule with one end bound to DNA polymerase. There are four ORFs in the HBV genome that result in the transcription and translation of...
different types of proteins. The core (C) gene encodes capsid proteins HBcAg and HBeAg; the P gene encode viral DNA polymerase; the S gene has three in-frame start codons and codes for three envelope proteins that differ in lengths of the N-termini (Fig. 2c). They are designated as large (L), major (M), and small (S) surface protein. L and M are surface proteins and are involved in hepatocyte receptor binding. The S surface protein is the protein detected in commercial assays as HBsAg. Finally, an X gene codes for a 16 kDa protein HBx, whose function is still not completely understood. It has been reported to modulate cellular signals, promoters, and cell-cycle pathways, some of which have been implicated in the development of hepatocellular carcinoma.

The eight genotypes of HBV (A-H) have a distinct geographical distribution (Table 1) and are classified based on >8% divergence in complete genome sequence. The

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Fig. 1. Global prevalence of chronic hepatitis B virus (HBV) infection in adults. Source: Centers for Diseases Control and Prevention, CDC 2010

Fig. 2. Structural and genetic organization of HBV. a, structure of HBV; b, compact organization of the HBV genome showing multiple overlapping open reading frames; c, expression of three envelope proteins from three in-frame start codons from the S-gene of the HBV genome.

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prevalence of HBV genotypes varies highly, with A and C being most common (>30%), and E and F being least common (<0.6%) in the US. Four of these (A, B, C, and F) are further divided into sub-genotypes based on genomic variability of >4%. Disease progression and response to anti-viral therapies differs in patients infected with different genotypes. 

**Life cycle and replication**

To understand current therapeutic approaches to HBV infection, knowledge of the HBV life cycle and replication is very important. HBV is a DNA virus, but it replicates like a retrovirus using reverse transcription. The initial steps of HBV entry into cells have been difficult to characterize. They are thought to involve cell membrane binding and endocytosis of viral particles. Several proteins have been proposed to assist in viral attachment to the cell surface and entry into the cell. For example, apolipoprotein H, poly-human serum albumin, interleukin-6, and carboxypeptidase D (gp 180) have all been proposed to play a role as HBV receptors. Recently, another 80 kDa protein has been identified that binds HBV surface proteins. To date, the exact mechanism of HBV entry into cells is still not well elucidated.

Once viral particles enter the cell, the viral nucleocapsid is uncoated and delivered to the cell nucleus. In the nucleus, partially double-stranded HBV DNA is repaired to form covalently closed circular DNA (cccDNA), which serves as a template for transcription by cellular enzymes. HBV DNA polymerase does not have repair mechanism activity. Also, unlike retroviruses, HBV DNA integration into host cell genome is not required for HBV replication. After re-circularization, HBV DNA starts to express transcripts required for HBV protein synthesis and pre-genomic RNA (pgRNA) required for viral replication. Two types of transcripts are expressed: sub-genomic and genomic. Sub-genomic, smaller transcripts, serve as templates for surface proteins and HBx expression. Genomic transcripts longer than one genome in length serve as templates for e, core, and polymerase expression. The HBV DNA polymerase selects genomic transcripts lacking the start ‘ATG’ codon at the 5’ terminus as pgRNA for packaging into virions. Sub-genomic and genomic transcripts are then transported and translated in the cytoplasm. Binding of DNA polymerase protein to pgRNA results in encapsidation and formation of RNA-containing nucleocapsids. After encapsidation, reverse transcription is then initiated upon interaction of the C-terminus of the DNA polymerase with core protein. Polymerase bound to pgRNA starts to reverse transcribe pgRNA into (-) DNA strand using a bulge in the stem loop of pgRNA. The RNase H activity of the polymerase degrades pgRNA after (-) DNA strand synthesis, leaving 13–20 nt on the 5’ end that serve as primers for (+) DNA strand synthesis. Following the synthesis of viral DNA and nucleo-capsid packaging, HBV surface proteins aggregate in cellular Golgi bodies, forming vesicles around viral nucleocapsids that result in enveloped HBV particles. These particles are then secreted as mature virions by exocytosis. This occurs along with secretion of non-infectious particles, viral surface protein aggregates without enclosed nucleo-capsid. Although several steps of the HBV life cycle have been well characterized, many aspects remain unclear and need to be explored in order to fully understand the life cycle of HBV.

**In vitro screening assays for HBV resistance**

Currently, there are no practical animal models for testing antiviral drug efficacy. Drugs are tested pre-clinically in vitro assays to determine both their efficiency and resistance profiles. In these assays, liver cells are infected with wild type or mutated HBV, and the effects of NAs are determined by measuring HBV replication or cytotoxicity. In vitro reverse transcription assays are performed to determine the effects of various mutations on resistance to NAs. Wild type or mutant HBV DNA polymerase is expressed from plasmids and tested for priming and elongation of viral DNA.

**Clinical drug resistance rates**

Determining the rate of the development of resistance from clinical experience varies considerably depending on the agents. For lamivudine, the one year resistance rate is 30%, and for telbivudine it is 15%. When used individually, entecavir and adefovir have higher barriers of resistance, with rates less than 2% after two years. No resistance to tenofovir has yet been reported. After five years of treatment, the rates of resistance were about 70% for lamivudine, 28% for adefovir, less than 1% for entecavir, and 0% for tenofovir and interferon.

**Current treatment and mechanisms of HBV resistance**

**Interferon therapy**

Conventional interferon and pegylated (long-acting) interferon alpha are two drugs currently available and approved for HBV therapy. Interferon is an immunomodulator, acting indirectly to eliminate HBV-infected host cells by activating various host genes and innate immune response pathways. Its use has a number of advantages including a finite treatment course, lack of drug resistance, and a decreased risk of cirrhosis and HCC with long term use. However, the sustained virological response rate is generally poor (6%) for PEG-interferon. However, there are substantial side effects, including fatigue, diarrhea, alopecia, flu-like symptoms, insomnia, and psychiatric complications, that often require additional medications to correct.

**Table 1. Geographic Distribution of HBV Genotypes**

| HBV genotypes | Geographic distribution |
|---------------|------------------------|
| A             | Northwest Europe, North America, Africa |
| B             | Asia                   |
| C             | Asia                   |
| D             | Worldwide distribution |
| E             | Africa                 |
| F             | South America, Central America (Hispanic origin) |
| G             | France, UK, Italy, Germany, USA |
| H             | Central America (Amerindian populations) |

Source: Ref [28] [145–153]
The presence of extremely stable cccDNA HBV in infected hepatocytes is the major obstacle to permanent elimination of HBV. The cccDNA acts as template for viral RNA genome production. The DNA-dependent DNA polymerase of HBV catalyzes synthesis of RNA, RNase H, and protein priming activities along with replication of HBV genome. Recently, NAs have been developed to directly inhibit the function of HBV DNA polymerase by either competitive binding to the enzyme or by terminating viral replication prematurely, which inhibits viral genome production. NAs currently available for HBV treatment are shown in Fig. 3.

**Lamivudine**

Lamivudine [(−)-β-L-2′-3′ dideoxythiacytidine] or 3TC was the first oral NA approved for the treatment of HBV (Fig. 3). 3TC is a cytidine analogue, and is phosphorylated intracellularly to 5′-triphosphate active metabolites. It inhibits DNA polymerase function by competing with a natural substrate (dCTP), resulting in DNA chain termination. Treatment with 3TC has been shown to not only suppress viral replication and HBV serum levels, but also to reverse fibrosis to varying degrees with long term use. Furthermore, it has been shown to decrease the risk of HCC. Lamivudine is a potent drug, but it unfortunately also has a high rate of drug resistance, which increases with duration of treatment. According to molecular model studies, residues F88, L180, and M204 play indirect, but crucial roles in the formation of a hydrophobic pocket and other non-covalent interactions in the DNA polymerase of HBV. These have been shown to be involved in the binding of NAs to the polymerase. Although slightly different structurally from natural cytosine, 3TC interacts with the HBV DNA polymerase active site perfectly, inducing a fitting movement of the M204 residue that results in significant inhibition of viral replication (Fig. 4). Long term use of 3TC for HBV treatment frequently leads to mutation of the M204 residue in the HBV DNA polymerase gene and gives rise to 3TC-resistant HBV. Three major mutations (M204I, M204V, and L180M) individually or in combinations in HBV DNA polymerase result in significant resistance against 3TC anti-viral characteristics (Table 2). Mutation of L180 to M180 or M204 to I204 or V204 add a bulky group of branched residues to the hydrophobic pocket, creating steric hindrance that prevents accommodation of the 3TC ring (Fig. 4). This ultimately causes resistance by increasing the thermodynamic stability of binding of the natural substrate to HBV DNA polymerase compared to 3TC. Dual mutations (L180M + M204I or L180M + M204V) can further reduce the binding affinity of 3TC with HBV DNA polymerase by combining effects of reduced interaction (mutated L180) and steric effects (mutated M204) (Fig. 5).

**Adefovir dipivoxil (Adefovir, ADV)**

Adefovir (ADV), an acyclic adenosine analogue (Fig. 3), is phosphorylated to yield an active metabolite that inhibits HBV DNA polymerase activity by competing with the natural substrate (dATP). In doing so, it causes chain termination. Decreased potency of ADV is due to lower effective intracellular drug levels compared to other NAs. In addition, ADV has been associated with a dose-dependent nephrotoxicity and can lead to reversible renal impairment. Molecular modeling studies have shown that ADV interacts with HBV DNA polymerase in a way similar to dATP. Major
mutations in the HBV DNA polymerase gene that cause ADV resistance are A181V, A181T, N236T, and I233V.93–95 Other mutations (P237H, N238T/D, V84M, S85A, V214A, and Q215S) have also been reported to decrease sensitivity to ADV, although the exact mechanism remains unclear96 (Fig. 5). ADV binding to HBV DNA polymerase is not affected by mutations (M204I, M204V and L180M) that result in resistance to 3TC. As such, ADV is effective against lamivudine-resistant HBV polymerase.97,98

Entecavir

Entecavir (ETV) is a guanosine analogue (Fig. 3) that is phosphorylated intracellularly to an active metabolite. It inhibits multiple functions of HBV DNA polymerase, including polymerase priming, reverse transcription, and DNA synthesis.99–101 Unlike 3TC and ADV, ETV does not cause chain termination because it possess a 3′-hydroxyl group,102 so addition of nucleotides to the nascent chain continues. Molecular modeling studies have demonstrated that ETV forms most interactions with the active site of HBV DNA polymerase in a manner similar to the natural substrate (dGTP). It does not, however, require induced fit movements of any residue in the polymerase.89,100 This allows ETV to be more effective and less susceptible to the development of drug resistance.103–105 ETV inhibits HBV DNA elongation after addition of a few nucleotides by increasing steric hindrance and thereby preventing addition of more bases to the DNA.100 ETV is more potent than other NAs and has a higher rate of viral clearance.106–110 It also has a high barrier to the development of resistance because a single mutation is not sufficient to cause resistance. Rather, multiple mutations are required: I169T, T184G, S202I, or M250V in addition to L180M and M204V/I, the latter two result in resistance to 3TC.111–113 (Fig. 5). Therefore, the risk of resistance is higher with HBV already resistant to 3TC.111

Fig. 4. Increased resistance to NAs caused by inhibition of induced fitting movement by a mutation near the active site. a, HBV DNA polymerase can bind to either natural substrate for HBV DNA synthesis or NAs by induced fitting movement at the active site; b, mutated HBV DNA polymerase can bind to natural substrate for HBV DNA synthesis. A mutation near the active site inhibits induced fitting movement of polymerase resulting in steric inhibition of binding of NA to polymerase.
Telbivudine

Telbivudine (LdT) is an analog of thymidine (Fig. 3) with an L-configuration rather than the D-configuration of natural nucleosides. It competes with natural dTTP (2’-deoxythymidine 5’-triphosphate) for the HBV DNA polymerase active site after phosphorylation intracellularly, leading to premature chain termination. Although LdT is more potent and less prone to drug resistance than 3TC,114–118 the drug resistance rate is still relatively high.119,120 An M204I mutation greatly decreases LdT inhibition of HBV replication by altering the orientation and position of key methyl groups (Fig. 5). The L180M mutation results in the loss of another methyl group required for LdT affinity to the polymerase. Hence, dual mutations (M204I + L180M) make HBV DNA polymerase highly resistant to LdT. Other mutations L80I, A181T/V, and L229W/V have been linked to LdT resistance.5,121 Besides the high resistance rate, adverse reactions of LdT, such as myopathy,122 myalgia,123 creatine kinase elevations,124 and general weakness, have also been reported in various clinical trials. Mechanisms of these adverse reactions are not clearly understood, and further studies are required to further characterize them.

Tenofovir

Tenofovir disoproxil fumarate (TDF), 9-((R)-2-((Bis(((isopropoxycarbonyl)oxy)methoxy)phosphinyl)methoxy)propyl) adenine fumarate (PMPA) is an adenine analog (Fig. 3) that competes with dATP after intracellular phosphorylation, resulting in chain termination. TDF is more potent than ADV because of a high tolerance to increased doses of TDF compared to ADV.125–127 Despite long-term treatment, no evident resistance has been reported with TDF.128 However, some mutations, A181V and N236T, have been found to decrease susceptibility to TDF,129–131 although the exact mechanism by which this occurs is unknown. Mild renal toxicity132–134 and osteomalacia135,136 has been observed with long term TDF treatment.

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Table 2. Anti-HBV Agents and Reported Resistance Mutations

| Analog   | Type                        | HBV polymerase mutation |
|----------|-----------------------------|-------------------------|
| Lamivudine | Cytosine analog (–)-L-2’-3’ dideoxythiacytidine | M204I/V, L180M, L180M-M204V, L180M-M204I |
| Adefovir | Adenosine analog 9-[(2-[[bis((pivaloyloxy)methoxy]-phosphinyl)methoxy]ethyl]adenine | A181I/V, T236T, I233V |
| Entecavir | Guanosine analog 2-amino-1,9-dihydro-9-[[1(S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one, monohydrate | 1169T, T184G, S202I, M250V, M204I/V |
| Telbivudine | Thymidine analog 1-(2-deoxy-β-L-ribofuranosyl)-5-methyluracil | M204I, L80I/V, L180M, A181 T/V, L229W/V |
| Tenofovir | Adenosine analog 9-((R)-2-((Bis(((isopropoxycarbonyl)oxy)methoxy)phosphinyl)phosphinyl)methoxy)propyl) adenine fumarate (PMPA) | A181I/V, N236T |

Table 3. Reported Mutations and Associated Resistance Determined by in Vitro Assays

| Mutation | Lamivudine | Adefovir* | Entecavir | Telbivudine | Tenofovir* |
|----------|------------|-----------|-----------|-------------|-----------|
| I169T    | >1000      | 1.0       | >1000     | >100        | No data   |
| A181T    | 2–6        | 1–5       | No data   | No data     | 1–1.5     |
| A181V    | 2–6        | 1–5       | No data   | No data     | 2.9–10    |
| T184G    | >1000      | 2.0       | >1000     | >1000       | 0.6–6.9   |
| S202I    | >1000      | 2.0       | >1000     | >1000       | 0.6–6.9   |
| M204I (YMDD) | >1000 | 0.7       | 30        | >1000       | 0.7–3.4   |
| M204V (YMDD) | >1000 | 0.7       | No data   | 1.2         | 0.7–3.4   |
| V214A    | 10–20      | 7–10      | No data   | 7–10        | >10       |
| Q214S    | 10–20      | 7–10      | No data   | 7–10        | >10       |
| N236T    | 3–8        | 7–10      | 0.67      | 2.4         | 2.9–10    |
| M250V    | >1000      | 1.0       | >1000     | 1.0         | 0.6–6.9   |
| L80I + M204I | >1000 | >10      | No data   | >1000       | >10       |
| L180M + M204V | >1000 | 0.2      | 30        | 133         | 3.4       |

Source: Ref [59]
HBV infections every year, and many go on to develop chronic hepatitis. Introduction of NAs have significantly impacted HBV treatment. Although these agents are highly potent, there is potential for the selection of viral resistance to NA therapy because of the necessity for long-term treatment, possible exposure to sequential monotherapy, and possible non-compliance. Molecular modeling, in vitro, and clinical studies examining, at the molecular level, the interactions between NAs and other agents with HBV DNA polymerase will assist in the development of new therapeutic agents. These drugs will hopefully lead to complete clearance of HBV from hepatocytes without the risk of developing viral resistance. Overall, the data on drug resistance suggests that the rates of resistance are lower with combination therapy than conventional monotherapy.

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**Conflict of interest**

One of the authors (GYW) is a member of medical advisory boards for Gilead, and Bristol-Myers-Squibb.

**Author contributions**

Writing the paper (NG, MG), reviewing and editing the paper (CHW, GYW).

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