Phenylpropenamide Derivatives AT-61 and AT-130 Inhibit Replication of Wild-Type and Lamivudine-Resistant Strains of Hepatitis B Virus In Vitro

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Chronic hepatitis B virus (HBV) infection, a major cause of hepatocellular carcinoma and liver cirrhosis, affects about 5% of the world’s human population (19, 24). Chronic HBV infection is unresponsive to currently available vaccines and can only be controlled by chemotherapy (19). In most countries, only two drugs, alpha interferon (IFN-α), and lamivudine (LMV) [(−)-β-L-2′,3′-dideoxy-3′-thiacytidine], are approved for treatment of chronic HBV (19), but additional drugs are being developed (4, 8). IFN treatment results in a sustained response in only a minority of cases and may be associated with dose-limiting side effects (19). LMV, a deoxycytidine analog, is a safer and more effective inhibitor of HBV replication, but its effects are rarely sustained after short-term treatment, and its long-term use frequently results in the emergence of drug-resistant HBV strains (1, 3, 10, 11, 17, 18, 23, 26, 29). Specific mutations associated with resistance to LMV have been identified by sequence analysis and shown to affect the catalytic YMDD (tyrosine-methionine-aspartate-aspartate) motif of the viral polymerase (1, 11). Because they are genotype dependent, the numbering systems used to identify these mutations are confusing; as a result, the adoption of a standardized genotype-independent nomenclature has recently been proposed (28) and is used here. The LMV resistance mutations that have been observed most frequently in clinical isolates cause the methionine at amino acid residue 204 in the reverse transcriptase coding region of the polymerase protein (formerly amino acid 539, 549, 550, or 552, depending on the genotype) to be replaced with either isoleucine or valine. These changes are identified as rtM204I and rtM204V, respectively, by the new nomenclature. While the rtM204I substitution alone appears to be sufficient to confer resistance to LMV, rtM204V is rarely observed in the absence of a second mutation that causes an upstream substitution of methionine for leucine at reverse transcriptase residue 180 (rtL108M; formerly identified as polymerase residue 515, 525, 526, or 528) (1, 3, 10, 11, 17, 18, 23, 26, 29). Interestingly, the rtL108M change, by itself, has been associated with resistance to famciclovir, another nucleoside analogue that has undergone clinical trials against chronic HBV infection (2, 9, 14, 25, 30). Several independent studies have provided evidence that mutations that confer resistance to LMV and/or famciclovir also confer resistance to other nucleoside analogs (7, 12, 13, 20, 21, 31; reviewed in reference 8). Cross-resistance may therefore limit the clinical potential of some nucleoside analogs that are being developed as potential anti-HBV agents (8, 27). The identification of safe and efficacious nonnucleoside inhibitors of HBV replication would greatly improve prospects for overcoming the problems associated with nucleoside analog cross-resistance. Although several low-molecular-weight nonnucleoside anti-HBV compounds have been identified (reviewed in reference 4), very few have been tested in controlled clinical trials.

The phenylpropenamide derivative AT-61 (Fig. 1a) was re-
recently shown to be a potent inhibitor of in vitro HBV replication in stably or transiently transfected HepG2 cells (15, 22). AT-61 specifically inhibited the in vitro replication of HBV but had no effect on the in vitro replication of a variety of other viruses, including duck HBV, woodchuck HBV, or human immunodeficiency virus type 1 (HIV-1) (15). Moreover, AT-61 was found to be equally active as an inhibitor of HBV replication in the HepG2-derived HepAD38 and HepAD79 cell lines (15). In these cell lines, the expression of stably transfected HBV genomes (wild type [wt] in HepAD38 and rtM204V in HepAD79, respectively) is controlled by a tetracycline-sensitive promoter (16). It was also reported that combinations of AT-61 and LMV produced synergistic antiviral effects against HBV replication in AD38 cells (15). These data suggest that the specific anti-HBV activity of AT-61 is due to a mechanism(s) that differs from that of LMV. Furthermore, they imply that mutations that confer resistance to LMV and other nucleoside analogs may not confer cross-resistance to AT-61 or other phenylpropenamide derivatives (15).

AT-130 (Fig. 1b), a congener of AT-61, differs from AT-61 in that rings A and B contain ortho-methoxy and para-nitroso substituents, respectively; in addition, a bromine atom replaces the chlorine present in AT-61. AT-130 has also been shown to have anti-HBV activity in HepAD38 cells in vitro (22).

The present study sought to determine the relative in vitro potencies of AT-61 and AT-130 as inhibitors of wt and nucleoside analog-resistant HBV replication by using a recently described model that employs recombinant baculoviruses to efficiently transduce HepG2 cells with replication-competent HBV genomes (6, 7).
Recombinant baculoviruses that encode either 1.28-times genome length wt HBV (genotype A, subtype adw2) or one of three drug-resistant mutants were constructed and propagated as described previously (7). The three drug-resistant viruses harbored point mutations that coded for rtlL180M, rtM204I, or rtlL180M + rtL204V substitutions in the HBV polymerase sequence, respectively. AT-61 and AT-130 were synthesized as described previously (22), and stock solutions of each drug were freshly prepared in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the cell culture media was always <1% (vol/vol), a concentration that has no measurable effect on HBV replication. The experimental procedures used for drug sensitivity testing were described in detail previously (7). Briefly, replicate cultures of HepG2 cells were transduced at a multiplicity of infection of 50 PFU/cell with either mutant or wt HBV and then exposed continuously to five different concentrations of AT-61, AT-130, or LMV, beginning immediately after transduction. Culture media were changed on days 2, 4, and 6 posttransduction, and cells were harvested on day 7. Replicating viral DNA was extracted from cytoplasmic core particles and analyzed by Southern hybridization and autoradiography (5, 7). Image densities from suitably exposed autoradiographs of Southern blots (Fig. 2) were measured by computer-assisted densitometry (GC-67 scanning densitometer with Molecular Analyst software from Bio-Rad Laboratories, Hercules, Calif.). The amount of viral replication in drug-treated samples was expressed as a percentage of the amount of replication in drug-free controls. Where possible, logistic dose-response curves described by the equation $y = aI + (c/b)^x$ were fitted to each set of data and equation parameters were estimated as described previously with the aid of TableCurve2D, a software package from Jandel Scientific (San Rafael, Calif.) (5, 7). Drug concentrations that inhibited the replication of each mutant HBV by 50 or 90% of the average amount measured in the corresponding drug-free controls (IC50 and IC90, respectively) were estimated from individual dose-response curves. To assess the cytotoxicity of these compounds, additional sets of HepG2 cultures were continuously exposed to AT-61 or AT-130 for 7 days, after which cellular toxicity was assayed colorimetrically by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide cleavage. Cytotoxicity was also tested in Huh-7, another hepatic cell line of human origin.

Preliminary experiments confirmed that treatment with either AT-61 or AT-130 caused dose-dependent inhibition of wt HBV replication in HepG2 cells and that neither compound was measurably toxic to either HepG2 or Huh-7 cells at concentrations of up to 250 μM, the highest concentration tested (data not shown). Results from the recombinant baculovirus assay system produced estimated IC50s of 0.064 ± 0.020, 21.2 ± 9.5, and 2.4 ± 0.92 μM (mean ± standard deviation of triplicate assays against wt HBV) for LMV, AT-61, and AT-130, respectively. These IC50s are consistent with the potency ranking reported earlier (22) but are greater than those reported previously, i.e., 0.6 to 5.7 μM for AT-61, depending on the cell line used for the assay (15), and 0.13 μM for AT-130 (22).

Subsequent experiments compared the activities of AT-61 and AT-130 as inhibitors of both wt and mutant HBVs. The results, which are summarized in Table 1, indicate that the calculated IC50s and resistance factors for wt and mutant HBVs were not significantly different following either AT-61 or AT-130 treatment. They also confirmed that, on a molar basis, AT-130 was more effective as an inhibitor of in vitro HBV DNA replication than was AT-61. Calculated IC50s and IC90s were in the ranges of 19 to 27 and 63 to 97 μM, respectively, for AT-61. The corresponding ranges for AT-130 were 1.3 to 5.1 and 11 to 36 μM. To confirm that the phenylpropenamide derivatives were active against LMV-resistant HBV mutants and to confirm that these mutants showed a drug resistance phenotype in vitro, replicate assays were carried out in parallel with LMV as an internal control. The results presented in Table 2 confirm that mutations that confer LMV resistance do not confer cross-resistance to the phenylpropenamide derivatives.

Results presented here confirm and extend previously reported observations regarding the anti-HBV activities of AT-61 and related phenylpropenamide derivatives (15, 22).

<table>
<thead>
<tr>
<th>Drug type and drug</th>
<th>Curve fit parameters</th>
<th>Resistance parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>Wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT-61</td>
<td>105 ± 5</td>
<td>18.3 ± 3.9</td>
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<tr>
<td>AT-130</td>
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<td>rtlL180M</td>
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<td>AT-61</td>
<td>113 ± 7</td>
<td>24.0 ± 7</td>
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<tr>
<td>AT-130</td>
<td>100 ± 7</td>
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<tr>
<td>rtM204I</td>
<td></td>
<td></td>
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<tr>
<td>AT-61</td>
<td>99 ± 1</td>
<td>21.8 ± 1.5</td>
</tr>
<tr>
<td>AT-130</td>
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<td>4.2 ± 2.4</td>
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<tr>
<td>rtlL180M + rtL204V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT-61</td>
<td>103 ± 3</td>
<td>19.2 ± 4.1</td>
</tr>
<tr>
<td>AT-130</td>
<td>102 ± 5</td>
<td>1.3 ± 0.5</td>
</tr>
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</table>

*The curve fit parameters a, b, and c define a logistic dose-response curve that is described by the equation $y = aI + (c/b)^x$ where $y = \%\text{ inhibition}$, a is the curve’s amplitude, $b$ is the x value at its transition center, and $c$ is a parameter that defines the transition width (see reference 5, 7, and 27 for further information). $r^2$ (the coefficient of determination) is a measure of curve fit closeness ($r^2 = 1$ indicates a perfect fit). Results (means ± standard errors) are from one set of experiments in which AT-61 and AT-130 were tested (at five different concentrations each) in parallel as inhibitors of replication of wt HBV and each of its three mutant derivatives.

*The resistance factor is the mutant/wt IC50 ratio (%).

### Table 2. Relative sensitivities of wt and drug-resistant HBV to phenylpropenamide derivatives AT-61 and AT-130

<table>
<thead>
<tr>
<th>Drug</th>
<th>Wt</th>
<th>rtlL180M</th>
<th>rtM204I</th>
<th>rtlL180M + rtL204V</th>
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<tr>
<td>LMV</td>
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<td>14.3</td>
<td>&gt;14.0</td>
<td>&gt;14.3</td>
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<tr>
<td>AT-61</td>
<td>1.0</td>
<td>1.02</td>
<td>2.21</td>
<td>1.22</td>
</tr>
<tr>
<td>AT-130</td>
<td>1.0</td>
<td>3.71</td>
<td>0.33</td>
<td>2.29</td>
</tr>
</tbody>
</table>

*Results of a second set of experiments in which AT-61, AT-130, and LMV were tested (at five different concentrations each) in parallel as inhibitors of replication of wt HBV and each of its three mutant derivatives.

* See footnotes to Table 1.
Although the mechanism of action of this group of compounds is unknown, it appears to be independent of interference with the RNA- or DNA-dependent activities of the HBV polymerase. Observing that exposure to AT-61 decreased the amount of RNA-containing cytoplasmic HBV core particles, King and colleagues postulated that AT-61 inhibits the packaging of pregenomic viral RNA (15), a prerequisite for genome replication (24).

Our observation that the HBV mutants most commonly associated with LMV and/or famiclovir resistance remain sensitive to both AT-61 and AT-130 justifies the further development of these compounds or their derivatives for eventual clinical use. These compounds appear to have the potential to arrest the replication of known drug-resistant HBV strains and, thus, in combination with nucleoside/nucleotide analogs (4, 5) and/or other drugs, may help reduce, or perhaps even prevent, the development of drug resistance (27).

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REFERENCES