

Review

HBV drug resistance: Mechanisms, detection and interpretation

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1. Introduction

Besides interferon, only two drugs—lamivudine and adefovir dipivoxil—are approved as first-line therapy for chronic hepatitis B (CHB) in the EU [1,2]. Each is a nucleoside or nucleotide analogue that acts mainly as a specific inhibitor of the viral polymerase/reverse transcriptase (for convenience, the acronym Nucleos(t)ide Reverse Transcriptase Inhibitor (NRTI) will be used here to refer all nucleoside and nucleotide derivatives that act by inhibiting functions of the HBV polymerase). Several other NRTIs besides lamivudine and adefovir dipivoxil have been found to be effective against HBV [1]. They include entecavir, which is licensed in the USA [3], and tenofovir, which is used to treat HIV-1 infection [4,5]. In most cases, treatment of CHB with any single NRTI produces rapid suppression of HBV replication in the short-term, an effect that is not often sustainable due to the emergence of drug-resistant HBV strains [1,6]. Although a variety of other factors including adverse short-term side effects, long-term toxicity, previous sub-optimal treatment regimes, inadequate drug exposure (due to pharmacological properties of particular drugs, poor patient compliance with prescribed treatment, or host genetic polymorphisms) influence the efficacy of treatments for chronic HBV, drug resistance is emerging as the single most significant factor in treatment failure [7]. Failure of NRTI treatment presents a significant clinical challenge, because remaining treatment options are limited [2,8]. Here, we attempt to summarise the current state of knowledge of HBV resistance to NRTI, briefly describe available methods for detecting and quantifying drug resistance and discuss the interpretation of drug resistance and its clinical applications.

1.1. What constitutes drug resistance?

Resistance can be defined in technical, virological or clinical terms. Technically, resistance is defined by either (1) a statistically significant change in the test parameter (increase in EC_{50} , for example) or (2) the minimum change in a parameter that is required for statistical significance (for example, a single \log_{10} change). Technical definitions only describe the attributes of specific tests and have no biological or clinical significance.

Virological resistance is now commonly described as high-, intermediate- or low-level based on fold-changes in EC_{50} in vitro. Such ranking, unfortunately, does not relate directly to clinical observations. For example, lamivudine failure is associated with > 100 fold increase in EC_{50} in vitro, but HBV isolates responsible for adefovir resistance exhibit only low-level (< 10 -fold increase in) resistance in vitro.

Clinical resistance is most easily defined in terms of viral load fluctuations (based on results of assays for serum HBV DNA), which are currently the best available indicators of HBV replication in vivo. The following definitions have been proposed as a basis for further development [8]. An *antiviral effect* is defined as a minimum reduction in serum HBV DNA of $1 \log_{10}$ IU/mL from the pre-treatment baseline within the first 3 months. Failure to achieve this decrease constitutes *primary treatment failure*. A confirmed increase in serum HBV DNA of $1 \log_{10}$ IU/mL from the nadir following initially effective treatment constitutes *secondary treatment failure*. Genotyping and/or phenotyping of clinical isolates are required to confirm that treatment failure is due to resistant virus (see Figs. 1 and 2).

2. Resistance to NRTI

Resistance and cross-resistance to NRTIs is more or less structure-specific. Anti-HBV NRTIs can be separated into three main structural groups (see Fig. 3 and Table 1).

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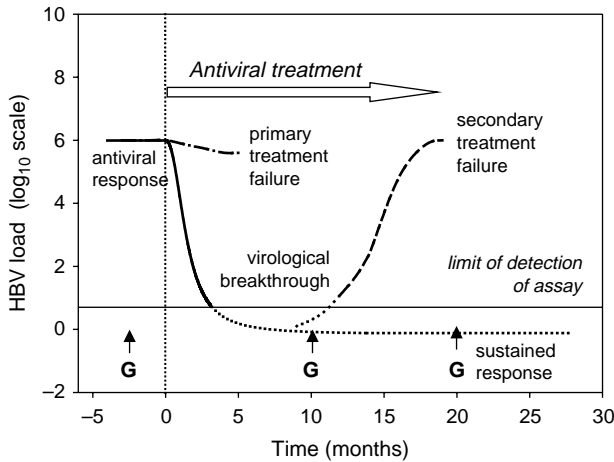


Fig. 1. Graphical illustration of different responses to antiviral therapy. Pre-treatment genotyping (G) is recommended and should be performed at six monthly intervals during treatment and follow-up (or more frequently in high-risk groups).

- (1) L-Nucleoside analogues, including lamivudine, its 5-fluoro-derivative emtricitabine, telbivudine (L-thymidine), tencitabine (L-deoxycytidine) and clevudine (L-FMAU).
- (2) Acyclic nucleoside phosphonates, represented by the dAMP analogues adefovir and tenofovir, together with others that have reached late stage clinical trials.
- (3) Deoxyguanosine analogues in which the deoxyribose moiety is replaced by a cyclopentane derivative—entecavir and abacavir (a prodrug for carbovir) or otherwise modified. The latter include diaminopurine dioxolane, (a prodrug for dioxolane guanine),

2',3'-dideoxy-3'-fluoroguanosine, and famciclovir (pro-drug for penciclovir).

Not surprisingly, primary resistance to any individual drug appears to confer at least some degree of cross-resistance to other members of its group, and may also diminish sensitivity to NRTI from other groups. Mutations that confer resistance to lamivudine confer cross-resistance to other L-nucleosides and reduce sensitivity to entecavir but not to adefovir or tenofovir [9]. Conversely, mutations that confer resistance to adefovir and/or tenofovir do not confer significant cross-resistance to L-nucleosides and entecavir, at least in vitro [10,11]. Multiple mutations in addition to those that confer resistance to lamivudine are required for high-level resistance to entecavir [12] (see Table 1).

3. Molecular modelling identifies mechanisms for resistance to NRTI

Laboratory studies of mechanisms for HBV resistance to NRTI have been frustrated by the inability to obtain sufficient quantities of purified polymerase. However, it has been possible to create three-dimensional models of the reverse transcriptase (rt) region of HBV polymerase based on its homology with related polymerases, including HIV-1 rt. Using these models, the amino acid changes resulting from mutations that confer antiviral resistance can be mapped to functional regions to provide a better understanding of mechanisms for resistance [13–15].

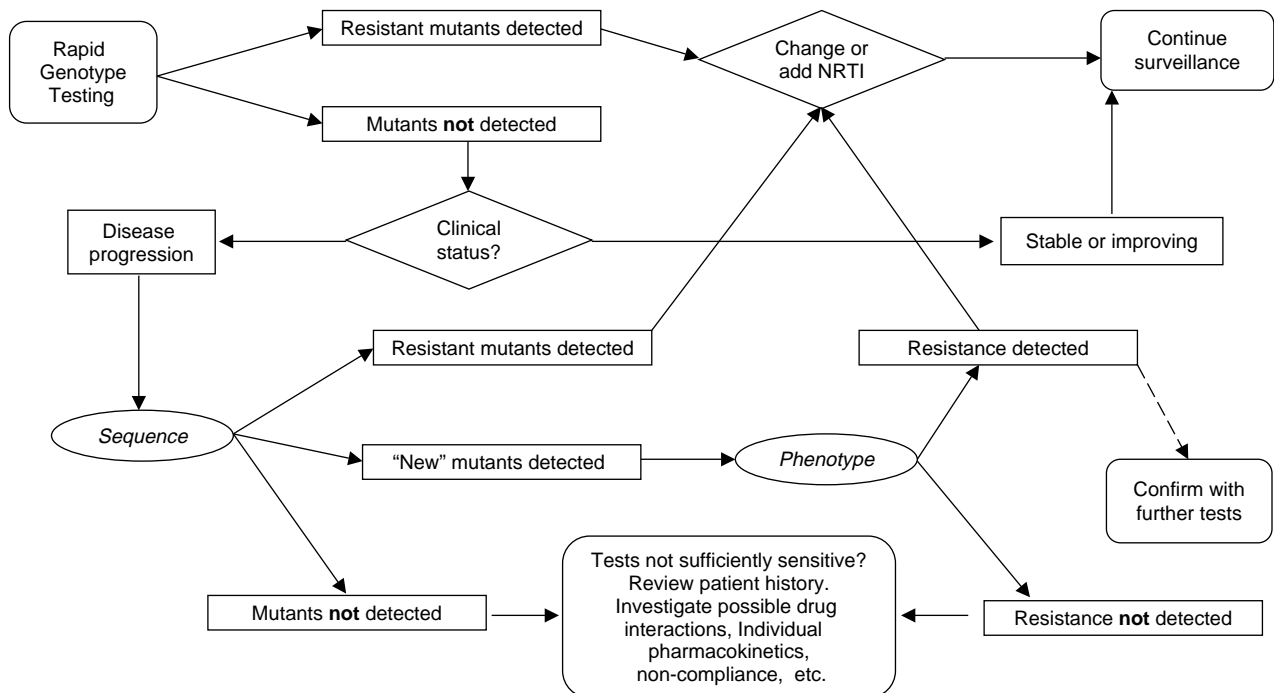


Fig. 2. Flow chart illustrating typical approach to detection and control of drug resistant mutants during anti-HBV therapy with NRTIs.

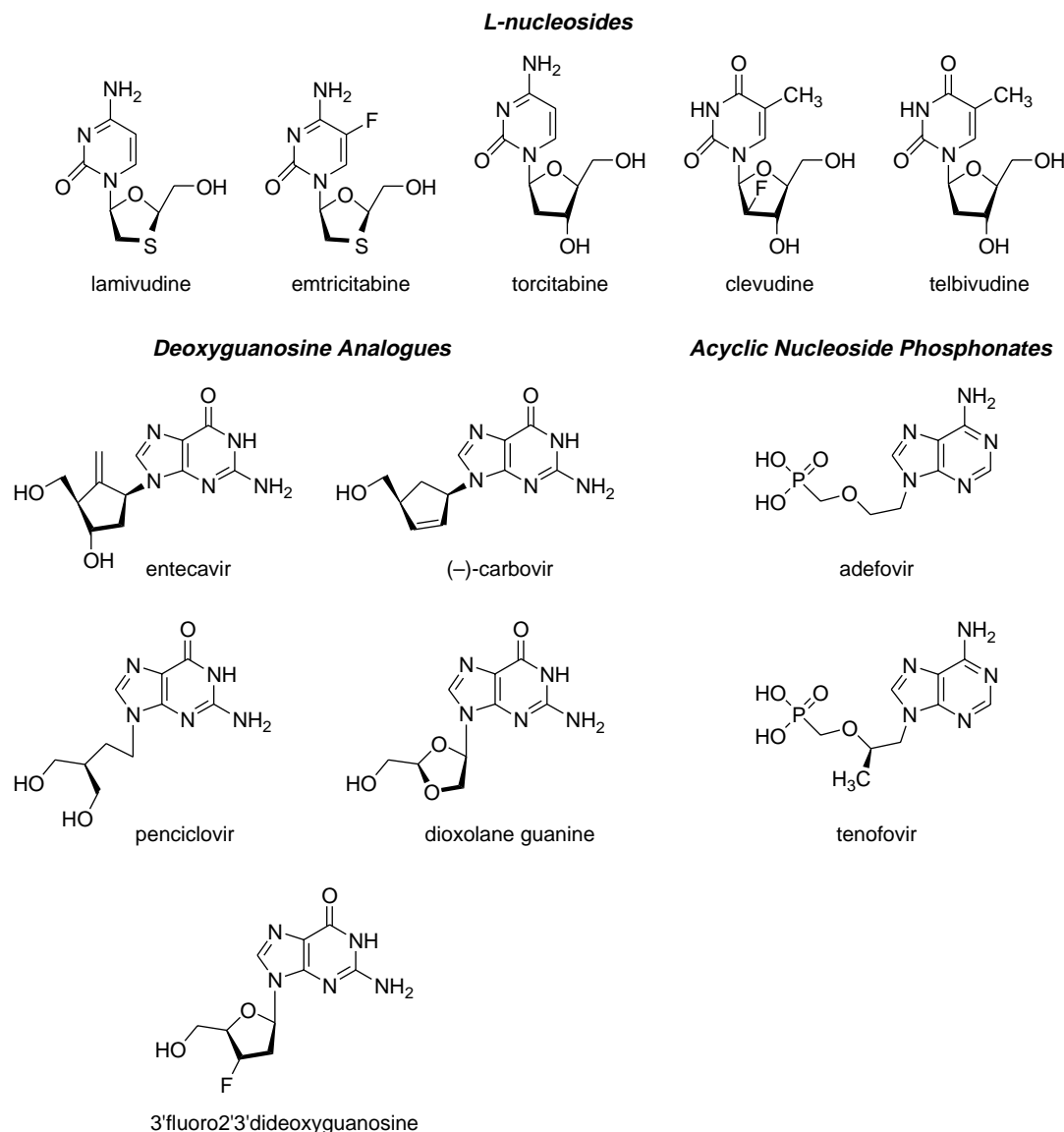


Fig. 3. Structural groups of NRTI. Note that all L-nucleosides are analogues of pyrimidine nucleosides, and lamivudine and emtricitabine, unlike the other 3 L-nucleosides, lack a 3'-OH group and are therefore obligatory chain terminators. All the deoxyguanosine analogues have the 'natural' D-conformation. Carbovir, dioxolane guanine and penciclovir are the active metabolites of abacavir, diamino purine dioxolane and famciclovir, respectively. Adefovir and tenofovir, which are both dAMP analogues, are active metabolites of adefovir dipivoxil and tenofovir diisopropyl fumarate, respectively.

3.1. Resistance to lamivudine and other L-nucleosides

Lamivudine resistance, which occurs at a cumulative rate of about 14–20% per year, is most frequent in individuals who are co-infected with HIV-1 and HBV [16]. Mutations that result in replacement of methionine in the tyrosine-methionine-aspartate (YMDD) catalytic site motif by valine, leucine or (rarely) serine are necessary and sufficient to confer resistance to lamivudine [17,18]. These changes are designated rtM204V, rtM204I and rtM204S, respectively, using the genotype-independent nomenclature proposed in 2001 [19]. The rtM204I substitution has been detected in isolation, but rtM204V/S are found only in association with other changes, some of which may be compensatory, in

particular rtL180M/C and rtV173L [20–22]. Genotyping of HBV mutants identified in clinical isolates implicated in lamivudine resistance reveals numerous amino acid substitutions in the polymerase protein besides rtM204I/V/S [23]. Phenotyping of individual HBV mutants is incomplete, but the majority of mutations may not contribute significantly to drug resistance, probably merely compensating for enzymatic deficiencies associated with resistance.

Molecular modelling suggests that lamivudine resistance conferred by rtM204I/V/S is due to both steric hindrance and electrostatic repulsion, since the substituted amino acids decrease the size of the dNTP binding pocket as well as changing the surrounding charge distribution [24]. Phenotyping shows that rtL180M/C alone is insufficient to confer

lamivudine resistance, but that when present in association with rtM204I/V/S it enhances both replication and lamivudine resistance [21–23,25,26]. Molecular modelling predicts that the presence of rtL180M decreases affinity for the oxathiolane ring of lamivudine-triphosphate by increasing local electronegativity around the deoxyribose-binding site, allowing better discrimination between lamivudine triphosphate and dCTP [24]. Better discrimination is manifest as higher copying fidelity in vitro [27].

Phenotyping also shows that rtV173L, which has been detected in 9% of cases of lamivudine resistance and is more common in liver transplant patients, compensates for replication defects of lamivudine resistant HBV mutants [20]. Modelling indicates that rtV173L may alter either the alignment of the nucleic acid template and/or the environment around catalytic site in such a way as to increase polymerisation efficiency.

By contrast, rtL80V/I, which was initially detected in genotype C HBV isolates from patients with severe hepatitis due to apparent lamivudine failure [28], has been found to enhance replication without contributing to lamivudine resistance (N. Warner, A. Bartholomeusz and S. Locarnini personal communication); it has also been associated with poor response to adefovir [29]. Modelling locates rtL80 within a beta sheet that interacts with a conserved alpha helix to form the enzyme's hydrophobic core, away from the active site but in close proximity to a conserved catalytic aspartic acid residue (rtD83), catalytic function of which is presumably altered by the rtL80V/I substitution. It seems likely that rtQ215S and rtA181T/V may also allosterically alter the geometry of the active site in a way that contributes to drug resistance by decreasing affinity for both lamivudine and adefovir.

The rtA181T substitution was first observed (surprisingly, without rtM204I/V) in clinical isolates from patients undergoing long-term lamivudine treatment [30], but has also recently been detected during treatment with adefovir [31] (see below). Similarly, rtI169T and rtT184S/G, which have been implicated in lamivudine resistance, may also contribute to entecavir resistance [12] (see below). These observations exemplify the way that prior treatment may compromise or reduce options for further treatment.

Clinical resistance to L-nucleosides other than lamivudine has yet to be studied in detail, but the molecular mechanisms involved can be expected to be similar. Two observations justify this assumption (1) lamivudine resistance confers high-level cross-resistance to other L-nucleosides in vitro and (2) in clinical trials of other L-nucleosides, primary resistance, when observed has been found to be due to rtM204I/V [32].

3.2. Resistance to acyclic nucleoside phosphonates

3.2.1. Adefovir resistance

Adefovir resistance emerges more slowly than lamivudine resistance, occurring at a cumulative annual rate of

approximately 2.5% over 4 years [33,34], although a recent long-term (5 year) study indicates higher rates in HBeAg-negative CHB [35,36]; it may also be more frequent in individuals infected with genotype D HBV, especially after lamivudine failure [35a,36].

Single nucleotide changes that produce rtN236T and/or rtA181V/T substitutions are sufficient to cause clinical failure of adefovir [31,35,37,38]. Other rt sequence changes implicated in adefovir failure include L80V/I, V84M, V214A, S85A Q215S, P237H and N238T/D, which may appear alone or in conjunction with rtN236T/rtA181V/T.

Molecular modelling reveals that rtN236, which is in the D domain and is hydrogen bonded to rtS85, interacts with the gamma triphosphate of incoming dNTPs. Substitution of T for rtN236 increases preference for the natural substrate dATP over its analogue adefovir diphosphate. Modelling shows that substitution of V or T for rtA181 causes an allosteric change in conformation of the catalytic site by forcing the repositioning of rtM204 [15]. Other substitutions in this region—rtV84M, rtS85A, rtP237H and rtN238T/D—occur infrequently and probably have a similar effect. Since changes in the HBV polymerase are detected in only a minority of clinical isolates during adefovir failure, additional unidentified factors are suspected of contributing to clinical adefovir resistance [39,40]. Although adefovir is active against lamivudine resistant HBV mutants both in vitro and in vivo [41], recent observations that lamivudine failure predisposes to more rapid development of clinical adefovir resistance is cause for concern [35a,42].

3.2.2. Tenofovir resistance

Tenofovir resistance, conferred by rtA194T in association with the changes that cause lamivudine resistance has recently been observed in individuals who were co-infected with HBV and HIV-1 [43]. Residue rtA194 is located in a loop at end of the B domain, which contains the alpha helix that interacts with the nucleic acid template. The rtA194T substitution may affect polymerisation efficiency by causing allosteric changes that result in misalignment between the template and dNTP-binding site. Alternatively, since rt194 is external in models of the reverse transcriptase region of the polymerase, it may affect interaction of the P protein with other components of the replication complex, which include cellular chaperones and the viral nucleocapsid [15]. Results of recent studies suggest that tenofovir is more efficacious in the long-term than adefovir, especially in patients infected with lamivudine-resistant HBV [44–47].

3.3. Resistance to deoxyguanosine analogues

Famciclovir, a prodrug for penciclovir, was abandoned as an anti-HBV agent because of poor efficacy in phase III clinical trials against CHB, probably due to inefficient activation in host cells. Long-term famciclovir treatment was found to select to rtL180M, which confers low-level resistance to penciclovir, although apparently sufficient to

cause clinical failure and partial resistance to lamivudine [48,49]. Resistance to other deoxyguanosine analogues other than entecavir has yet to be studied in detail.

3.3.1. Entecavir resistance

Only two instances of resistance to entecavir have been reported, to date, both in patients who had experienced lamivudine failure. Sequencing HBV isolates from each patient revealed mutations that caused pairs of additional amino acid substitutions in addition to the pair (rtL180M/rtM204V) associated with lamivudine resistance [12,50]. In the first case, these were rtM250V and rtI169T; in the second, rtT184G and rtS202I. Modelling revealed that both rtM250 and rtI169 could interact with either the nucleic acid primer or template, whereas substitutions for rtT184 or rtS202 could alter the geometry of the nearby dNTP-binding pocket. The occurrence of concomitant changes at residues 184 and 202 may be explained by their interaction [14]. As with adefovir, factors distinct from, or in addition to selection for resistant HBV polymerases may contribute to clinical failure of entecavir, since mutants have remained undetectable in nucleoside naive individuals [50].

3.4. Resistance to more than one NRTI

Simultaneous resistance to NRTI from different structural groups appears to be rare. Recent studies suggest that even in 'difficult' cases (for example, transplant patients and those who are co-infected with HIV-1 or otherwise immuno-compromised), long-term exposure to both lamivudine and adefovir is unlikely to result in resistance to both agents [51–53], probably because of severe replication deficiency conferred by resistance mutations in combination [10]. Furthermore, factors other than selection for adefovir-resistant HBV polymerase seem to be responsible for refractoriness to adefovir rescue therapy following lamivudine failure, since (1) poor response was found to be associated with re-emergence of wild-type, rather than adefovir-resistant HBV [39] and (2) lamivudine resistance increases copying fidelity of the polymerase [27]. Apart from the cases of entecavir/lamivudine resistance just described, the isolation of an HBV variant encoding rtV173L+rtL180M+rtA181T±rtN236T from a patient after failure of both lamivudine and adefovir has also been reported. Interestingly, the primary mutations responsible for primary lamivudine resistance (rtM204I/V) were undetectable [54]. Whilst rtL180M alone is insufficient to confer clinical resistance to lamivudine it must be sufficient when present together with rtA181T, which molecular modelling predicts alters the normal alignment of rtM204 [14]. These observations further emphasise that pre-existing mutations selected by prior therapy can modify subsequent options and outcomes.

4. Detection of drug resistance

A variety of different methods for detection, and in some cases, quantification, of drug resistance in HBV are now available, but they need to be improved and standardized, as interpretation and comparison of drug resistance data obtained by different methods is currently difficult and contentious. Drug resistant HBV can be detected by genotyping and/or phenotyping. Only the latter provides direct measurements of in vitro resistance to specific drugs, but each approach has inherent advantages and disadvantages [55].

4.1. Genotyping

Genotyping relies on either DNA sequencing or hybridization. Sequencing-based methods include cloning of PCR products and restriction fragment length polymorphism (RFLP) analyses. Direct DNA sequencing is typically not able to detect emerging drug resistance because it cannot detect minority populations of mutants. Cloning can overcome this problem, but analysis of large numbers of clones is required. Viral mutants that constitute as little as 5% of the total population can be detected by RFLP analyses, but separate sets of endonuclease reactions must be designed specifically for each mutant of interest. These methods are labour intensive, require highly skilled personnel and are not suitable for high-throughput screening [55]. They are used only for 'in house' assays; with the exception of the TRUGENE genotyping test developed by Visible Genetics [56], few have been commercialised or approved by regulatory bodies.

Hybridization-based genotyping methods, which can detect single nucleotide mismatches include:

- (1) fluorometric real-time PCR using the LightCycler instrument, which detects NRTI resistant HBV variants from differences in melting temperature [57,58]
- (2) mixed hybridization-sequencing-PCR ('mini-sequencing'), which involves the differential extension of multiple oligonucleotide primers with fluorescent ddNTPs
- (3) inhibition of primer extension, which relies on differences in efficiency of amplification of perfectly matched primer-templates and those with mismatched 3' termini, e.g. [59,60]
- (4) the commercially available INNO-LiPA line probe assay, which relies on differential hybridization of targets to a series of short membrane-bound oligonucleotide probes to discriminate between wild-type sequences and those of known drug-resistant mutants. LiPA assays can detect developing viral resistance when the mutants responsible constitute only a minor fraction of the total viral population, an advantage in cases where there is a high risk of disease progression [61,62].

The main limitation of all hybridization-based methods is their specificity: new sets of specific probes are required for every mutant and natural sequence variability in regions of interest reduces their discriminatory power. Furthermore, sequence context and secondary structures in the target can affect sensitivity and minor subpopulations (those constituting less than 10% of the total population) may escape detection.

Other genotyping methods that are still being developed rely on hybridization coupled to a variety of detection techniques including mass spectrometry [63,64], computer assisted microarray analysis [65–67], luciferase assays [68], or electrochemical methods based on oxidation of guanine [69]. All are rapid and accurate but require expensive equipment and highly trained personnel. Alternative, ‘low-tech’ approaches have also been investigated, including ‘reverse dot-blotting’ [70], fluorogenic ribonuclease protection assays and differential fluorescence of probe-target hybrids incorporated into nanostructures called ‘reverse micelles’ [71]. All the newer methods can detect minor subpopulations of HBV mutants, provided specific probes are available: their main disadvantage is that they cannot detect ‘new’ HBV mutants. For detection of known and ‘new’ mutants, genotyping using oligonucleotide microarrays appears to be the only viable alternative to direct sequencing, but since the number of clinically relevant HBV mutants is still relatively small and the technology is specialized and expensive, they are not cost-effective. Considering the diversity of new genotyping methods, it is difficult to predict which, if any, may eventually be regarded as the benchmark, but standardization will clearly become a challenge in the near future.

4.2. Phenotyping

Methods for drug resistance phenotyping of HBV rely on cell culture and associated techniques that were originally developed to study viral replication. All are labour-intensive and technically demanding and unsuited to high-throughput screening, limitations that have prevented more comprehensive phenotyping of HBV mutants.

4.2.1. Methods based on transient transfection

Two alternative phenotyping methods are based on transient transfection. The first relies on site-directed mutagenesis to generate point mutations known or suspected of being associated with drug resistance in well-characterized ‘laboratory’ strains of HBV [23,72]. Cell lines capable of supporting replication are transiently transfected with constructs by means of plasmid vectors. Replicate cultures of transfectants are then exposed to antiviral drug(s) and the phenotype conferred by specific mutations is deduced by comparing the effect of their presence and absence on drug sensitivity. Using this approach, effects of mutations are studied in isolation from their natural genetic

context, an advantage for research but a diagnostic disadvantage.

To increase clinical utility of phenotyping, the effects of mutations can be studied in their natural genetic background by amplifying full-length HBV genomes from clinical isolates and use them—rather than laboratory generated mutants—for transient transfection [73,74]. This method is more appropriate for diagnostic purposes and for long-term studies of viral quasispecies evolution in individual patients.

Uncontrollable variations in transfection efficiency is the major disadvantage common to all transient transfection methods; also, to enhance viral replication and facilitate antiviral assays, the expression of cloned pregenomic HBV RNA is driven by powerful promoters, making it impossible to obtain meaningful information about replication efficiency.

4.2.2. Transduction using recombinant baculovirus or other viral vectors

Alternative phenotyping strategies use recombinant baculoviruses (or other viral vectors) to deliver replication competent HBV genomes to HepG2 cells [75]. Use of viral vectors results in very efficient transduction, permitting much greater levels of HBV replication than is achievable by transient transfection. Since many drug-resistant HBV mutants are replication deficient, this can be a significant advantage for phenotyping. Following baculovirus-mediated transduction, most known drug-resistant HBV mutants replicate sufficiently well in HepG2 cells to making quantification of dose-dependent inhibition by antiviral agents (when it occurs) possible. Another advantage of transduction is that meaningful information about replication efficiency can be obtained, since HBV gene expression is usually controlled by endogenous rather than foreign promoters. We have used baculovirus-mediated transduction successfully for studies of both antiviral resistance [49,76] and replication efficiency [77]. Its main disadvantage is the need to continually generate, maintain and standardize stocks of viral recombinants.

4.2.2.1. Phenotyping integrated HBV. Transformed cells, especially the HepG2.2.15 and HB611 lines, which have HBV DNA stably integrated into their nuclear genomes have been used extensively for antiviral screening potential anti-HBV drugs for activity, but they cannot be used for drug resistance phenotyping, because the integrated virus is wild-type. Problems caused by the need to control for efficiency of transfection, transduction and viral replication efficiency has prompted several groups of researchers to create new sets of cell lines specifically for drug resistance phenotyping.

The HepAD38 and HepAD79 lines, for example, carry integrated copies of a wild-type HBV genome and its (rtM204V) mutant derivative, respectively. Viral gene expression is controlled by a tetracycline repressible cytomegalovirus (CMV) immediate early promoter [78].

The usefulness of this pair of cell lines is limited because rtM204V rarely occurs by itself in clinical HBV isolates, probably because it confers only partial lamivudine resistance. A trio of cell lines generated by the Yale University group is more representative of clinical isolates, since they express wild-type, rtL180M and rtL180M/M204V variants but other than in the initial publication [79], results of their use for HBV phenotyping have not appeared. Workers at the University of Alberta have also generated a similar trio of stable cell lines with stably integrated wild-type, rtM204I or rtL180M/M204V HBV [80]. Scientists at Gilead Sciences have created stable cell lines that express (in addition to the wild-type parent HBV), the mutants that encode the four most frequently observed amino acid profiles implicated in lamivudine failure, namely: rtL180M/M204V, rtV173L/L180M/M204V, rtM204I and rtL180M/M204I. They used these cell lines to assess resistance to eleven different anti-HBV NRTI, confirming that lamivudine resistance confers high-level cross-resistance to other L-nucleosides and reduced sensitivity to entecavir, but does not significantly affect sensitivity to adefovir or related drugs [11]. The main advantages of using continuous cell lines for phenotyping are that viral gene expression is predictable and reproducible; disadvantages are that new sublines must be created for each mutant of interest and the integration of viral HBV DNA into the cellular genome can affect the expression of both viral and cellular genes.

4.3. Virtual phenotyping

An alternative to ‘real’ phenotyping by laboratory assays has been named ‘virtual phenotyping’. This approach is relatively new for HBV, but has been used extensively to predict phenotypes of HIV isolates [81,82]. It relies on computer-assisted analysis and correlation of information from large databases of genotypic, phenotypic and clinical data. The sequence of individual clinical isolates is fed into an analytical program that interrogates its database for the best matches amongst sequences known to confer particular phenotypes. This information is collated and reported as the phenotype(s) most likely to be associated with the input sequence, without the need for direct phenotypic testing. As a simple example, mutations that cause a V or I substitution at rtM204 would be interpreted as conferring a lamivudine resistant phenotype; if present in conjunction with mutations that encode the rtV173L substitution, the virtual phenotype would be reported as lamivudine resistant and replication enhanced. Virtual phenotyping of HBV will need to become more sophisticated in the future when the introduction of new drugs will drive increasing genetic complexity. The inevitable co-accumulation of compensatory mutations that do not directly contribute to drug resistance can be expected to further complicate the virtual phenotyping process but the sophistication of techniques for

data management, data mining and analysis will undoubtedly increase correspondingly.

4.3.1. SeqHepB

SeqHepB is a program that has been developed specifically for virtual phenotyping HBV isolates. It aims to provide a large, globally available database for rapid computer-assisted analyses based on input of either genomic (nucleic acid) or amino acid sequences of clinical HBV isolates [83]. Registered users can access the program online, and input either genomic (nucleic acid) or amino acid sequences of clinical HBV isolates for analysis. SeqHepB will search for homology between input sequences and others already stored in the database, correlate the results with phenotypic data and clinical histories, determine the genotype and serotype of the isolate, report on any mutations detected, highlighting those having known clinical significance. Presently, the latter include mutations associated with antiviral resistance, vaccine escape and hepatitis B e antigen status. As the system develops, it should be possible to use SeqHepB to obtain up-to-date information about recommended drug therapy for individual patients and classes of patients and to review case histories that have been entered into the database. The database currently holds data derived from approximately 17,200 clinical isolates from 1500 patients and includes more than 3000 different HBV sequences in which approximately 90,000 variations have been identified. Various data mining algorithms are being developed to streamline the process of virtual phenotyping by SeqHepB. Programs such as SeqHepB will enable physicians to individualise patient management, cope with the explosion of antiviral associated HBV mutations, and to conduct cross-sectional or longitudinal studies. Evolving information technology should allow the rapid and efficient identification of new markers of drug resistance and other key virological parameters as well as prediction of genotypic and phenotypic trends in specific populations. In addition to genotyping, serotyping and predicting phenotypes, SeqHepB and similar programs should ultimately be able to provide a guide to their interpretation and recommendations about optimal treatment.

5. Interpretation of genotypes and phenotypes

Prediction of phenotypes from genotypes is likely to be difficult when multiple resistance and compensatory mutations are present, a situation that is common in HBV isolates from individuals who have been treated for long periods with a variety of antiviral drugs. Rather than being dichotomous, antiviral resistance and susceptibility are extremes of a continuous variable that is modified by, and modifies, replication fitness. The major advantage of phenotyping is that it provides quantitative results that should (ideally) allow HBV isolates to be located in

relationship to each other in the continuum. Phenotyping should be considered in addition to genotyping in cases where it may become necessary to use drugs that may not exhibit their maximum efficacy because of possible pre-existing resistance. Genotyping will identify the specific mutations that are associated with drug resistance in individual clinical isolates and analyses using sufficiently large databases and programs such as SeqHepB can predict the probable net effect of any detected mutations on phenotypes, but it cannot yet reliably predict phenotypes when the mutational profile is complex. In such cases, genotypes have to be interpreted in the context of complete treatment histories, including evidence of prior resistance, since ‘archived’ resistance mutations are unlikely to be detected in the absence of the appropriate selection pressure. For example, pre-existing lamivudine resistance predisposes to development of entecavir resistance [12], so consideration of entecavir salvage therapy after adefovir failure requires examination of treatment history for possible earlier lamivudine treatment and evidence of archived lamivudine resistance.

5.1. Influence of replication fitness on drug resistance phenotypes

A large body of evidence accumulated from decades of clinical experience support the hypothesis that (as in HIV-1 infected individuals) viral replication fitness is an important determinant of disease progression for CHB. In general, mutations that confer resistance to NRTI reduce replication fitness, at least in vitro, but secondary compensatory mutations that restore replication fitness are common and may also modify drug resistance phenotypes (see above). Although, intuitively it might be assumed that replication fitness is directly correlated with severity of outcome, this is not always the case. Furthermore, because pharmacological dose-response relationships are invariably non-linear and phenotyping relies on assays for HBV replication, which have finite detection limits, viral replication efficiency can influence results of in vitro phenotyping. It is therefore unwise to assume that drug resistant mutants that are replication deficient in vitro are likely to be benign. It is advisable, if possible, to assess relative replication efficiency and drug resistance in parallel assays. Unfortunately, it is not currently possible to perform infection/competition assays to assess the relative replication fitness of HBV mutants (as it is for HIV-1) because of the lack of in vitro cell culture systems capable of supporting complete cycles of HBV replication. However, by using appropriate controls, it is possible to determine the relative replication rates (‘replication yield phenotypes’) of different HBV mutants in HepG2 cells following baculovirus-mediated transduction [77].

6. Resistance testing for treatment optimisation

As more drugs become available and databases such as SeqHepB continue to expand, resistance testing is likely to guide therapeutic decision-making for all treatment-experienced CHB patients [36]. The probability of primary antiviral resistance occurring by transmission, although low, is not negligible, so suppressing apparently ‘benign’ replication-deficient multi-drug resistant mutants in experienced patients may be necessary. Designing new regimes that have the greatest chance of achieving virological suppression in individuals who have experienced treatment failure despite use of all available drugs will be a challenge. Once resistance to a particular anti-HBV drug occurs, the potential antiviral activity of most or all the other agents of its type (including those that the patient has never been exposed to) is likely to be attenuated. Phenotyping could be used to identify drugs and drug combinations that are likely to exhibit the greatest antiviral activity in this setting. Phenotyping of clinical isolates in such cases has a clear advantage over genotyping, since (despite limitations) it can be used as an index of the probable in vivo efficacy of individual drugs. On the other hand, genotyping is likely to play an increasingly important role in the management of treatment-naive individuals since determination of baseline genotypes will indicate how suitable specific drug(s) are likely to be for treating individual cases.

7. Preventing drug resistance and management of NRTI-resistant infection

There are three main ways to minimise development and spread of drug resistance: (1) avoiding unnecessary therapy, (2) careful choice therapy and (3) continuous surveillance for drug resistance.

7.1. Avoiding unnecessary therapy

A majority of individuals suffering with CHB will probably not benefit from current antiviral therapy. The quasispecies nature of populations of HBV in vivo implies that minor populations of drug resistant mutants are present even in treatment-naive patients, so institution of therapy is bound to force their selection and amplification. The AASLD, EASL, APASL, NIH and other professional bodies regularly issue guidelines to assist with recognition, diagnosis, prevention, and management of CHB: these are unanimous in recommending that therapy should be considered only for individuals with more active or advanced liver disease and others most likely to respond. Algorithms have also been developed to identify cases most likely to benefit from treatment and to determine when to initiate treatment [8,84–86].

7.2. Choice of therapy

Generation and amplification of mutant populations is *absolutely dependent on replication*, so antiviral therapy, once initiated, should aim to suppress viral replication as quickly and completely as possible [87]. This line of reasoning could be logically extended to argue for the use of the most potent available drug(s) as first-line therapy in every case, but is countered by the argument that such an approach would lead to more rapid and frequent development of resistance to the potent drug(s), with no recourse to salvage therapy using the less potent drugs. The relative merits of each argument will only become clearer when patterns of cross-resistance are properly documented and characterized. In the meantime, a pragmatic approach is advisable since the choice of therapy will depend on the clinical status of the individual patient, taking into account concurrent disease and/or treatment. For example, monotherapy with NRTI such as (lamivudine and adefovir) that activate against both HIV-1 and HBV is not recommended for co-infected patients because of the high risk of development of resistance [86,88].

7.3. Combination therapy

Provided appropriate drug combinations are used, combination therapy provides well-recognized benefits, including a reduction of the risk of drug resistance. Ideally, drugs used in combination should have different mechanisms of action and act additively or synergistically [89,90]. For example, combinations of L-nucleosides are theoretically unlikely to provide any benefit and may be antagonistic, because, in general, they compete for cellular activation mechanisms and viral targets. Despite this, results of recent clinical trials indicate that the combination of clevudine and emtricitabine may slow the development of resistance, particularly in HBeAg positive patients [91,92]. A more convincing case can be founded on the lack of cross-resistance between lamivudine and adefovir dipivoxil, *in vitro*, which is supported by an accumulating body of clinical data [40,41,53,93–98]. Combination of immunomodulators such as interferon with NRTI seems logical, and although early clinical trials of combinations of interferon and lamivudine were disappointing, recent results from extended trials using pegylated interferon and NRTI are more promising, especially in individuals with high HBV loads [99,100]. However, there is also evidence to the contrary [102], and one study found that although loss of HBeAg was significantly more frequent after combination therapy with pegylated interferon and lamivudine than after lamivudine alone (44 vs 29%; $P=0.01$), post-therapy relapse was also more common [102]. Choice of appropriate drug combinations will need justification from results of ongoing clinical trials and cost-benefit analyses and will vary on a case-by-case basis.

7.4. Surveillance and monitoring

Treatment efficacy should be monitored carefully so that drug resistance, if it occurs, is detected early, before viral breakthrough and consequent disease progression resumes [8,85,88]. Assays for serum HBV DNA and ALT should be performed 3–6 months after starting therapy to check for efficacy and compliance, the latter becoming a major contributor to primary treatment failure. Further, assays at 6-month intervals during the first 2 years of treatment are recommended for patients with milder liver disease. Three-monthly assessments are recommended after 2 years, when the probability of developing drug resistance increases. Consequences of resistance more rapidly manifest and more life threatening to individuals with advanced disease, for whom regular 3-monthly assessments are recommended [8,84,85].

7.5. Management of pre-existing NRTI resistance

To date, only lamivudine resistance has been extensively studied. There are arguments both for and against continuing lamivudine treatment of individuals with compensated liver disease and without evidence of cirrhosis [8]. Switching to adefovir dipivoxil with or without continued lamivudine are alternative options. Long-term add-on therapy with adefovir has been found to be consistently effective in several independent studies [39,40,103], even those involving HBeAg-negative cirrhotics [53] and patients who have been immunosuppressed for organ transplant or by co-infection with HIV-1 [52,104–107]. Switching to entecavir (at a daily dose of 1 mg rather than 0.5 mg recommended for treatment-naïve patients) after lamivudine failure has been shown to be effective in clinical trials [108], but this strategy may encourage development of resistance to entecavir [54]. Patients with severe disease should be given add-on combination therapy immediately on confirming the presence of drug-resistant virus, since stopping therapy or switching drugs are both regarded as too risky for this group. A recent UK costing study showed that second line treatment with adefovir following lamivudine failure, though expensive, was relatively cost-effective [109]. The relative clinical benefits and cost-effectiveness of other alternatives is still being assessed [110].

8. Conclusions

Ideally, treatment for HBV infection should begin at diagnosis, but this is not yet feasible because of limitations of existing drugs. Ongoing clinical trials and concurrent improvements in diagnostic technology guarantee that treatment options and opinions on patient management will continue to evolve. Problems caused by cross-resistance may eventually be solved by the

introduction of antiviral drugs that block stages of the viral life cycle distinct from those inhibited by NRTIs, but such drugs are unlikely to become available for clinical use in the near future. Until they do become available, an understanding of the molecular basis of NRTI resistance will become increasingly important and optimal use of NRTIs will depend on developing methods for defining, detecting and quantifying drug resistance and cross-resistance. Genotyping and phenotyping of clinical isolates of HBV is underway in many laboratories worldwide and the results can be expected to aid in design of therapeutic strategies that maximize the benefit from new antiviral drugs. Resistance testing will also make an increasing contribution to patient management, especially for individuals who have already failed treatment and those who have known risk factors associated with more rapid emergence of drug resistance. Although presently restricted to very few specialized laboratories, drug resistance phenotyping of HBV can be expected to become routine as more facile and accurate assay methods are developed along with algorithms for interpretation of results. In particular, standardization of tests and definitions of resistance/susceptibility that can be used to correlate laboratory results with clinical observations and outcomes are urgently needed.

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