Original article

Suboptimal response to adefovir dipivoxil therapy for chronic hepatitis B in nucleoside-naive patients is not due to pre-existing drug-resistant mutants

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Background: Adefovir dipivoxil (ADV) has demonstrated activity against wild-type and lamivudine-resistant hepatitis B virus (HBV). After 1 year of therapy, a median 3.5–4.0 log10 decrease in viral load is observed. Our aim was to characterize the different profiles of response to ADV in relation to the in vitro susceptibility of viral strains to ADV.

Methods: In an international Phase III randomized, placebo-controlled study of ADV in patients positive for hepatitis B virus e antigen (HBeAg), different profiles of virological response to ADV 10 mg/day were identified at week 48. The top 25% patients (quartile 1, Q1) showed >4.91 log10 reduction in serum HBV DNA at week 48, in Q2 patients demonstrated a 3.52 to 4.90 log 10 reduction of viral load, whereas in Q3 a 2.22 to 3.51 log 10 reduction in viral load was observed. The bottom 25% of patients (Q4) showed <2.22 log10 reduction in HBV DNA levels. The influence of baseline characteristics and drug compliance on response was investigated. The replication capacity and drug susceptibility of HBV genomes of selected clinical isolates that were considered representative of the treatment response quartiles were analysed using a phenotypic assay.

Results: The lowest quartile of response (Q4) appears to have worse compliance. Higher alanine aminotransferase levels at baseline are associated with improved response. Phenotypic analysis of viral strains in vitro in Huh7 and HepG2 cells showed that HBV genomes remained susceptible to ADV, regardless of treatment response observed in patients.

Conclusion: Suboptimal response to ADV might result from a host pharmacological effect or from patient compliance issues rather than from a reduced susceptibility of HBV to ADV.

Introduction

With the development of new nucleos(t)ide analogues targeting the reverse transcriptase activity of the hepatitis B virus (HBV) polymerase, major advances have been made in the management of antiviral therapy of chronic hepatitis B. Four nucleos(t)ide analogues are currently approved, lamivudine, adefovir dipivoxil (ADV), entecavir and telbivudine, while others are in clinical development (for example, tenofovir, emtricitabine and clevudine) [1]. Most clinical studies have shown the antiviral potency of these drugs and have demonstrated the correlation between serum viral load suppression and an improvement in serum alanine aminotransferase (ALT) levels, liver histology and increased rate of hepatitis B virus e antigen (HBeAg) seroconversion [1].

Because of the persistence of viral covalently closed circular DNA (cccDNA) in the nucleus of infected cells, long-term therapy with nucleos(t)ide analogues is required to prevent viral relapse [2]. As the viral polymerase activity is error-prone and has no proof-reading activity, such long-term treatments may lead to the selection of polymerase gene mutants that are resistant to antiviral drugs [3]. It was shown in clinical trials that the selection of drug-resistant mutants is
inevitable with nucleos(t)ide analogue monotherapy. The emergence of antiviral drug resistance is accompanied by a rise in serum viral load and a loss of clinical benefit with the progression of the liver disease [4,5]. The rapidity of selection of HBV drug-resistant mutants depends on the fitness of the mutants in the presence of the drug, the replication space available in the liver for the spread of the mutant, the level of resistance conferred by the mutation and the drug concentrations achieved in infected cells, and the specific host immune response against HBV-infected cells [3,6].

Furthermore, it was shown by several independent studies that incomplete viral load suppression during the first months of antiviral therapy may predict the subsequent selection of drug-resistant mutants [7–9]. ADV, an oral prodrug of adefovir, has demonstrated activity against wild-type and lamivudine-resistant HBV. In Phase III clinical trials, a median 3.5–4.0 log_{10} copies/ml decrease in viral load is observed after 1 year of therapy [10–12]. ADV therapy is associated with delayed and infrequent selection of drug-resistant viruses; levels of resistance rise progressively and resistance is present in 29% of patients after 5 years of therapy [8]. It was also shown in the early trials of ADV for HBeAg-positive chronic hepatitis B that the magnitude of viral load decline was dose-dependent, with a decline of 3.52 log_{10} copies/ml after 48 weeks of administration at 10 mg/day versus 4.76 log_{10} copies with 30 mg/day [11]. The range of virological response was not described, although it was observed that in some patients the virological response to ADV might be suboptimal. This could have clinical implications with the possibility of subsequent selection of adefovir-resistant mutants. This concern was highlighted by the observation that 49% of HBeAg-negative patients with an incomplete viral load suppression after 48 weeks of ADV administration (that is, >3 log_{10} copies/ml) developed antiviral drug resistance during the 5 year follow-up, versus only 6% of patients who had a viral load <3 log_{10} copies/ml at the same time point [8].

Therefore, our aim was to characterize the different profiles of virological response in patients who receive ADV and to identify factors that might influence response to the drug in relation to the \textit{in vitro} susceptibility of viral strains to ADV.

\section*{Methods}

\subsection*{Patients}

The study population comprised 152 HBeAg-positive and nucleoside-naive patients with chronic hepatitis B who completed the 48 weeks of administration of ADV at 10 mg/day in an international Phase III randomized, placebo-controlled study [11].

Four quartiles depending on response to ADV have been assigned in these patients, allowing identification of different profiles of response to ADV at a dose of 10 mg/day. The top 25% of patients (quartile 1, Q1) showed a greater than 4.91 log_{10} reduction in serum HBV DNA at week 48. In Q2, patients demonstrated a 3.52 to 4.90 log_{10} reduction of viral load at week 48. In Q3, a 2.22 to 3.51 log_{10} reduction in viral load was observed at week 48. The bottom 25% of patients (Q4) showed a less than 2.22 log_{10} reduction in HBV DNA levels at week 48. The HBV DNA assay used during the first year of the trial was the quantitative PCR Roche Amplicor Monitor assay (Roche Diagnostics, Indianapolis, IN) with a lower limit of detection of 400 copies/ml.

The influence of baseline characteristics, including serum HBV DNA and ALT levels, body weight, body mass index (BMI), liver fibrosis score, age, race, gender, as well as concomitant medications and drug compliance on response, was investigated by comparison of summary statistics across the quartiles. Drug compliance was analysed by pill count. The patients returned their bottles and the number of pills was noted in comparison to the dispensation date for accountability. Plasma samples from these patients were stored at -20°C until analysis. Clinical isolates from seven patients belonging to each quartile and taken at week 48 of therapy and/or at baseline were studied for drug susceptibility in tissue culture. Six of these patients were followed at the Liver Department of the Hôtel Dieu Hospital in Lyon, and one sample came from the clinical trial serum bank.

Determination of ADV concentration in patient serum

The method for extraction, HPLC analysis and fluorescence detection of ADV in serum has been published previously [13].

A standard curve was drawn from blank human serum spiked with known amounts of adefovir. The lower limit of detection was 25 ng/ml of serum. Measurements were performed in duplicate in two separate experiments without knowing treatment response or the time point during therapy in two patients, one with a good virological response and one without virological response during the first year of therapy.

Cloning of HBV clinical isolates at baseline and at month 12 of therapy

Replication-competent 1.1 HBV genome unit length vectors containing HBV genome isolated from the baseline and/or month 12 clinical samples were constructed. HBV DNA was extracted from the patient’s baseline serum sample using QIAamp Ultrasens virus kit (Qiagen, Hilden, Germany). Two PCR fragments were obtained and cloned in one step into a modified pTriEX vector (Novagen, Madison, WI, USA), as previously described in detail [14]. The sequence and the replication
capacity of these vectors containing HBV genome were verified. The polymerase gene sequence was analysed with primer 5′-AAAGCCCAAAGACCCAC-3′ allowing the analysis of the entire polymerase domain from amino acid position rt30 to rt270 [15,16].

Analysis of HBV genome replication and ADV susceptibility of clinical isolates in tissue culture
Huh-7 and HepG2 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Eurobio, Courtaboeuf, France) supplemented with 10% fetal bovine serum (FBS). ADV was obtained from Gilead Sciences. The analysis of drug susceptibility was performed after transient transfection of Huh7 or HepG2 cells with recombinant baseline or month 12 HBV clones. Sixty hours after transfection, the medium was changed and cells were cultured with drug-free medium or medium containing 6.25, 12.5, 25, 50, 100 μM of ADV. Treatments were renewed every day for 5 days. Intracellular HBV DNA was then purified following the protocol described by Summers et al. [17] and subjected to Southern blot analysis. The concentration of ADV required to inhibit intracellular viral DNA replication by 50% (IC50) in treated cells at the end of the treatment, compared with untreated cells, was determined by PhosphorImager analysis [14].

Statistical analysis
Statistical analysis of the baseline characteristics across the quartiles of virological response was performed using the Wilcoxon Rank Sum test. A P-value <0.05 was considered significant.

Results
Clinical parameters associated with different response profiles to ADV
The 152 patients receiving ADV at 10 mg/day were classified in four quartiles depending on their virological response, as described in the Methods. Of note, eight patients belonging to Q4 had a <1 log10 reduction of serum viral DNA levels at week 48. There was no significant difference in the patients’ median weight across the quartiles of virological response. A slightly higher BMI was observed in patients with the weakest virological response (Q4), but this did not reach statistical significance (P-value =0.406, Wilcoxon rank sum test). There was no correlation between gender, median age or ethnicity and the profile of virological response (Table 1).

Across the four quartiles of virological response, there was a statistically significant difference in the distribution of viral load: Q2 having a lower baseline viral load (P-value<0.0004). Median ALT levels at baseline were higher in the first quartile of virological response corresponding to the best responders (P-value<0.0001, Wilcoxon rank sum test; Table 1). The analysis of the distribution of the Knodell fibrosis score across the different quartiles of virological response could not identify significant differences (Table 1).

Viral genotype distribution did not vary across the four quartiles of virological response (Table 2). Furthermore, there was no significant difference in the decline of serum viral DNA at week 48 according to viral genotype: genotype A, -3.65 ±2.01 log10 copies/ml,

Table 1. Baseline patient characteristics

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>Virological response Q1 (best response) n=38</th>
<th>Virological response Q2 n=38</th>
<th>Virological response Q3 n=38</th>
<th>Virological response Q4 (worse response) n=38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median weight, kg (range)</td>
<td>73 [43.6–100.5]</td>
<td>62.8 [48–117.4]</td>
<td>70 [43–104.3]</td>
<td>74.4 [45.4–117.5]</td>
</tr>
<tr>
<td>Median BMI (range)*</td>
<td>23.4 [18.4–32.4]</td>
<td>23 [16.3–41.8]</td>
<td>24.8 [17.9–33.3]</td>
<td>26.5 [17.4–43.1]</td>
</tr>
<tr>
<td>Male gender, %</td>
<td>79</td>
<td>71</td>
<td>71</td>
<td>84</td>
</tr>
<tr>
<td>Race</td>
<td>White, %</td>
<td>39</td>
<td>21</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Asian, %</td>
<td>58</td>
<td>76</td>
<td>58</td>
</tr>
<tr>
<td>Median ALT, IU/l† (range)</td>
<td>208 [54–1,270]</td>
<td>85 [38–1,286]</td>
<td>76 [46–273]</td>
<td>81 [45–229]</td>
</tr>
<tr>
<td>Knodell fibrosis score</td>
<td>0, %</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1, %</td>
<td>55</td>
<td>68</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>2, %</td>
<td>32</td>
<td>24</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>4, %</td>
<td>11</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Not assessable</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

Wilcoxon rank sum test: *P=0.406; †P=0.0004; ‡P<0.0001. ALT, alanine aminotransferase; BMI, body mass index; HBV, hepatitis B virus.
genotype B, -3.45 ± 1.54 log10 copies/ml; genotype C, -3.63 ± 1.43 log10 copies/ml; genotype D, 3.41 ± 1.50 log10 copies/ml; genotype E, -4.51 log10 copies/ml.

Documented drug compliance during the trial, assessed by the percentage of days taking ADV, was associated with the type of virological response. Patients with the worse virological response (Q4) had a lower documented drug compliance of 97% compared with other patients (P-value 0.01, Wilcoxon rank sum test; Table 3).

Adefovir concentration in serum was determined on stored serum samples of selected patients. Figure 1 shows the evolution of viral load determined by quantitative real-time PCR assay and adefovir serum concentration of one patient (patient 2) who did not show a significant antiviral response (Q4). This patient showed consistent adefovir concentrations at the three time points when drug monitoring was carried out, suggesting that he did comply with the treatment schedule. His serum drug levels were in the expected range, as presented in the US package insert on Hepsera® and as measured in two other HBV patients who did show a good antiviral response to ADV.

Phenotypic analysis of HBV clinical isolates shows a similar in vitro susceptibility to adefovir across treatment response groups

We wanted to investigate whether the HBV clinical isolates circulating in patients could affect treatment response. Therefore, we examined the phenotype of HBV genomes cloned from selected patients belonging to the four quartiles in tissue culture experiments, as described in the Methods. HBV isolates were studied at baseline and after 48 weeks of therapy to see whether ADV administration might have selected more recalcitrant viral genomes during the treatment period. Furthermore, the phenotypic analysis was performed in both HepG2 and Huh7 cells, which are hepatoma cell lines with different cellular differentiation characteristics. Comparison was made with a cloned wild-type HBV genome used as a reference laboratory strain. These experiments allowed us to ask whether drug susceptibility of the same viral strain was dependent on cell type. Single-clone and polyclonal analysis of drug susceptibility was performed to mimic more closely the phenotype of the viral quasi-species circulating in patients, and to examine the potential role of virus interference in drug susceptibility.

Polyclonal analysis of HBV genomes obtained at week 48 from four patients with a Q1 (patient 1), Q2 (patient 3) and Q4 (patients 2 and 4) virological response, respectively, showed that these HBV genomes encoded unchanged susceptibility to adefovir in Huh7 cells (Figure 2). Furthermore, we studied in HepG2 cells the phenotype of five clones and the polyclonal mixture of patient 2 belonging to Q4, in comparison with the polyclonal mixture of patient 1 belonging to Q1 both harbouring a D genotype. The sequence analysis of all clones showed the absence of known resistance mutations. Again, this study showed no difference in the adefovir susceptibility of the single clones versus the polyclonal mixture at week 48 of therapy (Figure 3). These results indicate that, at week 48 of therapy, there

Table 2. Breakdown of virological response according to viral genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Virological response Q1 (best response) (n=38)</th>
<th>Virological response Q2 (n=38)</th>
<th>Virological response Q3 (n=38)</th>
<th>Virological response Q4 (worse response) (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype A, n (%)</td>
<td>14 (36.8)</td>
<td>5 (13.2)</td>
<td>7 (18.4)</td>
<td>14 (36.8)</td>
</tr>
<tr>
<td>Genotype B, n (%)</td>
<td>6 (15.8)</td>
<td>9 (2.37)</td>
<td>9 (2.37)</td>
<td>8 (21.1)</td>
</tr>
<tr>
<td>Genotype C, n (%)</td>
<td>14 (36.8)</td>
<td>18 (47.4)</td>
<td>14 (36.8)</td>
<td>11 (28.9)</td>
</tr>
<tr>
<td>Genotype D, n (%)</td>
<td>3 (7.9)</td>
<td>5 (13.2)</td>
<td>8 (21.1)</td>
<td>4 (10.5)</td>
</tr>
<tr>
<td>Genotype E, n (%)</td>
<td>0</td>
<td>1 (2.6)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Genotype G, n (%)</td>
<td>1 (2.6)</td>
<td>0</td>
<td>0</td>
<td>1 (2.6)</td>
</tr>
</tbody>
</table>

Table 3. Documented drug compliance: percentage of days without taking ADV

<table>
<thead>
<tr>
<th>Days without taking ADV</th>
<th>Virological response Q1 (best response) (n=38)</th>
<th>Virological response Q2 (n=38)</th>
<th>Virological response Q3 (n=38)</th>
<th>Virological response Q4 (worse response) (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median, %</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>97*</td>
</tr>
<tr>
<td>Range, %</td>
<td>86–100</td>
<td>41–100</td>
<td>91–100</td>
<td>70–100</td>
</tr>
</tbody>
</table>

Wilcoxon rank sum test: *P<0.01. †This patient is an Asian male, aged 22 at study entry. Alanine aminotransferase (ALT) was 167 U/l and hepatitis B virus (HBV) DNA was 14,686,300 copies/ml at baseline. From the drug administration listing, the patient missed multiple doses. Despite such poor compliance, he met the criteria for quartile 2 group. Long-term follow up of this patient showed that, despite continuously poor compliance, he had an hepatitis B virus e antigen (HBeAg) seroconversion and remained with a viral DNA level <1,000 copies/ml.
was no selection of resistant mutants that could have been already present before therapy.

We also analysed, in Huh7 cells, the phenotype of an HBV genome polyclonal mixture obtained at baseline from four patients, each belonging to one of the four quartiles of virological response (patients 4, 5, 6 and 7). The investigation showed no significant difference in the ADV IC50 in the four clinical isolates corresponding to the four profiles of virological response (Figure 4).

Interestingly, viral polymerase gene sequence analysis of all clones available for the four patients at baseline did not show the presence of the rtL233V mutation, and showed that mutation rtL217R was not associated with a specific treatment response. Indeed, the mutation rtL217R was found in four out of five clones of patient 1 (Q1 response), all clones of patient 4 at baseline and month 12 (Q4 response), patient 5 (Q1 response) and patient 7 (Q3 response), whereas the wild-type amino acid residue at position rt217 was found in all clones of patients 2 at baseline and month 12 (Q4 response), patient 3 (Q2 response) and patient 6 (Q2 response).

Discussion

In this study we have examined the profile of virological response to ADV in HBeAg-positive patients in a Phase III clinical trial. Unlike most studies that report virological response as a median decline of viral load [11,12,18,19], we studied the virological response according to its distribution in four quartiles. This analysis showed that 25% of patients had a >4.91 log10 reduction in serum HBV DNA at week 48, but also showed that 25% of patients had a <2.22 log10 reduction in HBV DNA levels at week 48. This is of major clinical interest, as it was shown by recent studies that the magnitude of viral load decline may predict the outcome of antiviral therapy (that is, the chance of seroconversion) and that persisting viraemia during treatment is associated with a higher risk of developing antiviral drug resistance [1,9].

Therefore, we analysed the clinical and virological factors that might be associated with the different types of antiviral response (Table 1). Baseline viral load was lower in patients with a Q2 virological response by comparison with the other groups. A higher baseline serum ALT value was associated with a better virological response, and this was also shown in the interferon-α trials [20,21]. This may indicate that the concerted action of a cytolytic T-cell response together with the direct antiviral effect of ADV contributed to an enhanced viral suppression in these patients [22].

Baseline BMI was slightly higher, but not significantly, in patients with a Q4 virological response, most probably because there was a higher proportion of males in this group of patients. A worse compliance was associated with a weaker antiviral effect. It is interesting to see that a difference of 2% of days taking the drug may translate into a significant difference in terms of virological

Figure 1. Evolution of viral load and ADV concentration in serum in patient 2 infected with HBV genotype D and with a quartile 4 virological response

Figure 2. Multiclonal phenotypic analysis of HBV isolates in Huh7 cells at week 48 of therapy

Viral load was determined by a real-time PCR assay [2] and ADV concentrations were measured at baseline, week 12, week 24 and week 36 [12]. ADV, adefovir dipivoxil; HBV, hepatitis B virus.

Antiviral response profiles to adefovir
Methods. An HBV wild-type genotype D clone was also transfected into HepG2 cells. A comparison was made with a polyclonal mixture obtained from patient 1 infected with a genotype D strain and presenting a Q1 response. The drug susceptibility of the isolates was analysed as described in the Methods. An HBV wild-type genotype D clone served as a control. ADV, adefovir dipivoxil.

Figure 3. Monoclonal and multiclonal phenotypic analysis of HBV isolates in HepG2 cells at week 48 of therapy

Five hepatitis B virus (HBV) clones obtained at week 48 from patient 2 with a Q4 response and infected with a genotype D strain, and a mixture of these clones, were transfected into HepG2 cells. A comparison was made with a polyclonal mixture obtained from patient 1 infected with a genotype D strain and presenting a Q1 response. The drug susceptibility of the isolates was analysed as described in the Methods. An HBV wild-type genotype D clone served as a control. ADV, adefovir dipivoxil.

Figure 4. Polyclonal phenotypic analysis of HBV clinical isolates obtained at baseline

Polyclonal mixtures from four patients belonging to the four quartiles of virological response (Q1 to Q4) and obtained at baseline were transfected in Huh7 cells: Q1, patient 5, mixture of eight clones of hepatitis B virus (HBV) genotype A; Q2, patient 6, mixture of eight clones of hepatitis B virus (HBV) genotype E; Q3, patient 7 mixture of 18 clones of HBV genotype A; Q4, patient 4, mixture of 10 clones of HBV genotype A. Adefovir susceptibility was assessed as described in the Methods. An HBV genotype D clone with wild-type genome served as a standard. ADV, adefovir dipivoxil.

Polyclonal mixtures from four patients belonging to the four quartiles of virological response (Q1 to Q4) and obtained at baseline were transfected in Huh7 cells: Q1, patient 5, mixture of eight clones of hepatitis B virus (HBV) genotype A; Q2, patient 6, mixture of eight clones of hepatitis B virus (HBV) genotype E; Q3, patient 7 mixture of 18 clones of HBV genotype A; Q4, patient 4, mixture of 10 clones of HBV genotype A. Adefovir susceptibility was assessed as described in the Methods. An HBV genotype D clone with wild-type genome served as a standard. ADV, adefovir dipivoxil.

Polyclonal mixtures from four patients belonging to the four quartiles of virological response (Q1 to Q4) and obtained at baseline were transfected in Huh7 cells: Q1, patient 5, mixture of eight clones of hepatitis B virus (HBV) genotype A; Q2, patient 6, mixture of eight clones of hepatitis B virus (HBV) genotype E; Q3, patient 7 mixture of 18 clones of HBV genotype A; Q4, patient 4, mixture of 10 clones of HBV genotype A. Adefovir susceptibility was assessed as described in the Methods. An HBV genotype D clone with wild-type genome served as a standard. ADV, adefovir dipivoxil.

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This indicates that pharmacological factors are important in determining the potency of the antiviral effect in patients. Drug level measurements were performed in some patients belonging to quartiles 1 and 4, and showed that the patients with the worse virological response indeed took the drug, at least at the time of the visit (Figure 1). More detailed analysis of pharmacokinetics and intracellular levels of the active metabolite of ADV would be interesting to perform in these different categories of patients to gain more insight into how the pharmacodynamics of the compound may affect antiviral efficacy.

Viral factors were also analysed in detail. There was no correlation with specific polymerase gene mutations or viral genotype, determined by population sequencing in the whole patient population (Table 2) [23,24] and by clonal analysis for a subset of patients in our own study, and the different virological response profiles. This is consistent with other results of polymerase gene sequence analysis in patients receiving ADV [2,25–27].

In contrast to a recent report [28], in our study population of nucleoside-naive patients we did not identify the rtL233V mutation in the group of patients with the worse virological response. The rtL217R mutation was observed in several viral clones, but was not associated with any type of virological response 

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showing that suboptimal response to ADV at 10 mg/day can be overcome by increasing the dose to 20 mg/day [31]. Altogether, our findings provide the rationale for a precise monitoring of viral load during ADV administration [32] and an early treatment modification in case of partial response, as suggested by the recent results of clinical trials [33].

In conclusion, the results of this study suggest that primary suboptimal response to adefovir and acquired resistance to adefovir are most likely to be due to different factors. Host and pharmacological factors are considered to be important in determining the virological response to adefovir, while the selection of polymerase mutants is associated with acquired resistance to the drug. However, incomplete viral suppression might be an important factor contributing to the subsequent selection of resistant mutants.

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Disclosure statement

The authors declare no conflicts of interest.

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Antiviral response profiles to adefovir