= Abstract =

A high rate of viral turnover, combined with an error-prone polymerase, results in an increased frequency of mutational events during hepatitis B virus (HBV) replication, resulting in a diverse population of progeny virus (quasispecies). Not surprising then, particular selection pressures, both from within (host immune clearance) or from outside (vaccines and antivirals) the host, readily select out new “escape” mutants resulting in treatment failure. The introduction of nucleos(t)ide analogue (NA) therapy for chronic hepatitis B has resulted in the emergence of antiviral drug resistance which has itself become the major factor limiting treatment effectiveness. Furthermore, due to the overlap of the viral polymerase and envelope reading frames in the circular HBV DNA genome, NA-resistance associated mutations selected within the catalytic domains of the polymerase usually result in significant changes to the neutralising antibody binding domains of the hepatitis B surface antigen, including the emergence of antiviral drug associated potential vaccine escape mutants (ADAPVEM’s). The main reason for this is that the neutralisation domain, the “a” determinant, is a conformational epitope. The public health significance of APADVEM’s may then be very considerable in terms of the global program for control of hepatitis B via universal infant immunisation. Thus, prevention of resistance requires the adoption of strategies that not only effectively control active HBV replication but also prevent the emergence of APADVEMs.

Introduction to antiviral drug resistance

Two key concepts are critical to an understanding of the development of antiviral drug resistance. First, viral infection is typically characterised by high levels of viral production and turnover. Second, the viral population in an infected person is highly heterogeneous. In an infected individual, there is a cycle of “viral reproduction”, viral mutant generation, genetic diversity, antiviral drug selection pressure leading to “survival of the fittest”, directly as a consequence of quasispecies dominance.1

Antiviral drug resistance also depends on at least six factors:
1. Viral mutation frequency.
2. The magnitude and rate of virus replication.
3. Intrinsic mutability of the antiviral target site (usually a viral enzyme).
4. Selective pressure (potency) of the drug.
5. Amount of replication space.
6. Fitness of the resistant mutant.

Other factors that can play a role include the “genetic barrier” of the drug which can be considered in the context of the number of specific mutations required for drug resistance to develop.2,3

One of the major classes of antiviral agent is the nucleos(t)ide analogues (NA), which are competitive inhibitors of the viral DNA polymerase enzyme and most NAs block viral replication by premature chain termination, since they lack a 3'-OH group.4 This class of drug has been extensively studied in both HIV as well as HBV-disease, and several mechanisms of NA resistance have been identified.2,4,5

1. Steric hindrance where the associated resistance substitution alters the ability of the viral enzyme to bind NA relative to the natural substrate.
2. Reduction in catalytic efficiency where the resistance substitution results in sub-optimal nucleophilic attack geometry for the subsequent information of NA into the newly replicating viral genome.
3. Increased excision of the NA as chain terminator by the process of pyrophosphorolysis.

By understanding the main processes involved in selection of drug resistant viruses, it is possible then to implement ways to prevent it:3
1. Maximise antiviral activity.
2. Maximise genetic barriers to resistance.
3. Maximise pharmacologic barriers.

Thus in this way, the emergence of resistant viruses should be minimised. In order to overcome resistance, the best approach has been to use combination chemotherapy; in hepatitis B, on first virological breakthrough, an add-on strategy using an agent with a complementary cross-resistance profile is the preferred approach.6

**HBV virology and pathways to resistance**

The lifecycle of the hepatitis B virus (HBV) revolves around two key processes: (a) generation of HBV covalently closed circular (ccc) DNA from genomic relaxed circular (RC) DNA and its subsequent processing by host enzymes to produce viral RNA; and (b) reverse transcription of the pregenomic (pg) RNA within the viral nucleocapsid to form RC DNA, thereby completing the viral life cycle. In the patient on a daily basis, the replication phases of HBV are marked by a high frequency of mutational events resulting from an enormous viral turnover rate combined with the error-prone reverse transcriptase/polymerase, producing a quasispecies pool comprising a particular HBV population that is dominant at any one point in time.1 Not surprisingly then, the introduction of nucleos(t)ide analog (NA) therapy has seen the emergence of antiviral drug resistance, which has become the main factor limiting the long-term application of antiviral agents such as NA for patients with chronic hepatitis B (CHB).

To date, there are eight codons associated with primary antiviral drug resistance in CHB, which map to four
Several major HBV evolutionary NA-resistance pathways (rtM204I/V, rtN236T and rtA181T/V) have now been characterised associated with these eight codons. The first or rtM204V/I pathway is responsible for resistance to the L-nucleosides such as lamivudine (LMV) and telbivudine (LdT), and also entecavir (ETV) as rescue therapy in LMV-experienced patients. The L-nucleoside pathway is associated with clusters of secondary mutations that can affect subsequent treatment with NAs (rtT184G, rtS202I) such as ETV. The second or the rtN236T pathway, accounts for adefovir (ADV) and tenofovir (TFV) resistance. The third pathway, rtA181T/V, is associated with resistance to LMV and ADV and is a potential multi-drug resistance (MDR) pathway and will probably impact on TFV sensitivity, as well, either alone or with the rtN236T. In naïve patients only treated with ETV, a fourth pathway has been described where at least 3 mutations are need to be selected out at the same time: rtL180M+rtM204V plus either one of rtT184 or rtS202 or rtM250 codon changes. Finally, in highly experienced NA treated patients, other MDR pathways are being increasingly recognised such as rtA181T+rtN236T+rtM250L. Sequential monotherapy treatment with NAs promotes multi-drug resistance. Thus, the prevention of resistance will require the adoption of strategies that effectively control virus replication and exploit an understanding of the mechanisms and processes that drive the emergence of drug resistance namely, high replication rates, low fidelity of the HBV reverse transcriptase/polymerase, selective pressure of the NA, genetic barrier of the drug, role of replication space (liver turnover) and fitness of the mutant.

Cross-resistance

Cross resistance is defined as resistance to drug(s) to which a virus has never been exposed. The NA resistance-associated mutations selected by particular groups of NA (eg: L-nucleosides, Acyclic Phosphonates or D-Cyclopentane) may diminish the antiviral activity of other drugs. This should be considered before any antiviral drug is prescribed and the physician should plan for eventual treatment failure. The initial selection and subsequent rescue therapies should be based on a knowledge of cross-resistance, so that the second agent lacks cross-resistance with the failing agent. Preferably by using the add-on/combination approach the advantage of using combinations of NA with complementary cross-resistance profiles has recently been highlighted and a summary of cross-resistance profiles based on the viral resistance “pathways” approach is shown in Table 1.

Public health relevance of resistance

The viral envelope (HBsAg) gene overlaps completely within the reverse transcriptase gene and so NA resistance can result in changes in HBsAg. This Pol-Env overlap is important for a number of reasons since it
Table 1. Cross-resistance analysis for the nucleos(t)ide analogues approved for chronic hepatitis B*

<table>
<thead>
<tr>
<th>Resistance mutation*</th>
<th>LVD/LdT-resistant (L180M +/- M204V/I)</th>
<th>ADV-resistant (N236T)</th>
<th>ADV-resistant (A181T/V)</th>
<th>ETV-resistant</th>
<th>LdT-resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation confers some degree of reduced sensitivity to listed drugs</td>
<td>• Entecavir</td>
<td>• Tenofovir</td>
<td>• Lamivudine/ Telbivudine/ Tenofovir</td>
<td>• Entecavir</td>
<td></td>
</tr>
<tr>
<td>Mutation confers complete resistance</td>
<td>• Telbivudine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drugs remaining fully active</td>
<td>• Adefovir</td>
<td>• Entecavir</td>
<td>• Entecavir</td>
<td>• Adefovir</td>
<td>• Adefovir</td>
</tr>
<tr>
<td></td>
<td>• Tenofovir</td>
<td>• Lamivudine</td>
<td>• Tenofovir</td>
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<td>• Telbivudine</td>
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*Modified from References 6 & 7.
*First virological breakthrough should be managed with an add-on strategy (combination), not switch (sequential mono-therapy).

has been shown that common LMV resistant HBVs such as (rtV173L+rtL180M+rtM204V) have important and significant changes in HBsAg (sE164D+sI195M) which significantly reduce anti-HBs (vaccine-associated) binding in vitro.

Likewise, in ADV failure, the rtA181T HBV can be found either by itself or in association with rtN236T, in up to 40% of cases. The rtA181T in rt results in a sW172 [stop] in the overlapping HBsAg and this mutant is defective in virion secretion, is retained in the cell, and acts as a dominant negative mutant for wild-type HBV secretion.11 The clinical implication of these observations is that the virological case definition of drug resistance, >1.0 log IU/mL from nadir in two consecutive samples taken 1 month apart,9,10 does not apply if this mutant is (co)-selected. The practical implication of this finding will be the need for HBV genotyping and polymerase sequencing, as well as HBV viral load monitoring in patients undergoing antiviral therapy.

These resistance patterns also have implications for managing patients chronically infected with hepatitis delta. For example, the rtM204I LMV associated change can result in sW196L/S/stop to the HBsAg, and the rtM204V results in sI195M; the ADV associated resistance mutations can also affect HBsAg. Current approaches to treating hepatitis delta virus (HDV) associated disease are focused on exploiting host cell targets such as with interferon-alpha. However, NA such as LMV and ADV do not inhibit hepatitis delta virus (HDV) RNA replication directly, but interestingly, LMV resistance mutations affecting the HBsAg, such as sW196L/S (rtM204I) inhibit secretion of HDV particles, whilst sI195M (rtM204V) does not affect secretion.12 Presumably, the same phenomenon will occur with the HBsAg changes observed in ADV resistance. Such differential efficiencies of HBsAg proteins expressed by NA-resistant HBV to support HDV secretion may have consequences for clinical prognosis as HBV-HDV co-infected patients are treated with antiviral agents generally.

These in vitro studies have been now extended to in vivo analyses, where, the common LMV-resistant mutation rtV173L+rtL180M+rtM204V,13 which displays the sE164D+sI195M change in HBsAg, successfully infected hepatitis B immunised chimpanzees that had high titres of circulating anti-HBs pre challenge.13 The
chimpanzee HBV transmission study also established the genetic stability of the rtV173L+rtL180M+rtM204V variant in a non-immunised chimpanzee, in whom no revertants to wild type (WT) were detected over time compared to infection with the sG145R vaccine escape mutant which quickly back-reverted to WT. This latter observation demonstrates the important role of compensatory mutations in “fixing the genetic archive”, especially in the setting of transmission of NA-resistance.

Molecular pathogenesis and oncogenic potential

Several HBV proteins are involved in the development of HCC, transcribed from either integrated HBV DNA or the HBV genome. The HBV surface gene (pre-S1/pre-S2/S) encodes three coterminal surface proteins designated L (large: pre-S1+pre-S2+S genes), M (middle: pre-S2+S genes) and S (small: S gene). Pre-S2/S genes truncated at the 3’ end (C-terminal) have been isolated from integrated HBV DNA sequences in HCC. The L and M proteins with C-terminal truncations have transcriptional transactivation potential, a function not exhibited by the full-length forms. Truncated HBV surface proteins have been implicated in the progression to HCC as they possess transactivational activity, demonstrated by increased nuclear factor (NF)-κB or activator protein (AP)-1 promoter activity.

The recent observation that NA therapy selects for HBV mutants that encode truncated surface proteins has substantial clinical relevance since they could theoretically accelerate the progression to HCC. Treatment of CHB with all the NAs can result in the selection of HBV variants with point mutations in the polymerase gene that not only confer NA resistance but also result in changes to HBsAg. In particular, the point mutation that causes the rtA181T change in the polymerase also encodes a stop codon (sW172*) in the overlapping surface proteins, resulting in truncation of the last 55 amino acids of the C-terminal hydrophilic region of the HBsAg. In vitro analysis of rtA181T/sW172* HBV has show that it is defective in secretion of viral particles resulting in intracellular retention of surface proteins and has a dominant negative effect on WT virion secretion resulting in lower viral loads extracellularly. Two recent reports have now provided evidence for involvement of HBV encoding the rtA181T/sW172* mutation in the pathogenesis of, and progression to HCC. Analyses of HBV DNA from patients who developed HCC despite LMV therapy revealed stop codon mutations in the envelope gene in 7 of 8 patients compared with the control group, in which no patients developed HCC. Using expression constructs encoding the HBV surface proteins, these investigators demonstrated that surface proteins truncated at amino acids sL21, sW156 or sW172 (the latter of which equates to the surface proteins expressed from rtA181T/sW172*) transactivated the c-myc and SV40 promoters. NIH-3T3 cells transfected with these constructs were also tumourigenic when injected into nude mice, whereas the WT full-length surface proteins were not. Another common LMV resistance mutation is rtM204I/sW196* stop which is observed in up to 10% of LMV-resistant patients, but to date, there is no data available concerning its effects on viral replication or hepatocyte biology.

Thus, it seems that although NA therapies significantly decrease viral load and improve patient survival in the short term, they might unexpectedly select for HBV variants that are potentially oncogenic, negating the
overall efficacy of NAs in preventing hepatocarcinogenesis, the main long-term goal of antiviral therapy in CHB. Future challenges in the treatment of CHB involve the development of antiviral therapies that do not select for potentially oncogenic drug-resistant HBV as well as the development of treatment strategies that effectively inhibit HBV replication eliminating the risk of drug resistance and therefore the emergence of these C-terminally truncated HBsAg HBV variants.

Conclusion

The current patterns of antiviral drug resistance in CHB are complex. However, four major pathways can be defined in most cases (rtM204V/I; rtN236T; rtA181T/V, and entecavir [rtL180M+rtM204V plus one of rtT184, rtS205. rtM250]) with the emergence of multidrug resistance a clear cause for concern in the longer term. Furthermore, broad clusters of compensatory mutations during LMV therapy will compromise future rescue therapy options with the newer more potent drugs such as ETV. The best cost-effective strategy is to prevent or avoid the emergence of antiviral drug resistance in the first place. This is especially relevant with the developing public health problem that most antiviral drug resistant HBVs have an altered envelope (HBsAg) and have the potential to behave as vaccine escape mutants, be transmitted and infect immunised individuals. Furthermore, there is preliminary evidence that these viruses can be associated with increased oncogenicity. The global program for control of hepatitis B is built on the foundation of universal infant immunisation and will continue to reduce new incident infections of hepatitis B. The challenge of drug resistance on this highly successful campaign requires timely and effective involvement of public health groups co-operatively working with treating physicians to ensure that successful and appropriate therapy guidelines for hepatitis B are achieved and implemented and that this strategy minimises or eliminates antiviral drug resistance.

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References