NOTES

Synergistic Inhibition of Replication of Human Immunodeficiency Virus Type 1, Including That of a Zidovudine-Resistant Isolate, by Zidovudine and 2',3'-Dideoxycytidine In Vitro

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The combination of zidovudine (AZT) and 2',3'-dideoxycytidine synergistically inhibits human immunodeficiency virus type 1 (HIV-1) replication in vitro with AZT-sensitive and AZT-resistant clinical isolates and HIV-1IIIB. Synergy was determined by the median-effect principle and isobologram techniques. Cytotoxicity of the agents was not observed. Clinical trials are ongoing to define the combination's role in HIV-1 therapy.

Several nucleoside reverse transcriptase inhibitors, including zidovudine (AZT), 2',3'-dideoxycytidine (ddC), and 2',3'-dideoxyinosine (ddI), have activity against human immunodeficiency virus type 1 (HIV-1) replication in vitro (25–28) and in vivo (6, 9–11, 20, 24, 32, 34, 36). Nevertheless, progression of disease continues to occur despite treatment, and HIV-1 replication is incompletely suppressed by AZT monotherapy (9, 15). HIV-1 isolates resistant to AZT in in vitro assays (21) can be obtained from most patients receiving prolonged AZT therapy (30). Viruses with decreased susceptibility to ddI or ddC have been noted (7, 13, 31). Cross resistance to ddC has not been reported in viruses with AZT resistance mutations, and molecularly cloned viruses with ddC resistance were not cross resistant to AZT (13, 21, 31).

Combination antiretroviral therapy may prolong drug efficacy against HIV-1 (18, 27, 35) and may reduce the emergence of resistant viruses. Combination therapy may also help decrease toxicity if lower doses of each agent can be used and if their toxicities are largely not overlapping, as is the case with AZT and ddC (8, 23, 24). A preliminary phase I clinical trial of this combination has shown promising results (23).

We performed a series of experiments to define interactions between AZT and ddC on the inhibition of HIV-1 replication by using HIV-1IIIB and clinical isolates that had previously been shown to exhibit AZT-sensitive and -resistant phenotypes in vitro (19). The resistant isolate contains reverse transcriptase mutations as the dominant genotype (19) at AZT resistance-conferring codons (i.e., codons 67, 70, 215, and 219) previously described (2, 21, 22).

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H9 cells provided by R. C. Gallo (National Cancer Institute, Bethesda, Md.) (29) were maintained as described previously (14). Peripheral blood mononuclear cells (PBMC) from individual HIV-1-seronegative donors were obtained by Ficoll-Hypaque density gradient centrifugation of heparinized venous blood for each experiment in which PBMC were used. Cells were treated with 10 μg of phytohemagglutinin (Difco Laboratories, Detroit, Mich.) per ml and propagated in medium supplemented with 10% interleukin 2 (Pharmacia Diagnostics, Silver Springs, Md.) as previously described (19). HIV-1IIIB was provided by R. C. Gallo (29) and propagated in H9 cells; the 50% tissue culture infectious dose per milliliter of cell-free supernatant was determined as described previously (14, 17). Paired HIV-1 clinical isolates were derived from an HIV-1-seropositive individual before (14a-4/87) and after (14a-6/89) 26 months of AZT monotherapy and were propagated and titrated as previously described (19).

AZT was obtained from P. A. Furman (Wellcome Research Laboratories, Research Triangle Park, N.C.), and ddC was obtained from I. Sim (Hoffmann-La Roche, Nutley, N.J.).

Three pairs of experiments were performed (Table 1). In experiments 1 and 2, 0.4 × 10⁶ H9 cells per ml of medium were exposed to 500 50% tissue culture infectious doses of HIV-1IIIB as inoculum per 10⁶ cells in flasks containing either single agents or the combination of AZT and ddC. In experiments 3 through 6, 10⁶ uninfected 4-day phytohemagglutinin stimulated PBMC per ml of medium were exposed to 1,000 50% tissue culture infectious doses of the HIV-1 isolate (either 14a-4/87 [experiments 3 and 4] or 14a-6/89 [experiments 5 and 6]) per 10⁶ cells in flasks containing drug. Untreated infected controls were maintained in duplicate, and drug-containing flasks were maintained in singleton. Uninfected drug-treated toxicity controls were maintained. Culture medium was changed twice weekly, maintaining original drug concentration(s). At medium changes, cell-free culture supernatant fluids were harvested for HIV-1 p24 antigen production starting on day 7 in culture. Cell proliferation and viability were assessed by the trypan blue dye exclusion method. Cell-free culture supernatant fluids were assayed by an HIV-1 p24 antigen enzyme-linked immuno-
TABLE 1. CIs for two-drug combination regimens of AZT and ddC against HIV-1 replication in vitro*  

<table>
<thead>
<tr>
<th>Expt</th>
<th>Conc (μM) of:</th>
<th>HIV-1 isolate</th>
<th>p24 antigen assay day</th>
<th>CI at HIV-1 inhibition of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AZT</td>
<td>ddC</td>
<td></td>
<td>50%  75%  90%  95%</td>
</tr>
<tr>
<td>1</td>
<td>0.04, 0.16, 0.64, 2.56</td>
<td>0.00125, 0.005, 0.02, 0.08</td>
<td>HIV-1111B</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>0.04, 0.16, 0.64, 2.56</td>
<td>0.00125, 0.005, 0.02, 0.08</td>
<td>HIV-1111B</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>0.0025, 0.005, 0.01, 0.02</td>
<td>0.02, 0.04, 0.08, 0.16</td>
<td>14a-4/87</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>0.0025, 0.005, 0.01, 0.02</td>
<td>0.02, 0.04, 0.08, 0.16</td>
<td>14a-4/87</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>0.125, 0.25, 0.5, 1.0</td>
<td>0.02, 0.04, 0.08, 0.16</td>
<td>14a-6-89</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>0.125, 0.25, 0.5, 1.0</td>
<td>0.02, 0.04, 0.08, 0.16</td>
<td>14a-6-89</td>
<td>7</td>
</tr>
</tbody>
</table>

* In all experiments the combination flasks used a fixed ratio of the single-agent concentrations. Using the data generated in each experiment, we obtained median-effect curves through computer estimation for each agent and the combination of agents. For each agent and the combination, median-effect curve parameters m (the slope) and Dm or IC50 (the x intercept) defined the shape of the curve over its entire length. These values were used to determine CIs by using the mutually exclusive CI equation. CIs of <1, 1, and >1 indicate synergism, additive effects, and antagonism, respectively (3-5, 14).

In each experiment, the dose-effect data for each single agent and the combination were used to plot a median-effect curve by computer estimation (5). These curves, which are log-log plots, are defined by the slope (m) and the x intercept (Dm, or 50% inhibitory concentration [IC50]). These values, m and Dm, are used for a computerized calculation of the combination index (CI) (Table 1) as described previously (3-5, 14). CI of <1, 1, and >1 indicate synergism, additive effects, and antagonism, respectively. The data were also analyzed by computer-generated classical isobolograms, which evaluate drug interactions by a dose-oriented geometric method. Data were subjected to quantitative computer analysis only if the linear correlation coefficient of the median-effect plot was high (r ≥ 0.75).

In experiments 1 and 2 performed on H9 cells, AZT and ddC as single agents inhibited HIV-1111B p24 antigen production in a dose-dependent manner (data not shown). The combination of agents demonstrated greater inhibition of p24 antigen production at all concentrations at which the single agents caused at least a 50% reduction of p24 antigen relative to that in the untreated infected control. At concentrations at which ddC had no effect, the effect of the combination in reducing HIV-1111B p24 antigen production was variable, and at two such datum points (one in experiment 1 and one in experiment 2), the combination resulted in less inhibition than AZT alone caused (data not shown). Median-effect plots showed good correlation with the dose-effect data, with linear correlation coefficients (r) of ≥0.9 for each drug treatment condition except AZT alone and ddC alone in experiment 1, where r values were 0.76 and 0.84, respectively. Combination indices from both experiments, calculated at the 50, 75, 90, and 95% effective doses, all determined synergy of the combination (CI < 1) (Table 1).

In experiments 3 and 4, the AZT-sensitive clinical isolate, 14a-4/87, was used to infect HIV-1-seronegative PBMC. In both experiments, each single agent inhibited HIV-1 replication in a dose-dependent manner. The isolate demonstrated an AZT-sensitive phenotype (IC50s of 0.008 and 0.01 μM). At all drug concentrations tested in both experiments, the combination of AZT and ddC more effectively suppressed p24 antigen production than either agent alone. Representative data from experiment 4 are shown in Fig. 1. The median-effect curves generated from the raw dose-effect data had uniformly high correlation coefficients (r values). The combination indices indicated synergy (CI < 1) at the 50, 75, 90, and 95% levels of drug effectiveness in both experiments (Table 1).

In experiments 5 and 6, the AZT-resistant clinical isolate, 14a-6-89 (19), was used. IC50s for AZT in these experiments were 0.89 and >1 μM, respectively, i.e., 100- to 1,000-fold higher than in the experiments in which the AZT-sensitive isolate was used. In contrast, ddC showed similar efficacy against both the AZT-sensitive (IC50 = 0.034 and 0.063 μM) and the AZT-resistant (IC50 = 0.063 and 0.028 μM) isolates. As in experiments 3 and 4, the combination suppressed HIV-1 replication more effectively than either agent alone at all concentrations tested. Representative data from experiment 6 are shown in Fig. 2. Computer-generated median-effect curves showed good correlation with the actual dose-effect data for each of the drug treatment conditions. Again, CIs at 50, 75, 90, and 95% effective doses in both experiments were all <1, indicating synergy (Table 1).

Synergistic inhibition of HIV-1 replication by AZT and ddC was also demonstrated in the six experiments using computer-generated dose-oriented classical isobolograms (data not shown).

No cytotoxicity of the combination was seen; in each pair of experiments 1, 2, 3, and 4, the viable cell numbers were obtained when the combination was used. The combination was more effective at the 200- to 400-fold higher concentrations of AZT and ddC than either agent alone.
of experiments, mean viable cell numbers in uninfected drug-treated flasks were at least 87% of the mean viable cell numbers of the uninfected untreated controls.

The combination of ddC and AZT in this study synergistically inhibited HIV-1 replication of both HIV-1_{IIIB} and clinically derived AZT-sensitive and AZT-resistant HIV-1 paired isolates, as determined by the multiple-drug-effect analysis of Chou et al. (3–5). At all drug concentrations tested against the clinical isolates (as low as 0.0025 μM AZT and 0.02 μM ddC), more-effective suppression of HIV-1 replication was seen with the two agents together than with either agent alone. Synergy was seen against replication of the AZT-resistant isolate despite a highly resistant genotype and phenotype. Similar synergistic interactions were reported previously for AZT combined with ddI against these clinical isolates (19). The mechanism of synergy by AZT-containing regimens against phenotypically AZT-resistant virus may involve inhibition of AZT-sensitive viruses in the mixture of viruses in a clinical isolate (2, 19). An alternative mechanism of synergy is that one chain-terminating nucleoside analog may potentiate the effect of another by stereoisomerically altering the agents’ binding to reverse transcriptase, resulting in more-complete viral inhibition within infected cells.

Synergy between AZT and ddC against replication of the laboratory strain, HIV-1_{IIIB}, in H9 cells was also seen. Additive to subsynergistic inhibition of HIV-1_{IIIB} replication by the combinatory drug had been reported previously, when different cell types, methods of virus quantitation, and data analysis were used (1). Our current study demonstrated synergistic interactions against the replication of HIV-1_{IIIB} at moderate to high levels of drug effectiveness. However, at suboptimal levels of effectiveness of the single agents in H9 cells (specifically when ddC alone had no effect), the combination’s benefit was variable compared with that of AZT alone. This phenomenon was not seen with either of the clinical isolates tested in PBMC, which may be a more clinically relevant cell type than the continuous H9 cell line.

The combination of AZT and ddC offers several advantages over monotherapy if the synergistic effect demonstrated here also occurs in vivo. Patients on long-term AZT monotherapy, particularly those with advanced disease, are likely to have isolates that exhibit some degree of AZT resistance (30). Our studies suggest that the addition of ddC would not only offer the antiretroviral effect of ddC, as cross-resistant isolates have yet to be identified, but would also potentiate the antiretroviral effect of AZT. More-complete suppression of sensitive virus would be expected as well. Patients who initiate therapy with a combination of agents may develop resistance more slowly, although this concept remains to be proven. Fischl et al. (12) offered preliminary evidence that resistance may develop more slowly in a cohort treated with AZT and a second synergic agent, alpha interferon (14), than in patients treated with AZT alone (30).

Finally, the combination of AZT and ddC may be particularly useful for therapy of HIV-1 infection because the toxicities of the agents do not appear to overlap substantially (23). The concentrations used in our study are achieved in human plasma after oral administration (33).

One preliminary trial suggested that the combination of AZT and ddC given to patients with advanced HIV-1 disease produced a greater and more sustained increase in mean CD4+ lymphocyte counts than that observed in historical controls treated with AZT alone (23). Large controlled clinical trials to further define the role of this combination in the management of HIV-1 infections are currently under way.

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