Antiviral Therapy 2013; **18:**77–85 (doi: 10.3851/IMP2294)

Original article

Clonal analysis of the quasispecies of antiviral-resistant HBV genomes in patients with entecavir resistance during rescue treatment and successful treatment of entecavir resistance with tenofovir

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Background: Clonal analysis of quasispecies of resistant HBV genomes in patients with entecavir (ETV) resistance receiving lamivudine (3TC) plus adefovir (ADV) rescue therapy has never been performed.
Methods: A sample of 10 patients with ETV resistance who were switched to 3TC+ADV treatment were analysed for changes in viral quasispecies. Serum samples at baseline, and at months 3 and 6 of 3TC+ADV treatment could be clonally analysed in 7 of 10 patients; 3–82 clones per sample (total 1,068 clones, mean 63) were sequenced.
Results: 3TC+ADV therapy led to a modest decline in HBV DNA. Almost all clones had L180M and M204V 3TC resistance mutations before and during combination therapy. All clones had ≥1 of the S202G, T184F, T184A, T184L, T184I and M250V ETV resistance mutations. The percentages of detected clones bearing 3TC (rtL180M and rtM204V) and ETV mutations did not change with rescue 3TC+ADV therapy. In 7 of 8 patients with detectable HBV DNA (median 5.17 $\log_{10}$ copies/ml) after a median 24 months of ADV therapy, HBV DNA became undetectable with 3TC plus tenofovir after 6 months of treatment.
Conclusions: In patients with ETV resistance tenofovir is effective. Clonal analysis data indicate no selection of specific HBV mutants during rescue 3TC+ADV.

Introduction

Chronic hepatitis B (CHB) virus infection continues to be a major health problem due to its high global prevalence. Sustained inhibition of HBV viral replication with remission of liver disease leading to prevention of liver-disease-related morbidity and mortality is the ultimate goal of treatment of CHB with nucleoside/nucleotide analogues (NAs). Lamivudine (3TC), adefovir dipivoxil (ADV), tenofovir (TDF), telbivudine (LdT) and entecavir (ETV) are the available NAs used in the treatment of CHB. With NAs, prolonged, if not indefinite treatment, is required to maintain viral suppression and to prevent viral relapse that almost invariably occurs after withdrawal of especially short-term antiviral therapy [1,2]. The main drawback of prolonged therapy is the possibility of the emergence of antiviral-drug-resistant HBV variants selected during treatment. Although with TDF in general and with ETV in treatment-naive patients resistance is of less concern today, optimal oral antiviral treatment is still either not available or not affordable in many parts of the world [3]. Lack of a proofreading function of viral reverse transcriptases and their intrinsic error-prone nature cause viral mutations to occur spontaneously during viral replication, resulting in emergence of multiple HBV variant quasispecies. The selection pressure exerted by the antiviral agent used determines the selection of HBV strains resistant to the NAs used. With prolonged treatment, the highest rate of resistance has been observed with 3TC, reaching up to 70% after 4 years of treatment [4,5]. ETV was one of the first NAs to be tested for its efficacy in 3TC-refractory CHB. Although *in vitro* studies revealed decreased sensitivity of ETV to 3TC-resistant HBV compared with wild type, the very efficient phosphorylation of ETV inside the cell was expected to compensate for the decrease in efficiency against 3TC-resistant strains compared with wild-type

HBV [6]. However, ETV led to increased rates of ETV resistance in 3TC-refractory CHB in the clinical setting, reaching a cumulative probability of ETV resistance of 51% at 5 years [7]. 3TC plus ADV combination had been suggested as rescue therapy for 3TC resistance [8]. However, HBV mutants conferring resistance to both NAs have been described in several distinct studies [9–12]. TDF appears now the ideal drug for treating patients with 3TC resistance [1,2].

ETV has a high genetic barrier to resistance, and resistance to ETV requires two pre-existing 3TC resistance substitutions at positions 180 and 204 in the HBV reverse transcriptase, plus one additional substitution at rtT184, rtS202 or rtM250 [6]. It has been shown *in vitro* that HBV with ETV-resistant substitutions remain susceptible to ADV and TDF [13]. This is most likely the case also in the human setting where this information is based on anecdotal reports only [13,14]. The primary aim of the current study was to analyse the effect of rescue therapy in patients with proven ETV resistance on the changes in viral quasispecies in 10 prospectively studied patients. We performed extensive clonal analysis to better characterize the effect of rescue therapy to the individual resistance patterns. A secondary aim of the study was to observe effectiveness of rescue therapy on HBV DNA decline.

Methods

Patients

The analysis is based on 10 patients who had participated in a Phase III study on the use of ETV in patients with 3TC-refractory CHB [15,16]. All 10 patients had experienced virological breakthrough (>1 $\log_{10}$ copies/ml above nadir) while on treatment with ETV (1 mg once daily). Of the 10 patients, all were followed at the Gastroenterology Section of the University of Ankara Medical School (Ankara, Turkey). These 10 patients were switched to 3TC+ADV combination treatment at the investigator's discretion. TDF was not used because it had not been licensed in Turkey at the time the patients were put on rescue therapy. Likewise, 3TC and not ETV was used in the combination rescue regimen because ETV was also not licensed. The study was approved by the Ethics Committee of the University of Ankara Medical School and informed consent was obtained from all patients.

HBV viral load measurement and genotype determination

Serum HBV DNA had originally been quantified by Roche COBAS Amplicor Monitor PCR assay version 2.0 (Roche, Pleasanton, CA, USA); lower limit of quantification was 300 copies/ml, as described previously [15]. For the clonal analysis experiments, HBV DNA levels were re-measured with a fully automated system that combines automated extraction of DNA on the COBAS AmpliPrep Instrument, coupled with a real-time PCR on the COBAS TaqMan Analyser using COBAS TaqMan HBV 48 test kit with a detection limit of 20 IU/ml (Roche Diagnostics, Mannheim, Germany). HBV genotypes were determined by direct sequencing as described previously [17].

Amplification of full-length HBV genomes

The full-length HBV DNA genome was amplified as previously reported by Günther *et al.* [18]. using a PCR system containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.35 mM Tris-HCl (pH 8.3), 200 μM dNTP, 5 U of Taq DNA polymerase and, 0.3 μM of HBV-P1 (5′-CCGGAAAGCTTGAGCTCTTC TTTTTCACCTCTGCCTAATCA-3′; nucleotides 1821-1841) and HBV-P2 (5′-CCGGAAAGCTTGAGCTC TTCAAAAAGTTGCATGGTGCTGG-3′; nucleotides 1825–1806) primers and 2 μl of extracted DNA in a total volume of 50 μl. PCR reaction was run for 40 cycles with denaturation at 94°C for 40 s, annealing at 60°C for 1.5 min and elongation at 68°C for 3 min, with an addition of 2 min after each of 10 cycles.

Direct sequencing of PCR products extracted from patients' serum

Last serum sample while on ETV treatment and 2 serum samples at months 3 and 6 of 3TC+ADV combination treatment, respectively, were clonally analysed. For the sequencing of the HBV *pol* region, a 700 base pair PCR amplification covering the catalytic domains of the polymerase gene was performed using the following primers designed in our laboratory: CLC188 5′-TCCCCAACCTCCAATCAC-3′ and CLC887 5′-AAACCCAAAAGACCCACAA-3′. The PCR reaction was run for 35 cycles with denaturation at 95°C for 45 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min. Amplicons were then sequenced using the Big Dye Terminator version 3.1 Cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA) in an ABI Prism 3130 XL Genetic Analyzer (Perkin Elmer, Foster City, CA, USA) according to the manufacturer's instructions.

Clonal analysis of HBV variants and cloning of full-length HBV genomes in an expression vector

The amplified 700 base pair HBV polymerase region was cloned into a TA vector by using TOPO-XL-PCR cloning kit (Invitrogen, Carlsbad, CA, USA) and the constructs were sequenced for clonal analysis as described above. For the cloning of full-length HBV genomes, our previously published methodology [9] was used, in which full-length HBV genomes were first cloned into a TA vector with the TOPO-XL-PCR cloning kit and subsequently subcloned into a pHY106

Table 1. Baseline and lamivudine plus adefovir on-treatment characteristics of patients

Patient	Age, years	BT under ETV, weeks	HBV DNA, log_{10} copies/ml					
			Baseline[a]	Month 3	Month 6	Month 12	Month 18	Month 24
FA	53	120	8.84	4.09	3.61	4.08	NA	NA
AY	21	72	8.46	7.45	7.38	6.25	NA	5.95
EY	23	36	8.27	6.75	6.40	6.24	NA	NA
DYO	31	192	8.54	5.98	5.71	4.80	Switch[b]	Switch[b]
OK	34	36	9.75	6.46	5.51	5.13	NA	5.15
NK	30	48	8.68	5.91	5.17	5.18	Switch[b]	UD
MHU	47	120	8.26	7.10	4.66	5.12	NA	4.75
HB	59	168	4.68	UD	UD	2.71	UD	UD
GC	24	72	9.42	6.23	5.44	4.90	NA	4.48
CS	43	168	4.75	UD	2.53	2.69	4.51	3.67
Mean ±SD	–	–	7.97 ±1.78	5.49 ±1.83	4.89 ±1.60	4.71 ±1.24	–	–

[a]Baseline refers to the time point of lamivudine plus adefovir treatment commencement. [b]Patient switched to tenofovir. Month 3 versus baseline HBV DNA P=0.0001. Months 6 and 12 versus baseline HBV DNA P<0.0001. BT, breakthrough; NA, not available; UD, undetectable.

vector after sequencing. The pHY106 expression vector, which was previously designed in order to facilitate the cloning and expression of amplified full-length HBV genomes [19], allows in-frame insertion of full-length HBV clinical isolates. In order to subclone the HBV genomes into the phHY106 vector, the product of TA cloning carrying a full-length HBV genome with mutations to be tested was digested with *Sap*I restriction endonuclease. The full-length HBV genome was then gel purified using a QIAquick Gel Extraction kit (Qiagen, Basel, Switzerland) according to the instructions provided with the kit and ligated into the *Sap*I-digested pHY106 vector. The same cloning procedure was also applied for the TA cloning products bearing wild-type HBV full genome. The sequence and the in-frame insertion of the HBV genomes were further confirmed by direct DNA sequencing.

In vitro replication of pHY106-HBV full-genome constructs and antiviral susceptibility testing

The *in vitro* replication ability of cloned full-genome HBV DNA was measured by transient transfection of the clones into Huh7 cell lines. The 24-well plates were seeded with 5×10^4 Huh7 cells, and at 18 h post-seeding, the cells were transfected with 2 μg pHY106-HBV construct together with 0.3 μg green fluorescent protein containing plasmid, which was used to determine the transfection efficiency using FuGENE Transfection Reagent (Roche Diagnostics). At 8 h post-transfection, cells were fed with fresh medium alone to test the replication efficiency or with medium containing increasing concentrations (0.1, 1, 10 and 100 μM) of 3TC, ADV, TDF, ETV and embtricitabine (FTC) to test the antiviral susceptibilities. The supernatant of the cells fed with only fresh medium was collected every day during 5 days and the supernatant of the cells fed with antiviral-containing medium was collected at the end of the day 5. Viral DNA was extracted from 200 μl supernatant using a High Pure Viral Nucleic Acid kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. Real-time PCR was performed to quantify HBV DNA using Light Cycler-Fast Start DNA Master Hybridization Probes (Roche Diagnostics, Indianapolis, IN, USA) according to our previously published protocol [20]. Two independent experiments were performed for each drug.

Statistics

A paired Student's *t*-test or Fisher's exact test was used as appropriate for continuous and categorical variables, respectively, for assessing the effect of rescue treatment. A P-value <0.05 was considered as statistically significant.

Results

Characteristics of patients with entecavir resistance and response to rescue treatment

Baseline characteristics of the patients are shown in Table 1. Six patients were male, the median age was 32.5 years (range 21–59). All were infected with HBV genotype D, had hepatitis B e antigen-positive CHB, had compensated liver disease and had received 3TC in the past and were put on ETV for viral breakthrough under 3TC. Viral breakthrough under ETV treatment had developed after a median of 96 weeks (range 36–192).

3TC+ADV rescue therapy was modestly effective in all 10 patients (mean ±SD baseline versus months 3, 6 and 12 HBV DNA, 7.97 ±1.78 log_{10} copies/ml versus 5.49 ±1.83 log_{10} copies/ml [P=0.00014], 4.89 ±1.60 log_{10} copies/ml [P<0.0001] and 4.71 ±1.24 log_{10} copies/ml [P<0.001]; Table 1 and Figure 1), respectively. None of the patients displayed primary non-response to rescue 3TC+ADV therapy, that is, in all patients

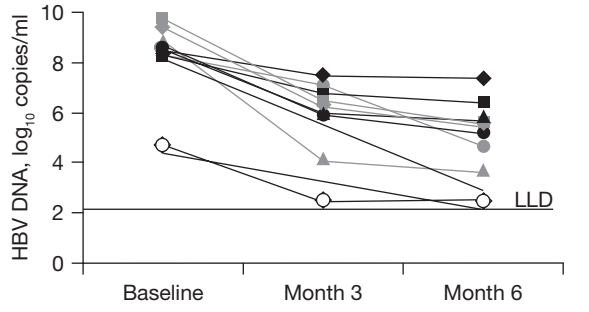
≥1 $\log_{10}$ decrease of HBV DNA was observed. During rescue treatment, no increase in serum creatinine levels or estimated glomerular filtration rate (calculated with the Cockcroft–Gault formula) was observed. In the eight patients whose HBV DNA was still detectable after a median 24 months (range 15–40) of ADV therapy, ADV was switched to TDF. At the time of switch to TDF, median HBV DNA in these patients was 5.17 $\log_{10}$ copies/ml (range 3.08–6.97). Out of these eight patients, seven became undetectable with 3TC+TDF after 6 months of treatment (P=0.0014; Figure 2).

Direct sequencing and clonal analysis of samples

Direct sequencing at the time of commencement of rescue therapy revealed the presence of ETV resistance mutations in all 10 samples at baseline. All patients had the L180M and M204V 3TC resistance mutations, six patients had S202G, and one each had T184A, T184L, T184F and M250V mutations. First detection of viral breakthrough under ETV treatment was 48–120 weeks for the T184 mutations, 36–192 weeks for the S202G mutation and 36 weeks for the M250V mutation.

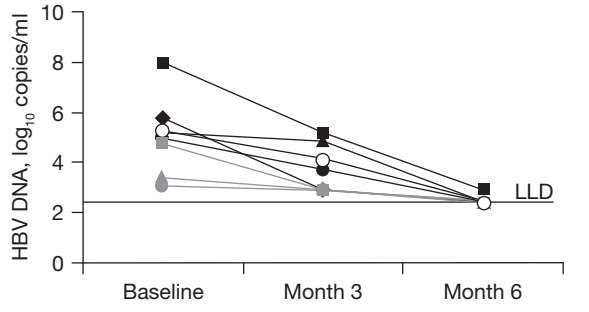
Sera of nine patients were available for clonal analysis. In two of the nine patients, baseline HBV DNA could not be amplified due to low viral load. In the remaining seven patients, clonal analysis was performed in serum samples obtained at commencement, and months 3 and 6 of rescue 3TC and ADV treatment. A total of 1,068 or 59 clones per serum sample (range 3–82) were analysed. Mutations detected in the PCR products amplified from the HBV DNA extracted from serum samples obtained at different time points and the cloning constructs are shown in Table 2. Sequence analysis of the clones revealed that almost all clones had L180M and M204V 3TC resistance mutations both before and during combination therapy. In addition, all clones were found to have ≥1 of the S202G, T184F/A/L/I and M250V ETV resistance mutations. No ADV resistance mutation was detected in any of the clones. Most of the clones were shown to have compensatory mutations, such as rtL80, rtL91, rtI169 and rtV173. During treatment with 3TC+ADV, almost no change in the proportion of the known 3TC and ETV resistance mutations occurred. In Table 3, the dominant viral strains at baseline and months 3 and 6 of rescue treatment are depicted. These dominant strains were named alphabetically for convenience. Without exception, all dominant strains contained the 3TC-resistant mutations L180M and M204V. During rescue treatment, the proportion of these dominant strains in the viral quasispecies pool practically did not change. Overall, 8 of the 10 dominant strains were used in the *in vitro* phenotyping assay. The *in vitro* drug susceptibility results of these dominant mutation patterns are shown in Table 4. More than 1,000-fold resistance compared with wild-type HBV was seen for all the dominant mutation patterns to 3TC, FTC and ETV. By contrast, ADV displayed only 1.9- to 2.8-fold resistance and TDF displayed almost no resistance.

Figure 1. Effect of lamivudine plus adefovir rescue therapy in 10 patients with entecavir resistance



LLD, lower limit of detection.

Figure 2. Effect of change from adefovir to tenofovir treatment in 7 patients with entecavir resistance



LLD, lower limit of detection.

Discussion

The results of this study confirm what has been known from anecdotal reports and non-systematic analysis [13,21,22] that 3TC+ADV rescue therapy is effective in ETV-resistant CHB, that is, from a functional point of view that ADV is effective against ETV resistance although the effect was modest. Further clonal analysis of samples performed in the current study at commencement of ETV treatment and during 6 months of ADV therapy suggests that ADV is effective in ETV-resistant CHB irrespective of the ETV-resistant dominant viral strain in the viral quasispecies pool. *In vitro* phenotyping data confirm the efficacy of both ADV and TDF against

Table 2. Mutations detected by direct sequencing of HBV DNA extracted from patients' serum samples and cloning constructs

Patient	Treatment[a]	VL[b]	Mutations detected, *n*/*n* total clones (%)															
			L80I/V	L91I	F122L	N123D	H124Y	I163V	L164M	I169V/T	V173L	L180M	T184L/I/A/F	S202G	M204V	L228F	L231V	N238D/S	M250V
GC	ETV	2.6×10^9	+	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–
			61/70	4/70	2/70	–	6/70	–	–	–	–	70/70	70/70	–	70/70	5/70	–	–	–
			(87.1)	(5.71)	(2.86)		(8.57)					(100)	(100)		(100)	(7.14)			
	Month 3	1.7×10^6	+	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–
			67/75	9/75	4/75	–	8/75	–	–	–	–	75/75	75/75	–	75/75	11/75	–	–	–
			(89.3)	(12.00)	(5.33)		(10.70)					(100)	(100)	–	(100)	(14.67)	–	–	–
	Month 6	1.7×10^5	+	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–
			63/66	12/66	2/66	–	6/66	–	–	–	–	66/66	66/66	–	66/66	9/66	–	–	–
			(95.45)	(18.18)	(3.03)		(9.09)					(100)	(100)		(100)	(13.64)			
DYO	ETV	3.5×10^8	–	–	–	+	+	–	–	–	–	+	–	+	+	–	–	–	–
			–	–	–	51/61	56/61	–	4/61	–	–	61/61	–	61/61	61/61	–	–	–	–
						(83.61)	(91.80)		(6.56)			(100)		(100)	(100)				
	Month 3	9.5×10^5	–	–	–	+	+	–	–	–	–	+	–	+	+	–	–	–	–
			–	–	–	46/54	52/54	–	2/54	–	–	54/54	–	54/54	54/54	–	–	–	–
						(85.19)	(96.30)		(3.70)			(100)		(100)	(100)				
	Month 6	4.1×10^4	–	–	–	+	+	–	–	–	–	+	–	+	+	–	–	–	–
			–	–	–	60/76	68/76	–	–	–	–	76/76	–	75/76	76/76	–	–	–	–
						(78.95)	(89.47)					(100)		(98.68)	(100)				
EY	ETV	1.9×10^8	–	–	+	+	+	–	–	–	+	+	–	–	+	–	–	–	+
			2/69	5/69	60/69	60/69	65/69	–	–	1/69	57/69	69/69	–	–	69/69	–	–	5/69	69/69
			(2.90)	(7.25)	(86.9)	(86.96)	(94.20)			(1.45)	(82.61)	(100)			(100)			(7.25)	(100)
	Month 3	5.7×10^6	–	–	+	+	+	–	–	–	+	+	–	–	+	–	–	–	+
			2/56	5/56	44/56	44/56	49/56	–	–	1/56	47/56	56/56	–	–	56/56	–	–	5/56	56/56
			(3.57)	(8.93)	(78.5)	(78.5)	(87.50)			(1.79)	(83.93)	(100)			(100)			(8.93)	(100)
	Month 6	2.5×10^6	–	–	+	+	+	–	–	–	+	+	–	–	+	–	–	–	+
			3/67	6/67	51/67	51/67	60/67	–	–	–	53/67	67/67	–	–	67/67	–	–	4/67	67/67
			(4.48)	(8.96)	(76.1)	(76.1)	(89.55)				(79.10)	(100)			(100)			(5.97)	(100)
FA	ETV	8.31×10^8	–	+	–	–	+	–	–	–	–	+	+	–	+	–	–	–	–
			1/49	47/49	–	2/49	28/49	–	–	–	–	49/49	49/49	–	49/49	–	–	–	–
			(2.04)	(95.9)		(4.08)	(57.14)					(100)	(100)		(100)				
	Month 3	6.8×10^7	–	+	–	–	+	–	–	–	–	+	+	–	+	–	–	–	–
			5/77	70/77	–	5/77	69/77	–	–	–	–	77/77	77/77	–	77/77	–	–	–	–
			(6.49)	(90.9)		(6.49)	(89.61)					(100)	(100)		(100)				
	Month 6	3.5×10^3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

[a]Months 3 and 6 of lamivudine plus adefovir rescue therapy are shown. [b]Viral load (VL) is copies/ml. ETV, entecavir; NA, no amplification; +, positive by direct sequencing; –, no data.

Table 2. Continued

Patient	Treatment[a]	VL[b]	Mutations detected, *n*/*n* total clones (%)															
			L80I/V	L91I	F122L	N123D	H124Y	I163V	L164M	I169V/T	V173L	L180M	T184L/I/A/F	S202G	M204V	L228F	L231V	N238D/S	M250V
OK	ETV	5.6×10^9	–	–	–	–	–	+	+	–	–	+	–	+	+	–	–	–	–
			–	1/43 (2.33)	–	–	2/43 (4.6)	24/43 (55.81)	43/43 (100)	–	–	43/43 (100)	–	43/43 (100)	43/43 (100)	–	–	–	–
	Month 3	2.9×10^6	–	–	–	–	+	–	–	–	–	+	–	+	+	–	–	–	–
			–	1/32 (3.13)	–	–	32/32 (100)	2/32 (6.25)	2/32 (6.25)	–	–	32/32 (100)	–	32/32 (100)	32/32 (100)	–	–	–	–
	Month 6	1.4×10^5	–	–	–	–	+	–	–	–	–	+	–	+	+	–	–	–	–
			–	–	–	–	3/3 (100)	–	–	–	–	3/3 (100)	–	3/3 (100)	3/3 (100)	–	–	–	–
MHU	ETV	1.8×10^8	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–	–
			–	4/63 (6.35)	–	–	3/63 (4.7)	–	–	–	–	60/63 (95.2)	–	63/63 (100)	63/63 (100)	–	–	–	–
	Month 3	4.5×10^6	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–	–
			–	5/53 (9.43)	–	–	4/53 (7.5)	–	–	–	–	49/53 (92.4)	–	53/53 (100)	53/53 (100)	–	–	–	–
	Month 6	7.8×10^3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
NK	ETV	1.6×10^8	–	+	–	–	–	–	–	+	–	+	+	–	+	+	+	+	–
			–	73/82 (89.0)	–	–	–	–	–	60/82 (73.2)	–	82/82 (100)	82/82 (100)	–	82/82 (100)	66/82 (80.49)	66/82 (80.4)	36/82 (43.90)	–
	Month 3	1.2×10^6	–	+	–	–	–	–	–	+	–	+	+	–	+	+	+	–	–
			–	53/72 (73.6)	–	–	–	–	–	68/72 (66.7)	–	72/72 (100)	72/72 (100)	–	72/72 (100)	38/72 (52.8)	38/72 (52.8)	14/72 (19.44)	–
	Month 6	8.2×10^4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

the observed ETV-resistant dominant viral strains and the lack of effect of other NAs. Finally, while ADV was modestly effective, the switch to TDF was associated with rapid and robust decline of HBV DNA of patients with ETV-resistant CHB who continued to have detectable HBV viraemia under ADV therapy. Although a head-to-head comparison is lacking, our data suggest superior antiviral efficacy of TDF compared with ADV in ETV-resistant CHB. The superior efficacy of 3TC+TDF compared with 3TC+ADV combination therapy in the human setting in variance with *in vitro* data can be explained by the higher dose of TDF that can be given to patients compared with ADV where nephrotoxicity precludes optimal dosing.

In the *in vitro* phenotyping assays, ADV was found to display 1.9- to 2.8-fold resistance to the dominant ETV-resistant mutation patterns compared with wild-type HBV, whereas TDF displayed 1- to 2-fold resistance

Table 3. HBV mutations on the same genome

Patient	Treatment[a]	Existing mutations	Mutation patterns	Clones, *n*/*n* total (%)
GC	ETV	L80I+L180M+T184F+M204V	–	52/70 (74)
	Month 3	L80I+L180M+T184F+M204V	–	55/75 (73)
	Month 6	L80I+L180M+T184F+M204V	–	51/66 (77)
DYO	ETV	N123D+H124Y+L180M+S202G+M204V+Y257H	A	39/61 (64)
	Month 3	N123D+H124Y+L180M+S202G+M204V+Y257H	A	34/54 (63)
	Month 6	N123D+H124Y+L180M+S202G+M204V+Y257H	A	51/76 (55)
EY	ETV	F122L+N123D+H124Y+V173L+L180M+M204V+M250V	B	42/69 (61)
	Month 3	F122L+N123D+H124Y+V173L+L180M+M204V+M250V	B	36/56 (64)
	Month 6	F122L+N123D+H124Y+V173L+L180M+M204V+M250V	B	39/67 (58)
FA	ETV	L90I+L180M+T184A+M204V+C256S	F	39/49 (81)
		L90I+H124Y+L180M+T184A+M204V+C256S	G	28/49 (57)
	Month 3	L90I+L180M+T184A+M204V+C256S	–	70/77 (91)
		L90I+H124Y+L180M+T184A+M204V+C256S	–	65/77 (84)
	Month 6	NA	–	–
OK	ETV	I163V+L164M+L180M+ S202G+ M204V+ C256S	C	24/43 (56)
		L164M+L180M+S202G+ M204V+C256S	D	38/43 (88)
	Month 3	H124Y+L180M+S202G+M204V+Y257H	E	29/32 (90)
	Month 6	H124Y+L180M+S202G+M204V+Y257H	E	3/3 (100)
MHU	ETV	L180M+S202G+M204V+Y257H	–	56/63 (89)
	Month 3	L180M+S202G+M204V+Y257H	–	45/53 (85)
	Month 6	NA	–	–
NK	ETV	L91I+I169T+L180M+T184L/I +M204V+L228F+L231V	H	52/82 (63)
	Month 3	L91I+I169T+L180M+T184L/I +M204V+L228F+L231V	H	42/72 (58)
	Month 6	NA	–	–

[a]Months 3 and 6 of lamivudine plus adefovir rescue therapy are shown. ETV, entecavir; NA, no amplification.

Table 4. 50% Inhibitory concentrations and fold resistance values of mutation patterns tested for drug susceptibility in Huh7 cell culture

	3TC		ADV		TDF		ETV		FTC	
HBV	FR	IC_{50}	FR	IC_{50}	FR	IC_{50}	FR	IC_{50}	FR	IC_{50}
WT	1.00	0.29	1.00	0.64	1.00	0.56	1.00	0.52	1.00	0.62
Pattern A[a]	>1,000	>1,000	2.02	1.29	1.46	0.82	>1,000	>1,000	>1,000	>1,000
Pattern B[a]	>1,000	>1,000	2.52	1.61	1.98	1.11	>1,000	>1,000	>1,000	>1,000
Pattern C[a]	>1,000	>1,000	2.08	1.33	1.70	0.95	>1,000	>1,000	>1,000	>1,000
Pattern D[a]	>1,000	>1,000	2.19	1.40	1.52	0.85	>1,000	>1,000	>1,000	>1,000
Pattern E[a]	>1,000	>1,000	2.80	1.79	1.92	1.08	>1,000	>1,000	>1,000	>1,000
Pattern F[a]	>1,000	>1,000	2.45	1.57	0.98	0.55	>1,000	>1,000	>1,000	>1,000
Pattern G[a]	>1,000	>1,000	1.9	1.22	1.59	0.89	>1,000	>1,000	>1,000	>1,000
Pattern H[a]	>1,000	>1,000	2.83	1.81	1.6	0.91	>1,000	>1,000	>1,000	>1,000

[a]Pattern A, N123D+H124Y+L180M+S202G+M204V+Y257H; Pattern B, F122L+N123D+H124Y+V173L+L180M+M204V+M250V; Pattern C, I163V+L164M+L180M+S202G+M204V+C256S; Pattern D, L164M+L180M+S202G+M204V+C256S; Pattern E, H124Y+L180M+S202G+M204V+Y257H; Pattern F, L90I+L180M+T184A+M204V+C256S; Pattern G, L90I+H124Y+L180M+T184A+M204V+C256S; Pattern H, L91I+I169T+L180M+T184L/I+M204V+L228F+L231V. ADV, adefovir; ETV, entecavir; FR, fold resistance (mutant 50% inhibitory concentration [IC_{50}]/wild type [WT] IC_{50}); FTC, emtricitabine; TDF, tenofovir; 3TC, lamivudine.

to ETV-resistant viral strains. Although TDF performed slightly better when compared with ADV in the *in vitro* phenotypic assay, the difference was rather small, and it is likely that the clinical antiviral superiority of TDF compared with ADV is a result of its more potent antiviral efficacy as a result of the possibility of higher dosing, as pointed out above, and unlikely to the difference in performance in the phenotypic assay. Data on ADV are in-line with previous anecdotal reports of ADV not being cross-resistant to ETV-resistant mutants [12,21,22]. The modest antiviral efficacy of ADV in the doses used in clinical practice has been extensively reported both in NA-naive and 3TC-resistant CHB [1,2].

All clones had ≥1 of the rtS202, rtT184 or rtM250 ETV mutations. These mutations were always associated with the L180M and M204V 3TC resistance mutations in all clones except seven (all in one patient, who did not reveal the L180M mutation and carried the H124Y mutation instead; Table 1). However, the proportion of this strain represented <10% in the quasispecies pool of this patient and is unlikely to be of functional significance. Compensatory mutations, such as rtL80, rtL91, rtI169 and rtV173 mutations, were also detected in the clonal analysis (rtL80 in 204/1,086 clones [19%], rtL91 in 295/1,086 clones [27%], rtI169 in 130/1,086 clones [12%] and rtV173 in 157/1,086 clones [14%]). In addition to these well-documented major and compensatory mutations, two mutations, N123D and H124Y, were detected with a high rate among the clones, namely, in 319/1,086 (29%) and 511/1,086 (47%) clones, respectively. These mutations were part of mutation patterns A, B and E and overall they did not appear to affect response to ADV (Table 3). Nonetheless, the significance of these mutations may need to be further validated. Finally, a number of different substitutions were observed at position 184 in contrast to the situation with other NAs where only a select set of substitutions occur at sites associated with resistance to the antiviral used [23].

Although viral loads of the patients decreased significantly (Table 1), no significant difference in the proportion of clones bearing 3TC (rtL180M and rtM204V) and ETV (rtS202G, rtT184F/A/L/I and rtM250V) resistance mutation patterns occurred during 6 months of 3TC+ADV treatment. This provides proof that ADV was effective at a similar level on all dominant viral strains.

The limitations of the study are the small number of patients and that cloning beyond 6 months of 3TC+ADV rescue therapy was not performed due to lack of available serum samples. Thus, emergence of ADV resistance mutations during prolonged treatment cannot be ruled out. It needs to be remembered, one such mutation, the rtA181T mutation, may occur without leading to viral breakthrough [23]. This mutation may be associated with a stop codon in the overlapping surface gene leading to truncation of the last 55 amino acids of the C-terminal of the surface proteins. It may cause defective virion secretion and lead to retention of surface proteins with potential cytotoxicity [24]. Furthermore, rtA181 mutations may be associated with cross-resistance between NAs. Hence, these points further underline the need to consider TDF and not ADV for the treatment of patients with ETV resistance. In conclusion, ADV is effective in 3TC-resistant CHB although TDF appears to be the drug of choice in treating ETV-resistant CHB. This is also in-line with the recent reporting that TDF was effective in patients with suboptimal response to ETV although those patients did not have ETV resistance [25]. The proportions of the detected clones bearing 3TC mutations (rtL180M and rtM204V) and ETV mutations (rtS202S, rtT184F/A/L/I and rtM250V) did not change significantly in the clonal analysis during the 6 months treatment course with 3TC+ADV (Table 2). The clonal analysis, thus, suggests that there is no selection of specific HBV mutants during rescue treatment regimens with 3TC+ADV in 3TC-refractory patients with ETV resistance.

Acknowledgements

This study was financially supported by Bristol–Myers Squibb. It was also funded partly by the University of Ankara, scientific research project number 08A330004.

Disclosure statement

CY is a member of advisory boards or is in the speakers' bureau belonging to Roche, Merck, Gilead and Novartis Pharma. All other authors declare no competing interests.

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Accepted 17 June 2012; published online 10 August 2012