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Telbivudine, a nucleoside analog inhibitor of HBV polymerase, has a different in vitro cross-resistance profile than the nucleotide analog inhibitors adefovir and tenofovir

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ABSTRACT

Telbivudine, a nucleoside analog inhibitor of the viral polymerase of hepatitis B virus (HBV), has been approved for the treatment of chronic HBV infection, along with the nucleoside inhibitors lamivudine and entecavir, and the nucleotide inhibitors adefovir and tenofovir. The resistance profiles of these agents were investigated via drug treatment of HepG2 cells stably transfected with wild-type or mutant HBV genomes bearing known resistance mutations. Telbivudine was not active against HBV strains bearing lamivudine mutations L180M/M204V/I but remained active against the M204V single mutant in vitro, potentially explaining the difference in resistance profiles between telbivudine and lamivudine. Against HBV genomes with known telbivudine-resistance mutations, M204I and L80I/M204I, telbivudine, lamivudine and entecavir lost 353- to >1000-fold activity whereas adefovir and tenofovir exhibited no more than 3–5-fold change. Conversely, against HBV cell lines expressing adefovir resistance mutations N236T and A181V, or the A194T mutant associated with resistance to tenofovir, telbivudine remained active as shown by respective fold-changes of 0.5 (N236T) and 1.0 (A181V and A194T). These in vitro results indicate that nucleoside and nucleotide drugs have different cross-resistance profiles. The addition of telbivudine to ongoing adefovir therapy could provide effective antiviral therapy to patients who develop adefovir resistance.

1. Introduction

Hepatitis B virus (HBV) is a DNA virus that can infect the liver of humans. More than 350 million people world-wide are chronically infected with HBV, and chronic HBV infection is the leading cause of cirrhosis and hepatocellular carcinoma. The risk of developing progressive liver disease has been linked to HBV DNA level in chronically infected individuals (Chen et al., 2006). In the past decade, the prognosis of chronic hepatitis B has been significantly improved by the emergence of a number of nucleoside and nucleotide analogs that effectively suppress the HBV virus by specifically inhibiting the activity of HBV polymerase (Zoulim, 2004). The oral nucleoside and nucleotide analogs approved for the treatment of chronic HBV infection include the nucleoside analogs lamivudine, telbivudine, entecavir, and the nucleotide analogs adefovir and, most recently, tenofovir (Marcellin et al., 2007; Heathcote et al., 2007a; Locarnini et al., 2004).

As with all small molecule antiviral drugs, the development of resistance to these anti-HBV compounds during monotherapy is expected. To date, a number of mutations in the HBV polymerase have been identified to be responsible for resistance to specific nucleoside or nucleotide analogs. Most of these occur within conserved motifs that are responsible for the enzymatic function of the polymerase (Zoulim, 2004). For example, the primary mutations responsible for lamivudine resistance, M204V and M204I, are located in the YMDD catalytic motif in the C domain at the polymerase active site (Lai et al., 2003). These primary mutations are often accompanied by secondary changes at codon L180 and/or L80 (in domain B and A, respectively, of the polymerase). Genotypic resistance to telbivudine is associated with the M204I mutation, but not the M204V mutation. The M204I mutation is accompanied by a secondary L80I mutation in more than half of telbivudine-resistance cases (unpublished results from the telbivudine GLOBE registration trial). M204V/I mutations also provide the basis for entecavir resistance, but one or more additional mutations (I169, V173, T184, S202, and/or M250) appear to be required to produce high-level resistance (Jardi et al., 2007).

In contrast, HBV polymerase nucleotide analog inhibitors are associated with different resistance mutations. In the clinic,

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adefovir resistance is caused primarily by the N236T mutant (Angus et al., 2003) and/or the A181V mutant (Hadziyannis et al., 2005). By 5 years, 29% of HBeAg negative- (Hadziyannis et al., 2005) and as high as 42% of HBeAg positive-(HEPSERA package insert. Gilead Sciences Inc., Foster City, CA, USA) patients treated with adefovir have developed adefovir-induced N236T and A181V resistance, and in those who have greater than 6 $\log_{10}$ copies/ml after 48 weeks of therapy, the resistance rate is greater than 66% (Locarnini et al., 2005). In a small number of patients, an A194T mutation has been associated with resistance to tenofovir (Sheldon et al., 2005). In addition, the presence of adefovir mutations (N236T and/or A181V) at baseline in patients treated with tenofovir led to a suboptimal virologic response to this nucleotide analog (Tan et al., 2008), and some patients required rescue therapy with tenofovir combined with emtricitabine.

To date, only a limited number of studies have been conducted to characterize the cross-resistance profiles of these HBV drugs (Brunelle et al., 2005; Qi et al., 2007; van Bomme et al., 2004; Yang et al., 2005). The scarcity of information has impeded development of effective salvage or combination therapy. This study aims at providing an extensive characterization of the cross-resistance profile of telbivudine. Using an in vitro system in which cell lines were stably transfected with mutant or wild-type HBV virus, we assessed the anti-HBV activity of telbivudine against mutant lines of HBV that have been implicated in resistance to other nucleoside or nucleotide inhibitors. Additionally, we evaluated the antiviral efficacy of other nucleoside or nucleotide inhibitors against telbivudine-resistant HBV mutants. Our data demonstrate that telbivudine has a different cross-resistance profile than nucleotide analogs, and specifically, that telbivudine remained fully active against lines of HBV carrying clinically reported nucleotide analogues resistance mutations, including N236T and A181V to adefovir, as well against A194T to tenofovir. Conversely, both nucleotide analogs remained active against lines of HBV carrying the telbivudine signature resistance mutations L80I/M204I.

2. Materials and methods

2.1. Generation of mutant and wild-type plasmids

All constructs were made using an overlength HBV genome, genotype D (subtype ayw), cloned under the control of a CMV promoter element. HBV plasmids containing point-mutated polymerase genes were derived by site-directed mutagenesis using pCMVhbv as a parent (kindly provided by Dr. C. Seeger, Fox Chase Cancer Institute) and the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) as described previously (Allen et al., 1998) using the primers listed in Table 1. The following HBV plasmids were generated: pCMV-wt, pCMV-A194T, pCMV-N236T, pCMV-M204V, pCMV-M204I, pCMV-L180M/M204V, pCMV-L180M/M204I, pCMV-L80I/M204I, and pCMV-A181V. The plasmid pCMVneo confers resistance to the G-418 antibiotic (neomycin) and was used to select transfected cells. This plasmid contains the backbone of pEGFP-N1 (Clontech, Mountain View, CA) with the SV40-driven Kan^r/Neo^r expression cassette but without the EGFP expression cassette.

2.2. Generation and selection of stable cell lines

Human hepatoma HepG2 cells (ATCC, Manassas, VA) were plated in 6-well plates in HepG2 growth media (1X EMEM, supplemented with 10% fetal bovine serum, L-glutamine, sodium pyruvate, non-essential amino acids, 1.5% sodium bicarbonate, penicillin, and streptomycin) and incubated overnight at 37 °C. Cells were transfected with either a wild-type or mutant HBV plasmid along with a neomycin resistance plasmid using Fugene (Roche, Indianapolis, IN) according to the manufacturer's suggested protocol. The plates were incubated overnight at 37 °C, and HepG2 growth/selection media (HepG2 growth media with geneticin) was added the following day. Transfected cells were fed twice a week for 2.5 weeks until distinct G418-resistant colonies had formed. Colonies that appeared to be clonal were picked off the 6-well plate and transferred to a 96-well plate containing HepG2 growth/selection media. HBV-expressing colonies were identified by testing the culture supernatant for presence of HBeAg via ELISA (see below). Positive colonies were subcloned by limiting dilution in 96-well plates and culture supernatant was screened by ELISA 2 weeks later (media was changed every 3–4 days). Positive wells were expanded, and frozen stocks were produced. Each cell line was then subjected to at least two rounds of subcloning via limiting dilutions. Cell lines that produced high levels of HBeAg were then tested for the production of functional, replication-competent nucleocapsids from cell lysates as well as secreted virus particles in cell supernatants using the endogenous polymerase assay (EPA) (see below).

Table 1
HBV polymerase site-directed mutagenesis primer pairs (5′ to 3′).

L80I	
	Forward: CAACTTGTCCTGGATATCGCTGGATGTGTC
	Reverse: GACACATCCAGCGATATCCAGGACAAGTTG
L180M	
	Forward: CAGCCCGTTTCTCATGGCTCAGTTTACGAGTGCCATTTGTTCT
	Reverse: AGAACAAATGGCACTCGTAAACTGAGCCATGAGAAACGGGCTG
A181V	
	Forward: CCGTTTCTCCTGGTTCAGTTTACTAGTGC
	Reverse: GCACTAGTAAACTGAACCAGGAGAAACGG
A194T	
	Forward: GTGGTTCGTAGGACTTTCCCC
	Reverse: GGGGAAAGTCCTACGAACCAC
M204I	
	Forward: GCTTTCAGTTATATCGATGATGTGGTATTGGG
	Reverse: CCCAATACCACATCATCGATATAACTGAAAGCC
M204V	
	Forward: GGCTTTCAGTTATGTGGATGATGTGGTATTGGG
	Reverse: CCCAATACCACATCATCCACATAACTGAAAGCC
N236T	
	Forward: TCTTTGGGTATACATTTAACCCCTAACAAAACAAAGAGATG
	Reverse: CATCTCTTTGTTTTGTTAGGGGTTAAATGTATACCCAAAGA

2.3. HBeAg ELISA

The capture antibody, a mouse monoclonal anti-HBeAg antibody (Fitzgerald, Concord, MA) was used at 10 μg/ml in 50 mM sodium carbonate buffer (100 μl/well) and incubated on a 96-well plate overnight at 4 °C, then washed 3X with phosphate buffered saline containing 0.1% Tween 20 (PBS-0.1% Tween 20). 100 μl/well of culture supernatant was transferred from the 96-well cell culture plate to the ELISA plate and incubated at 37 °C for 1 h, then washed three times. Detection antibody was a polyclonal (rabbit) anti-HBcAg-IgG antibody (Dako, Glostrup, Denmark) at a 1:3000 dilution in tris-sodium chloride-EDTA buffer with 10% fetal calf serum (10% FCS/TNE; 100 μl/well). Plates were incubated at 37 °C for 1 h and washed 3X as above. 100 μl/well peroxidase-conjugated polyclonal (goat) anti-rabbit-IgG antibody (Zymed, San Francisco,

CA) at a 1:10,000 dilution in 10% FCS/TNE was used for colorimetric detection, incubated at 37 °C for 1 h and washed 3X as above. The substrate was 13 mg of o-phenylenediamine dissolved in 12 ml citrate/phosphate buffer (100 μl/well). Color development was stopped with 2N H_2SO_4 prior to absorbance reading at A_{490} in a Fusion plate reader (PerkinElmer, Waltham, MA).

2.4. Endogenous polymerase assay

Cells were grown in 12-well plates to confluency for 3–4 days, and nucleocapsid-containing lysates and supernatants containing secreted virions were collected following drug treatment. Cytoplasmic lysates were prepared in detergent containing lysis buffer and analyzed by endogenous polymerase assays as reported previously (Seifer et al., 1998). Intracellular HBV nucleocapsids were immunoprecipitated from the cytoplasmic lysates overnight at 4 °C with a polyclonal rabbit anti-HBcAg antibody (Dako, Glostrup, Denmark) and immobilized on protein A sepharose CL-4B beads. Secreted virions were immunoprecipitated from clarified cell media overnight at 4 °C with a monoclonal mouse anti-LS antibody (MA18/7, a gift from Dr. W.H. Gerlich, Germany) in the absence of detergent. EPA reactions were then performed on the HBV coated immunobeads as described below.

2.5. Drug susceptibility assays

Lamivudine and adefovir were obtained from Moravek Biochemicals (Brea, CA), telbivudine was obtained from Idenix Pharmaceuticals (Cambridge, MA), entecavir was obtained from Bristol-Myers Squibb (Princeton, NJ), and tenofovir was obtained from the NIH AIDS Research & Reference Reagent Program (Germantown, MD).

Drug stock solutions were made up freshly in 100% DMSO as 200X stocks, and five additional 4-fold dilutions were prepared from these stocks. 12-well plates were seeded with cells expressing wild-type or mutant virus at a density of $0.5–1 \times 10^6$ cells per well in 2 ml media. Once cells reached confluency (1 day after cells were seeded), drug treatment was initiated by adding 10 μl of drug dilution into 2 ml of fresh media. The no-drug control wells received only 10 μl of DMSO in fresh media. Cells were treated every-other-day with 2 ml of fresh drug/medium for a total of 8 days. Cell lysates were then collected on day 10 and subjected to EPA analysis.

Cells were lysed in 1 ml of lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.2% NP-40) for 30 min on ice. Lysates were spun for 5 min at 16,000 × *g* and room temperature to remove cellular debris. 500 μl of clarified lysate was then mixed with anti-HBcAg-coated protein A sepharose CL-4B beads and incubated overnight at 4 °C. Unbound protein was removed by 3 washes with EPA wash buffer. EPA reactions were assembled by adding 50 μl of EPA cocktail (50 mM Tris–HCl pH7.4, 75 mM NH_4Cl, 1 mM EDTA, 20 mM $MgCl_2$, 0.1 mM β-ME, 0.5% NP-40, 100 μM cold dGTP, TTP, dCTP, and 50 nM ^{33}P-dATP) to the pelleted beads and incubated overnight at 37 °C. Nucleic acids were deproteinized by incubation in 100 mM Tris–HCl (pH 7.6), 150 mM NaCl, 12.5 mM EDTA, 1% sodium dodecyl sulfate and 1 mg of proteinase K per ml for 1 h at 37 °C followed by two extractions with phenol/chloroform and ethanol precipitation in the presence of 4 μl of GlycoBlue™ (Applied Biosystems). Pelleted nucleic acids were air dried, dissolved in 10 μl of TE (10 mM Tris–HCl pH 8, 1 mM EDTA) and electrophoresed through 1% agarose gel in tris-borate buffer. Gels were blotted onto positively charged nylon membrane overnight at room temperature via capillary transfer in 0.4 N NaOH. Dried membranes were exposed to a PhosphorImager screen (GE Healthcare, Piscataway, NJ) overnight at room temperature, then scanned (Storm 860, GE Healthcare) and visualized with ImageQuant software (GE Healthcare).

2.6. Fold change calculations

The 50% effective concentration (EC_{50}) values and R^2 values were calculated from the resulting best-fit equations determined by XLfit 4.1 software (IDBS, Guildford, UK). Fold-resistance values were first calculated for each individual experiment by dividing the mutant HBV EC_{50} by the EC_{50} for the wild-type HBV. Then the mean fold change values and standard deviation were determined using all the individual values from a particular experimental series.

3. Results

We generated an extensive series of mutant HBV cell lines, each carrying one or two point mutations in the viral polymerase gene corresponding to mutations that have been associated with resistance to lamivudine, telbivudine, adefovir, or tenofovir in the clinic: M204V, M204I, L180M/M204V, L180M/M204I, L80I/M204I, A181V, N236T and A194T. Each HBV mutant was transfected into human hepatoma HepG2 cells, and HepG2 lines that stably expressed the virus were identified using ELISA and EPA.

To determine the susceptibility of each mutant line to drugs, between three and five independent EPA experiments were performed. In brief, confluent cells were treated for 10 days with a drug, and HBV wild-type or mutant nucleocapsids were harvested and subjected to an endogenous HBV polymerase assay (EPA). Radiolabeled HBV DNA was recovered from nucleocapsids, separated on agarose gels, and then visualized and quantified via PhosphorImaging (Fig. 1). The bands for three different full-length HBV DNA species (relaxed circular, double-stranded and single-stranded HBV DNA) were collectively used for quantitation and determination of EC_{50} values. The EPA strictly measures the residual active HBV polymerase remaining after drug treatment; in contrast, the PCR-based and Southern blotting methodologies used in most HBV resistance studies to date measure overall HBV DNA species that potentially includes non-replicative molecules such as chain-terminated HBV DNA.

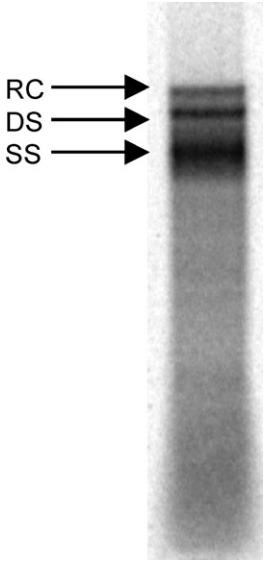

Fig. 1. Visualization of HBV DNA. HBV wild-type nucleocapsids were subjected to endogenous HBV polymerase reactions, and radiolabeled HBV DNA was recovered and separated on agarose gels. Full-length HBV replication species are indicated by arrows (RC = relaxed circular, DS = double-stranded, SS = single-stranded).

3.1. Lamivudine-resistance mutants

In a first series of experiments, the drugs telbivudine, lamivudine, and adefovir were tested against wild-type HBV virus and mutant HBV viruses carrying the M204V, M204I, L180M/M204V, or L180M/M204I mutations that are widely reported to be lamivudine signature resistance mutations (Locarnini et al., 2004). The PhosphorImage seen in Fig. 2 depicts the HBV replication levels and primary drug titration data typically obtained with the EPA and the mutants included in this study; the quality of the data fit and the resultant EC_{50} values can be judged from the R^2 values of ≥0.91. Most mutant HBV genomes were found to replicate 3–5-fold less well than the wild-type genomes (data not shown).

The mean EC_{50} values (in μM) obtained from three such experiments for telbivudine, lamivudine and adefovir against wild-type HBV virus were 0.65 ± 0.28 (telbivudine), 0.05 ± 0.03 (lamivudine) and 0.33 ± 0.17 (adefovir). Importantly, it is our experience that the antiviral activity of telbivudine in cell culture can vary widely among different experiments, and particularly among different cell lines (Seifer et al., unpublished results). Therefore the EC_{50} values of each drug determined against wild-type and mutant HBV viruses should only be compared in parallel experiments and should not be compared across the different experimental series.

Table 2 summarizes the EC_{50} values obtained for all mutants tested in this report as well as the range of EC_{50} values seen for wild-type HBV over all the experiments. Against the M204I single mutant, telbivudine and lamivudine were essentially inactive (EC_{50} > 1000 μM). Against the M204V single mutant virus the mean EC_{50} values (in μM) were 0.96 ± 0.36 for lamivudine and 0.85 ± 0.48 for telbivudine. The replication of the L180M/M204V and L180M/M204I mutant viruses was not measurably inhibited by either telbivudine or lamivudine, as indicated by EC_{50} values ranging from >823 μM to >1000 μM (Table 2). In contrast, adefovir retained significant activity against all of the lamivudine-resistant mutants, in agreement with prior reports (Delaney et al., 2001). The mean EC_{50} values (μM) seen for adefovir against individual mutations were 1.02 ± 0.22 (M204V), 1.6 ± 1.12 (M204I), 0.62 ± 0.3 (L180M/M204V), and 1.49 ± 0.3 (L180M/M204I) (Table 2). These values are in accord with other independent studies (Lada et al., 2004).

The impact of the lamivudine-resistant mutants on the observed efficacy of the drugs can best be seen from the corresponding fold-resistance values derived from the EC_{50} data (EC_{50} mutant virus divided by EC_{50} wild-type virus) as summarized in Table 3. Telbivudine and lamivudine were found to exhibit substantial fold resistance, ranging from >1049 to >22,922, when tested against the L180M/M204V and L180M/M204I double mutants, or against the M204I single mutant. However, in the case of the single M204V mutant, telbivudine exhibited essentially unchanged antiviral activity with a 1.2-fold change in susceptibility, whereas lamivudine showed a 24.8-fold change. A 153-fold shift was reported for the M204V mutant versus lamivudine in a previous study (Sheldon et al., 2005).

Adefovir was found to exhibit a moderate decrease in antiviral activity against the four lamivudine-resistant viruses as indicated by fold changes ranging from 3.3 ± 2.9 (L180M/M204V) to 4.6 ± 3.0 (M204I). It should be noted that changes of this magnitude should not necessarily be taken to imply drug-associated resistance as they probably lie within the inherent variability of the complex cell culture assay used in this study. In any case, the significance of such small in vitro changes with regards to the clinical antiviral effects of the drugs is unclear and adefovir is reported to retain activity against lamivudine-resistant HBV in the clinic (Perrillo et al., 2000; Peters et al., 2003).

3.2. Telbivudine-resistance mutants

Next, the drugs telbivudine, entecavir, adefovir, and tenofovir were tested against wild-type HBV and mutant HBV strains carrying the telbivudine signature resistance mutations (M204I or L80I/M204I) that were defined by the telbivudine GLOBE trial (Lai et al., 2007). In this series of experiments, mean EC_{50} values (in μM) against wild-type HBV virus were 0.75 ± 0.16 (telbivudine), 0.33 ± 0.08 (adefovir), 0.40 ± 0.12 (tenofovir), and 0.004 ± 0.002 (entecavir).

As seen in Table 2, against the M204I single mutant telbivudine was inactive (EC_{50} > 1000 μM) and entecavir exhibited a profound loss of antiviral efficacy against the M204I mutant virus as indicated by the mean EC_{50} value of 3.10 ± 1.41 μM. On the other hand, adefovir and tenofovir exhibited only a mild loss of antiviral in vitro activity as seen by the mean EC_{50} values of 1.71 ± 0.72 μM and 1.95 ± 0.74 μM, respectively. A similar pattern emerged when the four drugs were evaluated against the L80I/M204I double mutant. Again, the mean EC_{50} value of telbivudine was greater than 1000 μM. For the other drugs, the mean EC_{50} values (in μM) were: 1.08 ± 0.20 (adefovir), 1.27 ± 1.01 (tenofovir), and 1.04 ± 0.56 (entecavir) (Table 2).

With respect to fold-resistance (Table 3), telbivudine was inactive against the M204I and L80I/M204I viruses as shown by fold-resistance values of >1379, while entecavir exhibited similar fold-resistance values of 1032 (M204I) and 353 (L80I/M204I). On the other hand, we found that both adefovir and tenofovir retained near-wild-type in vitro activity against the M204I or L80I/M204I mutant viruses, as indicated by moderate fold changes in drug sensitivity ranging from 3.1 to 5.4.

3.3. Tenofovir-associated mutant

The drugs telbivudine and tenofovir were next tested against wild-type HBV virus and a mutant HBV line carrying the clinically reported A194T tenofovir resistance mutation (Locarnini et al., 2005). In this experiment, the mean EC_{50} values for telbivudine and tenofovir against wild-type HBV virus were 0.61 ± 0.21 μM and 0.32 ± 0.23 μM, respectively. Tenofovir was found to be approximately 3 times more potent against wild-type HBV in this cell line than in HepG2.2.15 cells (Delaney et al., 2006). Against the A194T mutant virus, the EC_{50} values were similar to those obtained with both drugs against wild-type virus (0.56 ± 0.04 μM for telbivudine, and 0.30 ± 0.24 μM for tenofovir) (Table 2). Thus, both wild-type and A194T mutant HBV polymerases were effectively suppressed by telbivudine and tenofovir as indicated by the mean fold resistance values of 1.0 ± 0.3 and 1.8 ± 2.2, respectively (Table 3). These results are in accord with a previous in vitro study, in which A194T mutant virus was found not to be resistant to tenofovir (Delaney et al., 2006).

3.4. Adefovir resistance mutants

The drugs telbivudine, lamivudine, and adefovir were tested against wild-type HBV virus and a mutant HBV line carrying the N236T adefovir resistance mutation. The PhosphorImage seen in Fig. 3 shows representative primary data and inhibition bar graphs obtained with the N236T HBV cell line. In this experimental series, the mean EC_{50} values (in μM) for the three drugs against wild-type HBV virus were 0.03 ± 0.01 (lamivudine), 0.46 ± 0.22 (adefovir), and 0.43 ± 0.19 (telbivudine).

Against the N236T mutant virus the mean EC_{50} values (in μM) were 0.04 ± 0.01 (lamivudine), 1.76 ± 0.60 (adefovir), and 0.26 ± 0.29 (telbivudine) (Table 2). These numbers accord well with previously reported values (Yang et al., 2004). The corresponding

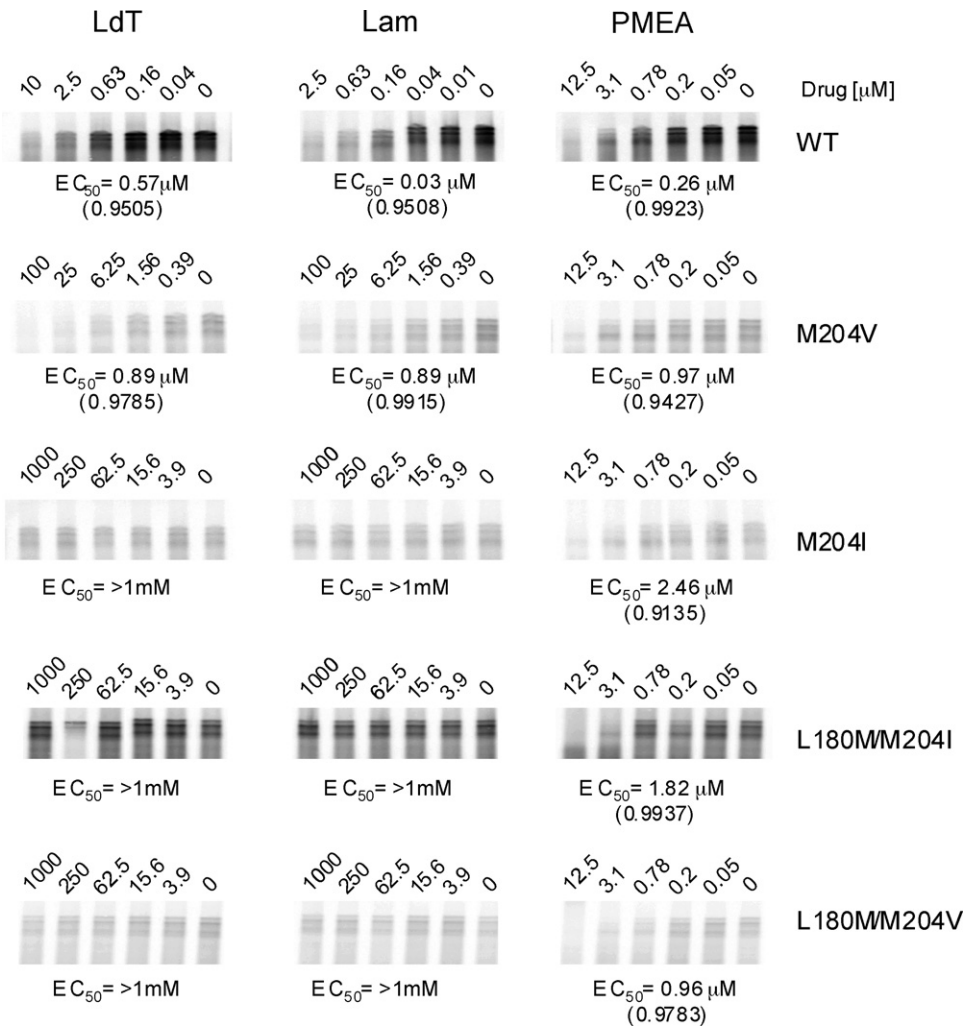

Fig. 2. Antiviral assessment of telbivudine (LdT), adefovir (PMEA), and lamivudine (Lam) against cell lines expressing HBV wild-type (WT) and lamivudine-resistant mutant nucleocapsids using endogenous polymerase assay. The primary data are derived from one out of three experiments. In each lane, full-length HBV DNA species were used for quantitation and curve fitting. EC_{50} values are shown below each PhosphorImager panel. R^2 values are given in parenthesis where applicable.

fold change values (Table 3) showed that adefovir was 3.9 ± 2.2-fold less effective in inhibiting replication of N236T virus while lamivudine gave a fold shift of only 1.5 ± 0.5. Among the three drugs, telbivudine exhibited the best activity against the N236T mutant as indicated by the fold change value of 0.5 ± 0.4 (Table 3).

In this study the fold resistance of the N236T mutant cell line to adefovir ranged from 2- to 6-fold, with a mean of 3.9 ± 2.2. These results accorded with other published values. Angus et al., 2003 described a 7.3-fold resistance to adefovir using serum-derived virus from a patient exhibiting clinical adefovir resistance, and a 23-fold resistance to adefovir using a molecular clone/transfection-based approach. Others observed 7.5- and 15-fold resistance to adefovir using similar molecular clone/transfection assays (Yang et al., 2004; Bartholomeusz et al., 2004). The slight differences in

Table 2
In vitro sensitivities of wild-type (WT) and mutant HBV viruses to telbivudine, lamivudine, entecavir, adefovir or tenofovir.

Selected by	Virus (WT)	Telbivudine (0.43–1.45)	Lamivudine (0.03–0.05)	Entecavir (0.004)	Adefovir (0.33–0.46)	Tenofovir (0.32–0.40)
Lamivudine	M204V	0.85 ± 0.48	0.96 ± 0.36	ND	1.02 ± 0.22	ND
	M204I	≥1000	≥1000	ND	1.60 ± 1.12	ND
	L180M/M204V	≥1000	≥1000	ND	0.62 ± 0.30	ND
	L180M/M204I	≥823 ± 307	≥1000	ND	1.49 ± 0.30	ND
Telbivudine	M204I	>1000	ND	3.10 ± 1.41	1.71 ± 0.72	1.95 ± 0.74
	L80I/M204I	>1000	ND	1.04 ± 0.56	1.08 ± 0.20	1.27 ± 1.01
Adefovir	N236T	0.26 ± 0.29	0.04 ± 0.01	ND	1.76 ± 0.60	ND
	A181V	1.46 ± 0.56	ND	0.002 ± 0.001	0.13 ± 0.40	0.37 ± 0.15
Tenofovir	A194T	0.56 ± 0.04	ND	ND	ND	0.30 ± 0.24

Antiviral data were generated by endogenous HBV polymerase assays and PhosphorImager analysis. EC_{50} values (in μM) were calculated from the resulting best-fit equations determined by XLfit and represent the mean and standard deviation of three to five independent experimental data sets.
ND = not determined.

Table 3
Fold changes in drug susceptibilities.

Selected by	Virus (WT)	Telbivudine (1)	Lamivudine (1)	Entecavir (1)	Adefovir (1)	Tenofovir (1)
Lamivudine	M204V	1.2 ± 0.4	24.8 ± 17.8	ND	3.8 ± 2.3	ND
	M204I	>1360 ± 363	>22,922 ± 9063	ND	4.6 ± 3.0	ND
	L180M/M204V	>1360 ± 363	>22,922 ± 9063	ND	3.3 ± 2.9	ND
	L180M/M204I	>1049 ± 226	>22,922 ± 9063	ND	3.6 ± 1.1	ND
Telbivudine	M204I	>1379 ± 298	ND	1032 ± 588	5.4 ± 2.1	5.2 ± 1.9
	L80I/M204I	>1379 ± 298	ND	353 ± 265	3.4 ± 0.4	3.1 ± 1.7
Adefovir	N236T	0.5 ± 0.4	1.5 ± 0.5	ND	3.9 ± 2.2	ND
	A181V	1.0 ± 0.4	ND	0.6 ± 0.2	0.5 ± 0.2	1.3 ± 1.1
Tenofovir	A194T	1.0 ± 0.3	ND	ND	ND	1.8 ± 2.2

Fold changes were first calculated as the ratio of mutant EC_{50} to the corresponding wild-type EC_{50} for that particular set of experiment. Then, the mean and standard deviations were determined for the entire experimental data set.
ND = not determined.

experimental values may also reflect experimental variations, such as the use of different HBV genomes, different transfection systems (e.g., transient versus stable), and different cell types (HepG2 cells in our studies versus Huh7 cells used by Angus et al. (2003)).

Additionally, we tested the drugs telbivudine, entecavir, adefovir and tenofovir against wild-type HBV and a mutant HBV line carrying the A181V adefovir resistance mutation. In this series of experiments mean EC_{50} values (in μM) against wild-type HBV virus were as follows: 1.45 ± 0.07 (telbivudine), 0.34 ± 0.22 (adefovir), 0.40 ± 0.20 (tenofovir) and 0.004 ± 0.002 (entecavir). Surprisingly, A181V HBV nucleocapsids expressed by this particular cell line largely displayed wild-type susceptibility to all drugs tested in this study, including adefovir. Mean EC_{50} values ranged from 0.002 ± 0.001 μM (entecavir) to 1.46 ± 0.56 μM (telbivudine). The corresponding fold changes ranged from 0.5 to 1.3 (Table 3). These results appear somewhat different from the results of Qi et al. (2007) which suggested that the A181V mutant gave a 4.3-fold shift with adefovir and a >27-fold shift with telbivudine. As noted above, a variety of factors might account for these differences, in particular the use of different cell lines among different investigators and the use of divergent assay systems with very different readouts. In support of our findings, we note that no resistance due to A181V mutants has been seen through 2 years of therapy in the GLOBE trial (Lai et al., 2007; Standring et al., 2007). For adefovir, the fold shifts seen in this report and by Qi et al. (2007) are quite small and probably lie within the experimental variation inherent in the complex in vitro assays used. These small effects are not readily related to the emergence of resistance in the clinic. Adefovir has exhibited more limited antiviral potency than telbivudine in the clinic (Hadziyannis et al., 2005; Lampertico et al., 2005), so it is possible

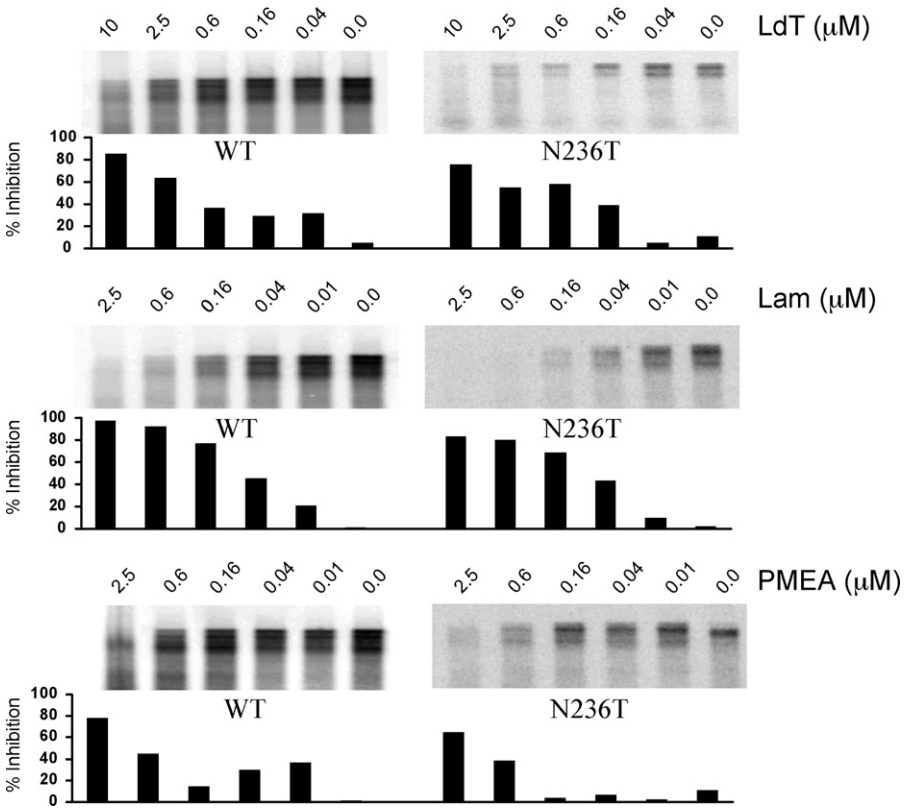

Fig. 3. Antiviral assessment of telbivudine (LdT), lamivudine (Lam) and adefovir (PMEA) against cell lines expressing HBV wild-type (WT) and adefovir resistant (N236T) mutant nucleocapsids using endogenous polymerase assay. The primary data are derived from one out of four experiments. In each lane, full-length HBV DNA species were used for EC_{50} calculations.

that even a small loss of activity for adefovir may compromise its antiviral efficacy in vivo. Moreover, variations between the replication of the wild-type and A181V mutant HBV genomes in vivo may contribute to the emergence of resistance; such variations may not be readily detected in in vitro systems.

In summary, telbivudine, adefovir, tenofovir, and entecavir were generally active against the A181V viruses in this in vitro assay system.

4. Discussion

Telbivudine is a nucleoside analog that suppresses HBV replication by preferentially inhibiting second strand HBV DNA synthesis (Seifer et al., 2005). The results of the pivotal GLOBE trial demonstrated potent antiviral efficacy of telbivudine against HBV in both HBeAg-positive and HBeAg-negative patients (Lai et al., 2007). Importantly, compared to lamivudine, significantly less resistance was observed with telbivudine at year 1 (Lai et al., 2007) and year 2 (Zeuzem et al., 2007). Telbivudine effectively suppresses the robust M204V resistance pathway seen with lamivudine; this leads to the halving of the resistance rate and denotes that the M204I mutation is the signature telbivudine-resistance mutation. The M204I mutation was accompanied in more than half of telbivudine-resistant patients (patients with a 1 $\log_{10}$ above nadir rebound in HBV viral load) by an L80I change and only a single case (0.1%) of the M204V mutation (as the L180M/M204V double mutant) was seen to be associated with viral breakthrough among 680 telbivudine patients in the pivotal trial after 2 years (Seifer et al., 2007). Therefore, M204I is the primary causative genotypic change associated with telbivudine resistance in the clinic.

In this study we investigated the in vitro cross-resistance profile of telbivudine by testing for telbivudine susceptibility in HBV viruses bearing mutations reported to confer resistance to adefovir, tenofovir, or lamivudine in the clinic. Conversely, we examined whether adefovir, tenofovir, or entecavir were active against the mutations responsible for resistance to telbivudine in vitro and in the clinic.

Telbivudine remained active in vitro against HBV genomes bearing the signature N236T and A181V adefovir resistance mutations. In addition, telbivudine was active against the clinically reported A194T tenofovir-associated HBV mutant. When other drugs were tested against the telbivudine-resistance mutations M204I or L80I/M204I in vitro, adefovir and tenofovir remained active. Interestingly, entecavir showed substantially diminished activity against these mutants; this point is discussed further below.

The most important finding from this study is that the cross-resistance profile for the nucleotide analog drugs (adefovir and tenofovir) differs from the nucleoside analog drugs (telbivudine, entecavir, and lamivudine). Resistance to nucleoside analogs appears to arise through mutation of residue 204 in the YMDD motif of the HBV polymerase, as nearly all HBV mutants that show resistance to a nucleoside analog appear to harbor mutations at codon M204. In the pivotal GLOBE trial, the M204V/I mutations emerged in 83.7% of lamivudine patients with viral breakthrough through 2 years of treatment. Although significantly less frequent, viral breakthrough in telbivudine patients was also predominately (86.8%) associated with a YMDD mutation, specifically M204I in response to telbivudine (Li et al., manuscript in preparation). To date in the GLOBE trial, no other genotypic changes have been unequivocally linked to telbivudine resistance in the absence of the M204I mutation within the YMDD motif. In contrast, resistance to nucleotide analogs appears to arise through a different mechanism. As showed by the present study and other reports (Xiong et al., 1998; Chin et al., 2001; Lada et al., 2004; Yang et al., 2004), the M204V/I mutations have little impact on the antiviral efficacy of nucleotide analogs adefovir and tenofovir in vitro, and the M204V/I mutations have not been selected by nucleotide analogs in the clinic to date. Moreover, adefovir has been shown to be effective against M204 HBV variants in the clinical setting.

The finding that telbivudine retains full activity against the M204V single mutation presumably explains the ability of telbivudine to suppress the emergence of the L180M/M204V resistance pathway in the clinic. The M204V mutation is critical for the development of lamivudine resistance, as it is thought to be the first step in the pathway that leads to the M180L/M204V double mutant (Gauthier et al., 1999; Nafa et al., 2000). The L180M mutation was suggested to evolve shortly after the M204V mutation as a compensatory change to help restore viral replicative capacity (Ono et al., 2001; Warner et al., 2007). The in vitro data are consistent with the notion that the robust suppression of the M204V mutant effectively eliminates the L180M/M204V resistance pathway. Interestingly, the L180M/M204V double mutant remains highly resistant to telbivudine when tested in vitro. This suggests that the L180M mutant plays more than a compensatory role in this setting.

Of note, it has been previously reported that HBV genomes with the M204V or M204I mutations remain sensitive to the nucleoside analog entecavir in vitro as well as in the clinical setting (Levine et al., 2002; Langley et al., 2007; Chang et al., 2005). For example, Langley et al. (2007) found only partial (8-fold) in vitro cross-resistance of the M204I mutant to entecavir and changes of 8–30-fold in activity against M204 variants are reported in the entecavir label (BARACLUDE package insert. Bristol-Myers Squibb, Princeton, NJ, USA). However, in our study entecavir was about 353–1000-fold less active against the M204I HBV viruses. Our findings support results reported by Qi et al. (2007), who described a 471-fold-resistance to entecavir for M204I mutant. The clinical significance of such large fold increases remains to be determined. For potent drugs such as entecavir and telbivudine, it may be that a substantial fold reduction in susceptibility is required for the emergence of resistance mutants. For drugs such as adefovir which achieve a lower viral load reduction, a lesser fold change in susceptibility may lead to development of resistance. While caution is warranted when translating in vitro data into clinical efficacy, our in vitro results and those of Qi et al. (2007) suggest that entecavir may not be the best option to treat patients with the M204I mutation in the clinical setting. Although entecavir appears to be initially effective against HBV bearing M204 mutations, resistance to entecavir emerges more readily in this context and was seen in more than 10% of lamivudine-refractory patients after 2 years (Tenney et al., 2007). As noted above, despite a modest loss (3–5-fold) of in vitro activity, adefovir appears to be as active or even more active against M204 HBV mutants in patients (Perrillo et al., 2000; Westland et al., 2005).

When thinking about the optimal therapies for HBV-infected patients, the distinct in vitro cross-resistance profiles between nucleotide analogs and nucleoside analogs support the use of these drugs in combination therapy. Recent studies have indeed supported the notion that adefovir is active in patients with M204 mutations. This idea is further supported by recent clinical data on telbivudine and adefovir (Heathcote et al., 2007b). Adefovir salvage therapy resulted in improved viral suppression as measured by viral DNA levels load drop when combined with telbivudine (Heathcote et al., 2007b). Recent clinical evidence of the need for rescue combination therapy (tenofovir plus emtricitabine) in some individuals who have a sub-optimal response to adefovir and have adefovir-associated mutations N236T and/or A181V suggests that any switch from adefovir to tenofovir monotherapy should be preceded with mutational genotypic sequencing (Tan et al., 2008), and that tenofovir should only be used in combination with another

agent in some adefovir experienced patients. The available clinical data also indicate that adding-on drugs is superior to switching drugs (sequential monotherapies) in patients (Benhamou, 2004; Lampertico et al., 2007). A number of trials evaluating combination HBV therapy have been completed and have demonstrated more potent HBV DNA suppression compared to monotherapies (Hui et al., 2008; Sung et al., 2008). More trials are needed to provide additional information on antiviral efficacy and emergence of genotypic resistance during combination therapy. Pending these results, our in vitro data suggests that adding-on the nucleoside analog telbivudine in patients already being treated with the nucleotide analog adefovir may provide a therapeutic benefit in term of viral load reduction and suppressing the emergence of resistance mutations. Moreover, telbivudine is particularly likely to provide benefit in patients with adefovir resistance mutations, such as N236T or A181V.

In conclusion, the present results support combining HBV nucleoside and nucleotide inhibitors with different resistance profile to provide an effective clinical strategy for treating HBV in the clinic. Further studies are warranted to determine the best combination to use and the optimal time to start combination therapy.

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