Cross-resistance testing of next-generation nucleoside and nucleotide analogues against lamivudine-resistant HBV

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Several next-generation nucleoside and nucleotide analogues are currently in clinical development for the treatment of chronic hepatitis B. However, the efficacy of newer agents against lamivudine-resistant hepatitis B virus (HBV) has not been fully explored. To investigate this in vitro, we generated novel stable cell lines expressing HBV encoding the four major patterns of lamivudine resistance mutations (rtL180M+rtM204V, rtV173L+rtL180M+rtM204V, rtM204I and rtL180M+rtM204I). Using these cell lines, we assessed the susceptibility of all four strains of lamivudine-resistant HBV to eleven nucleoside analogues in various stages of clinical development. Our studies indicate that lamivudine-resistant HBV remain sensitive to acyclic phosphonate nucleotides (adefovir, tenofovir, and alamifovir), have reduced susceptibility to entecavir, and have high-level cross-resistance to all L-nucleosides tested including emtricitabine, telbivudine, clevudine, and torcitabine.

Introduction

The development of safe and efficacious nucleotide and nucleoside analogue therapies has been a significant advance in the treatment of chronic hepatitis B. Two compounds in this class of antivirals are currently approved: adefovir dipivoxil (ADV), a pro-drug of the nucleotide adefovir, and the L-nucleoside lamivudine (3TC). ADV and 3TC overcome the major limitations of interferon-α (parenteral dosing and poor tolerability) and provide multi-log suppression of serum hepatitis B virus (HBV) DNA, enhanced HBeAg seroconversion, and remission of liver disease. However, a significant drawback of 3TC therapy is the selection of resistant viral mutants and the ensuing rebound of viraemia. Once viral load suppression is lost due to resistance, most patients lose the clinical benefits achieved during therapy [1,2].

Emergence of resistance during 3TC monotherapy occurs at a rate of approximately 20% of patients per year and involves non-synonymous mutations at several positions in HBV polymerase gene [3]. Primary 3TC resistance mutations include rtM204V and rtM204I which are located in the YMDD motif of HBV reverse transcriptase and are sufficient to confer high-level phenotypical resistance [4]. Two additional mutations observed in conjunction with rtM204V/I are rtV173L and rtL180M. In vitro studies have shown that these mutations compensate for the reduced replication phenotype of rtM204 mutant HBV [5–7]. Four major patterns of 3TC-resistance mutations have been observed in patients at the following frequencies: rtL180M+rtM204V (59%), rtL180M+rtM204V+rtV173L (17%), rtM204I (11%), and rtL180M+M204I (11%) [8]. ADV has been shown to effectively treat all four strains of 3TC-resistant HBV [8].

In contrast to 3TC, resistance to ADV occurs infrequently. During Phase III clinical trials of ADV, specific ADV-resistance mutations of rtN236T and rtA181V, were observed in 0%, 2%, and 4% of patients after 48, 96, and 144 weeks of therapy, respectively. Phenotypical testing of rtN236T and rtA181V mutant HBV isolated from patient sera or generated by mutagenesis of laboratory strains confirmed that these mutations conferred resistance to adefovir in vitro [9,10]. Importantly, in vitro and limited clinical data indicate that ADV-resistant HBV remains sensitive to 3TC [11,12].

Several new nucleotide and nucleoside analogues are being developed to treat chronic hepatitis B (reviewed by Lok [13]). Compounds in Phase III clinical trials include the L-deoxycytidine analogue emtricitabine (FTC) (currently approved for HIV therapy), the deoxyguanosine analogue entecavir, and the L-thymidine analogues telbivudine (L-dT) and clevudine (L-FMAU). Compounds currently in phase II studies include the L-cytidine analogue torcitabine (as the valyl prodrug valtorcitabine) and the acyclic purine phos-
phonate alamofovir (MCC-478). The deoxyguanosine analogue dioxolane guanine (DXG) is in clinical development for HIV (as the diaminopurine prodrug amadoxovir), but has also been reported to have anti-HBV activity in vitro [14,15]. Tenofovir disoproxil fumarate, a nucleotide prodrug of tenofovir, is approved for the treatment of HIV but has also demonstrated anti-HBV activity in patients during uncontrolled clinical studies [16–18].

It is likely that combination-based regimens for the treatment of chronic hepatitis B will be explored with increasing interest; this will be especially important for patients who develop resistance to current agents and for those who do not undergo complete antiviral suppression during monotherapy. Conceptually, treatment with two or more agents with distinct cross-resistance patterns may enhance viral suppression and reduce the frequency of drug resistance. While studies examining nucleoside and nucleotide combination regimens are only beginning for chronic hepatitis B, the success of this approach has long been realized in HIV therapy where regimens including two nucleosides and a third agent from a separate class are the current standard of care [19]. To develop rational combination regimens for chronic hepatitis B patients, it will be crucial to understand the cross-resistance pattern of newer antivirals. Therefore, we performed a comprehensive cross-resistance analysis of the four major strains of 3TC-resistant HBV to nucleoside and nucleotide analogues currently in development for chronic hepatitis B.

Materials and methods

Generation of plasmids expressing wild-type and 3TC-resistant HBV

Plasmid pHY108 encodes a wild-type 1.1 x unit length HBV genome (genotype A, serotype adw2) under the transcriptional control of the CMV promoter as well as a neomycin resistance gene under the control of the SV-40 promoter in a pBR322 backbone. 3TC resistance mutations were introduced into pHY108 by site-directed mutagenesis using a QuikChange kit (Stratagene, La Jolla, CA, USA). Mutagenesis primers used to generate the rtV173L, rtL180M, and rtM204V mutations have been described previously [7]. The rtM204I mutation was generated using primers HYHBV091 (5′-GCT TTC AGC TAT ATT GAT GTG GTA-3′) and HYHBV092 (5′-TAC CAC ATC ATC AAT ATA GCT GAA AGC-3′) (bolded sequence indicates mutant codon). To eliminate the possible presence of spurious mutations elsewhere in the HBV genome, a region of the polymerase gene containing the mutations was excised from mutagenized pHY108 using ApaI and HpaI and sub-cloned back into an unmanipulated stock of pHY108. The sub-cloned region, including the restriction junctions, was sequenced to confirm that only the intended mutations were present in the final HBV expression constructs.

Cell culture

HepG2 cells were obtained from the American type culture collection (ATCC, Manassas, VA, USA) and maintained in humidified incubators at 37°C and 5% CO₂. HepG2 cells were grown in minimal essential medium (ATCC) supplemented with 100 units/ml penicillin, 10 µg/ml streptomycin, and 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA, USA). Novel stable cell lines (described below) were maintained under conditions identical to HepG2 cells.

Generation of stable cell lines

HepG2 cells were transfected with plasmids encoding wild-type or mutant HBV and a neomycin resistance gene (described above) using Fugene 6 (Roche, Indianapolis, IN, USA). Three days after transfection, cells were selected in media supplemented with 800 µg/ml of G418 (Sigma, St. Louis, MO) for 2–3 weeks. G418-resistant colonies were transferred to 48-well plates and screened for HBeAg secretion using an ETI-EBk+ immunoassay kit (DiaSorin Stillwater, MN, USA). HBeAg+ colonies were expanded and screened for other markers of HBV replication including HBsAg secretion (DiaSorin ETI-MAK-2 immunoassay), extracellular HBV DNA (by PCR analysis of conditioned media according to the method of Gunther et al. [20] and intracellular viral replicative intermediates (by Southern blotting). PCR-amplified extracellular HBV DNA was also sequenced using the primer HBV-Seq-10 (5′-CTG GAT GTG TCT GCG GCG-3′) to confirm that each cell line expressed HBV with the intended reverse transcriptase mutations. Cell lines positive for all markers of viral replication were further expanded and frozen down.

Comparison of HBV expression in selected cell lines

To compare HBV expression levels in novel cell lines, confluent monolayers were trypsinized and 2 x 10⁵ cells were aliquoted into microcentrifuge tubes. Cells were pelleted by spinning at 750 xg for 1 min, resuspended in 200 µl of PBS, and adjusted to 0.3% NP-40 for lysis. Lysates were centrifuged at 14 500 xg for 1 min to pellet nuclei. 40 µl of each supernatant was transferred to a 96-well PCR plate (MJ Research, South San Francisco, CA, USA), and mixed with 60 µl of BuccalAmp extraction buffer (Epicentre, Madison, WI, USA). HBV DNA was released from the cytoplasmic core particles by heat extraction in an MJ Research PTC-100 thermocycler using the following programme: 30 min at 65°C, followed by 17 min at
94°C. Extracted DNA was quantified by real-time PCR as described below.

**Antiviral compounds**

Adefovir, tenofovir, FTC, DXG, clevudine, and entecavir were synthesized by Gilead Sciences (Foster City, CA, USA) (Figure 1). 3TC, telbivudine, torcitabine, and 3'-deoxyadenosine were purchased from Moravek Biochemicals (Brea, CA, USA). The F-1 metabolite (phosphonic acid) of alamovir-F1 was a kind gift of Dr Antonin Holy (IOCB, Czech Republic).

**Antiviral assays**

Cells were seeded in 96-well plates at a density of 2x10^4 cells per well and allowed to attach overnight. The following day (day one), cell culture medium was aspirated using a 12-well vacuum manifold and cells were treated with fresh media containing drug. Serial drug dilutions were prepared in media in 96-well plates using a Precision 2000 Automated Pipetting System (Bio-tek Instruments Inc, Winooski, Vermont). Cells were treated with fresh media containing drug on days three and five. Wild-type and 3tc-resistant cell lines were assayed in parallel using the same master stocks of each drug. On day seven, media were aspirated and cells were washed once with PBS and lysed in 100 ml of 0.3% NP-40 in PBS. The lysates were then transferred into 96 well V-bottom plates and spun at 500 xg for 5 min to pellet cellular nuclei. 40 ml of supernatant from each well were transferred to 96-well PCR plates and mixed with 60 ml of BucAmp extraction buffer. HBV DNA was released from the cytoplasmic core particles by heat extraction as described above. Extracts were stored at -20°C until real-time PCR quantification of HBV DNA was performed (see below). The resulting data were fitted to the three parameter logistic dose response equation y=a/(1+(x/b)^c) using Prism v 4.0 (Graphpad Software Inc. San Diego, CA, USA). Fold-resistance was calculated as the ratio of mutant EC50 to wild-type EC50.

**Real-time PCR quantification of HBV DNA**

Cytoplasmic extracts were analysed by real-time quantitative PCR (qPCR) using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR primers HBV-Taq1 (forward) and HBV-Taq2 (reverse) and the FAM/TAMRA labelled TaqMan probe BS-1 were used for HBV quantification as described previously [21]. PCR reactions were performed in a 20 µl volume using 10 µl of 2x TaqMan Master Mix (Applied Biosystems), 200 nM BS-1 probe, 300 nM HBV-Taq1 primer, 300 nM HBV-Taq2 primer, and 2 µl of extracted DNA. Plasmid pHBVEcoRI which encodes a single genome of HBV DNA was run as a standard.

A linear dynamic range from 10^1 to 10^7 copies was observed in agreement with previous reports for this primer/probe set [21].

**Cytotoxicity testing**

To assess cytostatic or cytotoxic effects of antiviral compounds, WT-42 cells were seeded into 96-well plates and treated with compounds using a protocol identical to the antiviral assay described above. Following the one week drug treatment, cell viability was assayed by MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reduction using the CellTiter 96 AQueous non-radioactive cell proliferation assay (Promega, Madison WI, USA) according to the manufacturer's instructions.

**Results**

Generation of stably-transfected cell lines expressing major patterns of 3TC-resistant HBV

To facilitate cross-resistance testing of anti-HBV compounds, we established novel cell lines that expressed wild-type HBV and the four major mutational patterns of 3TC resistance: rtL180M+rtM204V, rtV173L+rtL180M+rtM204V, rtM204I, and rtL180M+M204I. Plasmids encoding each viral genotype and a neomycin resistance gene were transfected into HepG2 cells and the cultures were treated with G418 to select clones with stably integrated HBV genomes. Individual clones were screened for expression of HBeAg, HBsAg, extracellular HBV DNA, and intracellular viral replicative intermediates. Multiple positive clones which ranged in expression level were identified for each viral genotype. One clone of each genotype was selected for further studies: WT-42 (wild-type), LMMV-40 (rtL180M+rtM204V), VLLMMV-24 (V173L+rtL180M+rtM204V), MI-91 (rtM204I), and LMMI-17 (rtL180M+M204I). Each of these cell lines expressed the expected pattern of intracellular HBV replicative intermediates by Southern blot analysis and was confirmed to express the correct viral genotype by DNA sequencing (data not shown). Expression levels of cytoplasmic HBV DNA in each novel cell lines was quantified by real-time quantitative PCR (qPCR) (Table 1). The number of integrated plasmids was quantified using primers and probes specific for the neomycin resistance gene (data not shown). Like cytoplasmic HBV expression, the number of integrated copies varied between cell lines. However, there was not a significant correlation between integrated copy number and cytoplasmic HBV replication, suggesting that additional factors influenced levels of viral replication in the cell lines.
Figure 1. Chemical structures of nucleoside and nucleotide analogues that inhibit HBV
Development and validation of a high throughput assay for anti-HBV testing

We chose real-time qPCR as an HBV DNA detection method since it has high sensitivity, can be run in a 96-well format, and does not require radio-isotopes. As a prerequisite for PCR quantification, we needed to develop an efficient method for extracting PCR-ready HBV DNA from the cytoplasm of cells without contamination from viral DNA integrated into the cellular genome. This was accomplished in a 96-well format by NP-40 lysis of cells, removal of nuclei by centrifugation and extraction of HBV DNA from cytoplasmonic core particles using a commercially available lysis buffer. To confirm that this protocol separated integrated nuclear HBV DNA from the replicating cytoplasmic virus, we assayed cytoplasmic extracts for the presence of the neomycin resistance gene which is present in the integrated HBV plasmids (Figure 2). In contrast to whole cell and nuclear extracts, which were positive for both HBV DNA and the neomycin resistance gene, cytoplasmic extracts were positive only for HBV DNA, indicating that the protocol successfully separated actively replicating virus from integrated viral genomes.

Using the WT-42 cell line, we assayed the antiviral activity of eleven approved or developmental nucleoside/nucleotide analogues against wild-type HBV. Nine drug concentrations (selected based on previously reported EC50 values) were assayed in triplicate during each experiment. Cytotoxicity assays were performed prior to antiviral testing to ensure that none of the compounds were being used at cytostatic or cytotoxic doses (data not shown). Under the extraction and quantification conditions, approximately $10^4$ copies of HBV DNA were detected per qPCR reaction, which was well within the linear range of the assay ($10^1$ to $10^7$). EC50 values generated using qPCR were highly reproducible between independent experiments (Table 2, Figure 3A) and were significantly correlated ($P<0.0001$, $R^2=0.94$) with previously reported EC50 values obtained using Southern blotting and nucleic acid hybridization (Figure 3B) [6,22–25].

Cross-resistance testing

We then assayed the sensitivity of each 3TC-resistant mutant to the 11 anti-HBV compounds using the LMMV-40, VLLMMV-24, MI-91 and LMMI-17 cell lines (Figure 1, Table 2). Similar to WT-42, each of the mutant cell lines produced a signal within the linear range of the qPCR assay (range for cell lines: $3\times10^3$ to $3\times10^5$ copies/cell line).

### Table 1. Quantification of cytoplasmic HBV copies in novel cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mutational pattern</th>
<th>Cytoplasmic HBV* copies/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-42</td>
<td>Wild-type</td>
<td>9.7 ± 1.7</td>
</tr>
<tr>
<td>LMMV-40</td>
<td>rtL180M+rtLM204V</td>
<td>88.4 ± 13.5</td>
</tr>
<tr>
<td>VLLMMV-24</td>
<td>rtV173L+rtL180M+rtLM204V</td>
<td>60.3 ± 3.6</td>
</tr>
<tr>
<td>MI-91</td>
<td>rtLM204I</td>
<td>14.7 ± 2.9</td>
</tr>
<tr>
<td>LMMI-17</td>
<td>rtL180M+rtLM204I</td>
<td>5.3 ± 1.3</td>
</tr>
</tbody>
</table>

*Cytoplasmic HBV was measured by qPCR as described in materials and methods. Values represent the mean ± standard deviation.
1.4x10^5 HBV copies/reaction). All three acyclic phosphonate compounds (adefovir, tenofovir, and alamaflovir-F1) had similar efficacy against wild-type HBV and the four 3TC-resistant mutants. All four of the 3TC-resistant mutants had reduced susceptibility to entecavir (range for mutants: 37 to 471 fold), however high doses of entecavir were still able to reduce viral replication below 50%. Interestingly, DXG had similar efficacy against wild-type HBV and 3TC-resistant mutants encoding the rtM204V mutation, but showed significantly increased EC50 values against mutants encoding rtM204I. DXG was the least potent compound tested (EC50=63 μM) and we were unable to use doses high enough to precisely quantify the resistance to M204I containing mutants. All four of the 3TC-resistant mutants had high level resistance (>140 to >2000 fold) to all of the l-nucleosides tested (3TC, FTC, clevudine, telbivudine, torcitabine and l-deoxyadenosine). In most cases, no dose response was detected up to the highest drug concentrations assayed for these compounds.

Discussion

Several next-generation nucleotide and nucleoside analogues are under advanced clinical development for the treatment of chronic hepatitis B. Since viral resistance development is currently a problem with 3TC and, to a lesser extent, with ADV, it will be crucial to understand the cross-resistance profiles of newer agents so that they can be used appropriately. Furthermore, a clear understanding of cross-resistance is essential for the rational design of combination therapies which should continue to be explored clinically as new agents become available. To our knowledge, the study reported here is the largest side-by-side comparison of developmental nucleoside and nucleotides against all of the 3TC-resistant HBV strains commonly observed in the clinic.

To perform our testing, we generated new stable cell lines expressing rtL180M+rtM204V, rtV173L+rtL180M+rtM204V, rtM204I, or rtL180M+M204I

Table 2. EC50 values for nucleoside and nucleotide analogues against wild-type HBV

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50 (μM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adefovir</td>
<td>0.55 ±0.30</td>
</tr>
<tr>
<td>Tenofovir</td>
<td>0.77 ±0.34</td>
</tr>
<tr>
<td>Alamaflovir-F1</td>
<td>0.04 ±0.02</td>
</tr>
<tr>
<td>Entecavir</td>
<td>0.001 ±0.0003</td>
</tr>
<tr>
<td>DXG</td>
<td>62.59 ±4.21</td>
</tr>
<tr>
<td>Emtricitabine</td>
<td>0.07 ±0.02</td>
</tr>
<tr>
<td>Lamivudine</td>
<td>0.06 ±0.02</td>
</tr>
<tr>
<td>Clevudine</td>
<td>0.15 ±0.12</td>
</tr>
<tr>
<td>Telbivudine</td>
<td>0.70 ±0.32</td>
</tr>
<tr>
<td>Torcitabine</td>
<td>0.38 ±0.14</td>
</tr>
<tr>
<td>l-deoxyadenosine</td>
<td>1.77 ±1.26</td>
</tr>
</tbody>
</table>

*EC50 values were calculated by nonlinear regression of antiviral data generated by qPCR analysis of HBV DNA in treated WT-42 cells. Values represent the mean ± standard deviation of two or more independent experiments performed in triplicate.

Figure 3. Intra- and inter-experimental variability during the EC50 assay and correlation of TaqMan EC50 with historical values

(A) qPCR was used to quantify HBV DNA during three independent tenofovir dose response experiments; each data-point represents the mean of three replicate wells with error bars indicating standard deviation. EC50 values of 0.6, 1.2, and 0.6 μM were obtained. (B) EC50 values obtained by qPCR (X-axis) were plotted against those reported in the literature using Southern blotting and nucleic acid hybridization (Y-axis, see text for references); the two data sets show a significant correlation (r²=0.94, P=<0.0001).
Table 3. Fold resistance of nucleoside and nucleotide analogues to HBV encoding the major lamivudine resistance mutations

<table>
<thead>
<tr>
<th>Compound</th>
<th>L180M+</th>
<th>V173L+</th>
<th>M204V</th>
<th>M204I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adefovir</td>
<td>1.1</td>
<td>1.1</td>
<td>1</td>
<td>1.8</td>
</tr>
<tr>
<td>Tenofovir</td>
<td>0.8</td>
<td>1.8</td>
<td>2.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Alamafovir-F1</td>
<td>3.1</td>
<td>1.6</td>
<td>3.8</td>
<td>1.5</td>
</tr>
<tr>
<td>DXG</td>
<td>2.2</td>
<td>1.3</td>
<td>&gt;5</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Emtricitabine</td>
<td>&gt;2000</td>
<td>898</td>
<td>&gt;2000</td>
<td>845</td>
</tr>
<tr>
<td>Lamivudine</td>
<td>&gt;700</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Clevudine</td>
<td>&gt;1600</td>
<td>&gt;1600</td>
<td>&gt;1600</td>
<td>&gt;1600</td>
</tr>
<tr>
<td>Telbivudine</td>
<td>&gt;322</td>
<td>&gt;322</td>
<td>&gt;322</td>
<td>&gt;322</td>
</tr>
<tr>
<td>Torcitabine</td>
<td>&gt;650</td>
<td>&gt;460</td>
<td>&gt;180</td>
<td>&gt;650</td>
</tr>
<tr>
<td>L-deoxyadenosine</td>
<td>&gt;140</td>
<td>&gt;140</td>
<td>&gt;140</td>
<td>&gt;140</td>
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</tbody>
</table>

*Fold-resistance calculated as the wild-type EC50 (Table 2)/mutant EC50.

The in vitro efficacy of adefovir against all four 3TC-resistant strains is in agreement with existing in vitro and clinical data. Previous modelling studies attributed adefovir’s efficacy against rtM204I/V mutants to the acyclic methoxy-ethyl linker used in place of cyclic ribose. The shorter, less bulky, and potentially more flexible acyclic linker appears to be accommodated in the sterically-restricted nucleotide binding pocket of 3TC-resistant HBV polymerase [26]. Consistent with this hypothesis, tenofovir, and alamafovir-F1, which share adefovir’s acyclic structure, also retained activity against all strains of 3TC-resistant HBV. Additional acyclic phosphonate analogues with activity against 3TC-resistant HBV have also been reported recently [27].

The reduced susceptibility of 3TC-resistant HBV to entecavir observed here agrees with data we and others have previously reported using transient transfection assays, [6,28]. In contrast to 3TC, entecavir has a cyclic sugar in the natural D-conformation. However, the carbocyclic ring of entecavir contains a vinyl substituent in the 6 position, making it bulkier than the natural deoxyribose ring which has an oxygen in the analogous position. The increased volume of entecavir’s sugar ring analogue provides a plausible basis for its reduced affinity to the restricted binding pocket of 3TC-resistant HBV polymerase [28]. Although entecavir has been shown to suppress viral load in 3TC-refractory patients, several lines of evidence suggest that the mutant virus has reduced susceptibility compared to wild-type in patients. Viral load suppression in 3TC-resistant patients appears to be less (-4.3 to -5.1 logs) [29,30] compared to treatment-naive patients (-7 logs) [31], despite the use of a higher dose in 3TC-refractory patients (1.0 mg vs 0.5 mg in naive patients) and the inclusion of some patients with wild-type HBV in the studies. 3TC resistance mutations are also maintained during entecavir therapy, suggesting an advantage for the M204I/V mutant over wild-type HBV; this is in contrast to ADV therapy which results in a progressive loss of 3TC-resistant HBV over time [32]. Finally, the presence of 3TC resistance mutations predisposes patients to the development of novel entecavir resistance mutations [24]. This is reminiscent of the accelerated development of 3TC resistance observed in patients that first failed famciclovir therapy after developing the rtL180M mutation [33,34].

All four patterns of 3TC-resistant HBV were highly cross-resistant to all of the L-nucleoside analogues we tested. These data confirm the cross-resistance to FTC which has been observed in vitro and clinically, but also provide novel information on several newer compounds in clinical trials. Interestingly, the rtM204I/V mutations which are selected by the deoxy-cytidine analogues 3TC and FTC also confer cross-resistance to l-thymidine analogues (telbivudine and clevudine) as well as to the l-purine analogue l-deoxyadenosine. Indeed, the emergence of the M204I resistance mutation was recently observed in 5% (2/44)
of telbivudine treated patients in a Phase II clinical study [35]. Unlike 3TC and FTC, telbivudine, clevudine, torcitabine, and L-deoxyadenosine also lack the 3′ sulfur atom which may contribute to resistance based on steric conflict with the β-methyl branch of valine 204 [26]. Overall, the data presented here suggest that the most important structural determinant for rtM204I/V-mediated resistance is the t-configuration and that modifications to the nucleoside base or sugar ring are not able to mitigate resistance significantly.

Our studies defined three groups of compounds with respect to 3TC-resistant HBV: 1) compounds that retain wild-type efficacy (adefovir, tenofovir, and alamafvir), 2) compounds with reduced susceptibility (entecavir) and 3) compounds with high level cross-resistance (includes all t-nucleosides). These in vitro data suggest that acyclic phosphonates may provide the best long-term option for treating 3TC-resistant HBV. Due to distinct cross-resistance patterns, the combination of acyclic phosphonates and t-nucleosides should also be considered in treatment-naïve patients that do not achieve full suppression of viral load (that is, PCR undetectable) during monotherapy.

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Cross-resistance profiles of anti-HBV nucleosides

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