Review

Genotypic determinants and phenotypic properties of antiviral-resistant HBV variants: insight from entecavir resistance studies

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Successful antiviral therapy results in cessation of disease progression and reversal of symptoms, and often can eliminate the virus infection. The emergence of virus with resistance to therapy, however, can abrogate these benefits; therefore, it is important to determine the pathways and frequency of resistance to antivirals. Often, resistance pathways can be elucidated in preclinical experiments through the isolation and characterization of resistant variants in cell culture or animals. For viruses without adequate cell culture systems, however, resistance pathways must await identification through analysis of isolates from patients failing therapy. Such is the case for antivirals used to treat chronic hepatitis B. Here, we detail the process used to define the pathways and frequency of resistance using genotypic and phenotypic assays during the development of entecavir, a novel therapy for chronic HBV infection. The scheme takes into account the need for rapid analysis of large numbers of clinical isolates through nucleotide sequencing and methods for phenotypic validation using cell culture and in vitro enzyme immunoassay formats.

HBV antiviral therapy and resistance

Successful antiviral therapy is defined by several parameters, including potent inhibition of virus growth and spread at clinically achieved concentrations, minimal toxicity, activity against the diversity of viruses that infect patients and durable suppression despite pressure that favours selection of drug-resistant virus variants. Virus susceptibility to therapy is typically assessed preclinically using cell culture or animal models. With many viruses, such as HIV and HCV, virus variants that foretell clinical resistance can be isolated preclinically in cell culture systems. In managing patients with resistance, it is crucially important to identify both the genetic changes that give rise to clinical resistance and their effect on viral replication (fitness), as well as potential cross-resistance to other therapies. The frequency of resistance emergence during therapy and the cross-resistance pattern are often important factors determining the benefit:risk ratio of a particular treatment. Factors including the error rate of viral genome replication, suppression through antiviral therapy, the number of mutations required to produce resistance, and the replication capacity of drug-resistant variants all combine to determine the likelihood of resistance [1–3].

With antiviral therapies for chronic hepatitis B (CHB), resistance must typically be defined in patients because no robust multicycle cell culture system for HBV exists. With lamivudine (3TC; Figure 1), the first specific antiviral approved for therapy of CHB by the US Food and Drug Administration (FDA; 1998), patients failing therapy harboured isolates with substitutions in the YMDD nucleotide-binding active site loop of the HBV polymerase reverse transcriptase (RT) domain. 3TC-resistant (3TCr) HBV contains YMDD methionine residue 204 changes to either YVDD (valine) or YIDD (isoleucine). These changes conferred high levels of resistance and loss of clinical virus suppression [4–7]. The M204V change was always accompanied by a leucine 180 to methionine substitution (L180M), whereas the M204I change is only occasionally accompanied by this change [8]. In vitro phenotypic experiments showed greatly reduced 3TC susceptibility with 3TCr HBV [7]. Changes at residue 80 [9] and 173 [10] can also accompany 3TCr changes. 3TCr virus was also replication-impaired in vitro [7,11]. The rapid replacement of resistant virus by the wild type, after therapy is withdrawn, also supports the notion that resistance impairs replication [12]. Subsequently, YMDD-substituted HBV
was also found to be resistant to the structurally-related CHB antiviral, telbivudine (LdT; Figure 1), which was approved for therapy in 2006 by the FDA [13], as well as experimental therapies emtricitabine (FTC) [8] and clevudine [14]. By contrast, HBV with changes at RT residues 181 from alanine to threonine or valine, or at 236 from asparagine to threonine, displays reduced susceptibility to adefovir (ADV) and tenofovir disoproxil fumarate, approved for CHB by the FDA in 2002 and 2008, respectively (Figure 1). Recently, A181 substitutions have also been associated with 3TCr in patients [15,16], and had been reported as the 3TCr pathway in the eastern woodchuck WHV model of HBV [17].

Entecavir-resistance studies

Entecavir (ETV; FDA-approved in 2005) is structurally unrelated to the other anti-HBV nucleoside/nucleotide analogues (Figure 1); thus, de novo studies during clinical trials for ETV were conducted to determine pathways and frequency of resistance. The goals of the resistance studies performed in conjunction with the Phase II and Phase III registrational clinical studies were several-fold; the primary objectives were to define genotypic changes that result in decreased susceptibility and treatment failure (genotypic ETV resistance; ETVr) and to determine the rate of ETVr in treated patients. Secondary objectives of the studies were to determine the effect of resistance on virus replication and to determine the mechanism(s) of resistance.

Resistance cohort and genotypic analysis

Patients included in the resistance cohorts were from five clinical studies and a rollover study. Nucleoside-naive cohorts (n=663) included patients treated with 0.5 mg oral ETV once daily who were seropositive for hepatitis B e antigen (Phase III study ETV-022 [18], ClinicalTrials.gov identifier NCT00035633) and those who were seronegative for hepatitis B e antigen (Phase III study ETV-027 [19], NCT00035789). Patients from 3TC-refractory cohorts (n=187) were treated with 1 mg ETV and were from a Phase III study ETV-026 ([20], NCT00036608), a Phase II study ETV-014 [21] and an orthotopic liver transplant recipient Phase II study ETV-015 [22]. These patients had previously documented 3TCr and/or continued viraemia during 3TC therapy.

Figure 1. Nucleoside/nucleotide analogues for chronic hepatitis B

The structures of the nucleoside/nucleotide analogues approved or in development (emtricitabine) for chronic hepatitis B, as well as deoxyuribose, are shown. The only differences between the structures of lamivudine and emtricitabine, and between adefovir and tenofovir, are marked by an asterisk. Three classes with respect to ribose replacements are present, l-nucleosides (lamivudine, emtricitabine and telbivudine), alkyl-phosphonates (adefovir and tenofovir) and cyclopentyl (entecavir). Note that the resistance groups appear to be driven according to the structure of the ribose replacement.
The steps followed for resistance analysis are outlined in Figure 2. Resistance surveillance included genotypic analysis whereby patient HBV isolate RT DNA was amplified and sequenced and changes in amino acids encoded on treatment were examined with respect to a database of wild-type HBV sequences, representing every HBV genotype. Population nucleotide sequencing was used for paired baseline and on-treatment isolates for all patients with PCR-detectable HBV DNA (Roche Cobas Amplicor, lower limit of quantification 300 copies/ml [approximately 50 IU/ml]) at yearly intervals, upon virological breakthrough (≥1 log₁₀ increase in HBV DNA from nadir), or upon discontinuation, through 5 years of therapy [23]. An extended genotypic analysis through year 6 was also performed [24]. Changes that emerged on treatment that differed from conserved residues in the database of 250 HBV RTs (conserved residues were 99% identical among database isolates) or varied from the residues found at polymorphic positions, were examined further through phenotypic analysis (see Phenotyping). The linkage of multiple emerging changes within individual virus genomes was determined by sequencing of individual plasmid clones of amplified patient RTs. Once ETVr residues were identified, the cumulative probability of resistance was calculated as described in Pawlotsky et al. [25], and as previously detailed [26].

Phenotyping
Phenotypic analysis included two schemes, depending upon the patient virological responses. Because virological breakthrough provides an opportunity to isolate and identify viral variants responsible for resistance, all breakthrough isolates were compared to paired baselines through genotypic and phenotypic studies. In addition to breakthrough isolates, all isolates with novel emerging changes on therapy were analysed phenotypically. For phenotypic analysis, patient HBV RT DNA was recombined into a greater-than-unit-length HBV expression plasmid, which expresses the HBV pregenomic RNA from the cytomegalovirus promoter [27]. This plasmid was used to transfect HepG2 hepatoma cells. At 5 h post-transfection, the HepG2 cells were seeded into 96-well plates and cultured in the presence of antiviral agents. Following 5 days of culture, media was collected, detergent treated, and HBV nucleocapsids immunocaptured using HBV core antibody [28]. HBV DNA was quantified by hybridization. Immunocapture of detergent-treated nucleocapsids containing only replicated DNA served several purposes. It enabled facile
and higher throughput quantitation of replicated DNA without the need to use arduous Southern blotting to separate the replicated DNA from the input. Furthermore, it could be used on cell lysates or supernatants. It also removed the effects of overlapping HBsAg envelope changes, as secreted unenveloped and detergent de-enveloped nucleocapsids were captured alike.

Susceptibility in cell culture was determined for ETV, and for 3TC and ADV, the antiviral agents approved for CHB infection at the time. These comparative studies enabled the cross-resistance profile of ETVr changes to be determined. For breakthrough isolates, the PCR-amplified patient RT population was tested as a whole population to determine the phenotype of the patient’s circulating quasispecies. For novel emerging changes, individual cloned isolates were obtained and used in the transfection to ensure the novel change was present in the transfected HBV DNA. While some phenotyping methods used full-length HBVs amplified from patient sequences [29,30], studies using full-length HBV vectors [31], as well as in vitro RT enzyme studies [32] showed that differential susceptibility to ETV was encoded within the patient RT domain, thus the RT recombinant clones were used in the comprehensive analysis of ETV patients.

Other HBV phenotyping systems include cell lines with constitutive [33] or inducible [34] expression of HBV, or infection of cells with recombinant baculoviruses expressing HBV sequences [35]. Although these systems are appropriate for repeated analysis of a limited number of HBV sequences, they require extensive preparation of each individual isolate and are therefore unsuitable for the phenotypic analysis of numerous isolates that comprised the ETV resistance surveillance. For novel emerging changes, the PCR-amplified patient RT population was tested as a whole population to determine the phenotype of the patient’s circulating quasispecies. For novel emerging changes, individual cloned isolates were obtained and used in the transfection to ensure the novel change was present in the transfected HBV DNA. While some phenotyping methods used full-length HBVs amplified from patient sequences [29,30], studies using full-length HBV vectors [31], as well as in vitro RT enzyme studies [32] showed that differential susceptibility to ETV was encoded within the patient RT domain, thus the RT recombinant clones were used in the comprehensive analysis of ETV patients.

Nucleocapsid reverse transcriptase assays
In addition to cell culture phenotypic assays, in vitro RT enzyme studies were used to complement information regarding ETVr [28,32,36]. These studies used detergent-treated HBV nucleocapsids, isolated through sucrose gradient centrifugation from lysed, transfected cells. Phosphonoformate was used during the culture [37,38] to ensure little elongation by the HBV polymerase during culture to increase the dynamic range of the assay and to ensure that the in vitro assay could detect elongation of both minus (first) and plus (second) strand DNA.

Results of ETV resistance surveillance
The first recognized ETVr emerged in Phase II patients who had extensive prior antiviral therapy [28]. Genotypic and phenotypic studies of isolates from these patients revealed that unique RT substitutions of residues T184, S202 or M250 conferred high levels of phenotypic resistance in the context of pre-existing 3TCr conferred by M204V and L180M. An additional ETV-specific change at residue I169 emerged upon prolonged therapy in the patients. This change did not appear to confer additional resistance and thus was potentially growth adaptive. The ETV-specific changes at 184, 202, or 250 did not confer substantial resistance in the absence of the 3Cr changes. ETVr HBVs were replication-impaired as shown by comparison of HBV DNA levels produced in culture relative to the wild type. Phenotypic ETVr seen in culture was reflected in results of in vitro RT studies with ETV triphosphate. As a result of basal 3Cr, the ETVr HBV was completely cross-resistant to 3TC and LdT. However, the ETVr HBV was not cross-resistant to ADV.

Analysis of the resistance cohort
Studies of ETV susceptibility in patient population quasispecies isolates revealed that similar susceptibility to ETV was exhibited across HBV genotypes [31]. In addition, those with 3Cr exhibited an average eightfold reduced susceptibility to ETV relative to wild type, either with the M204I or the M204V (plus L180M) genotype [31]. This was also seen with these changes engineered into laboratory HBV clones [39]. Thus, ETV exhibited nanomolar potency against both wild type and 3Cr HBVs [31], which translated to potent suppression of HBV DNA levels in both nucleoside-naive [18,19] and 3Cr-refractory [20,21] CHB patient studies.

Further analysis of the patients in the resistance cohort confirmed the positions of ETVr changes (Figure 3). All changes occurred in the presence of 3Cr at M204I/V with or without L180M. This profile differed from the resistance profiles of 3TC, LdT and ADV, in which only a single amino acid change was required for clinical resistance. Several other findings emerged from the resistance analysis: ETVr changes at positions T184, S202 or M250 were not found in the absence of 3Cr substitutions [22]. In addition, a small subset of 3Cr refractory patients (6%) had substitutions prior to ETV therapy at positions 184, 202 or 250. A number of other 3Cr refractory patients in which ETVr emerged on therapy were found to have low levels of ETVr at baseline, which could be detected by an ultrasensitive, allele-specific PCR method [40]. Lastly, a number of different substitutions arose in patients with ETVr at positions 184, 202 or 250 [22,23,39] in HBV with 3Cr substitutions (Figure 3). This result differed from studies with other HBV antivirals, where only a select set of substitutions at positions encoding resistance have been found (Figure 4). In the few nucleoside-naive patients in which ETVr emerged on therapy, all three substitutions, M204V plus L180M for 3Cr, and S202G for ETVr, emerged simultaneously [41,42]. This high genetic barrier in nucleoside-naive patients likely
contributes to the very low resistance to ETV observed in long-term therapy [23].

Using the in vitro HBV RT nucleocapsid assays, resistance to ETV was found to correlate with resistance in culture. The inhibition constant ($K_i$) for ETV triphosphate of ETVr HBV polymerases was significantly increased compared with wild type, although the Michaelis constant ($K_m$) for the natural substrate dGTP was less affected [43]. Molecular modelling was used to create a model of the HBV RT [32] based on the crystal structure of the HIV RT and template [44]. Modelling confirmed that ETV triphosphate was still able to access its binding pocket in 3TCr HBV RT [32]. In addition, studies showed that the ETVr substituted

Figure 3. Entecavir resistance substitutions in HBV

The HBV polymerase reverse transcriptase domain is schematically represented. The proposed structural motifs are labelled fingers, palm and thumb [8]. The conserved polymerase sequence domains (A–G) are also noted [8,47,48]. Entecavir resistance changes were identified in clinical isolates on therapy, as described [22]. 3TC, lamivudine.

Figure 4. HBV nucleoside/nucleotide therapy resistance groups and pathways

The different HBV nucleoside/nucleotide analogue therapies occupy three different resistance groups according to the structure of their ribose isostere. Entecavir (ETV) resistance arises from two different pathways depending upon whether the patient has lamivudine resistance (3TCr) or is nucleoside/nucleotide analogue treatment-naive (NA-naive). ADV, adefovir; FTC, emtricitabine; LdT, telbivudine; RT, reverse transcriptase; TDF, tenofovir disoproxil fumarate.
HBV RT with changes at positions T184, and S202 restricted the size of the ETV triphosphate pocket by further alterations of the 3′C YMDD loop [43]. The ETVr RT with changes at M250, however, altered the ETV triphosphate binding pocket through repositioning of the primer template [43]. This resulted in resistance being restricted to synthesis of the RNA-directed minus strand HBV DNA only, whereas ETVr with changes at positions 184 or 202 exhibited resistance during synthesis of both minus- and plus-strand DNAs. Modelling also was consistent with experimental results that showed a lack of cross-resistance of the ETVr HBV to ADV [32]. Phenotypic testing of patient breakthrough isolates with ETVr also failed to show cross-resistance to ADV [22,28], consistent with the findings of others [16,45,46].

The ETVr HBVs showed reduced [3H]-ETV incorporation in culture and the HBV RT enzyme exhibited reduced catalytic activity in vitro [43]. To understand if different levels of resistance and/or replication fitness were responsible for the observation of several different patient ETVr substitutions, a comprehensive analysis wherein each amino acid was substituted at positions associated with ETVr was conducted and the resulting HBVs phenotyped in culture. The results revealed that different substitutions accounted for diverse levels of replication and of ETV susceptibility [39]. Importantly, the ETVr substitutions that were found among isolates from ETV-treated patients, and especially those who experienced virological breakthrough, were those that resulted in the highest levels of combined replication and resistance [39].

Conclusions

Resistance surveillance was conducted during the clinical studies of entecavir for CHB therapy. These studies were structured to comprehensively identify any possible resistance: all patients were followed at baseline and at each year on continuous treatment using HBV DNA levels by PCR. The isolates from those who had detectable HBV DNA and those who experienced a virological breakthrough were genotyped using nucleotide sequencing. Breakthrough isolates were phenotyped in culture for susceptibility to ETV as well as 3TC and ADV. Novel substitutions that emerged in patients with detectable HBV DNA were also phenotyped. Additional phenotyping involved in vitro RT enzyme studies. The genotypic analyses were conducted over 6 years of therapy [24] and the combined genotypic/phenotypic analysis over 5 years [26]. The results of these comprehensive analyses suggested a unique resistance pattern for ETV, where multiple substitutions were required for phenotypic resistance. This genetic barrier to resistance, combined with potent suppression of HBV DNA in patients, resulted in low levels of resistance in long-term therapy of nucleoside-naive CHB patients.

Disclosure statement

DJT is employed by Bristol–Myers Squibb, which markets entecavir under the tradename Baraclute.

References


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