Lamivudine or Stavudine in Two- and Three-Drug Combinations against Human Immunodeficiency Virus Type 1 Replication In Vitro

Debra P. Merrill, Mona Moonis, Ting-Chao Chou, and Martin S. Hirsch

Two- and three-drug combinations of lamivudine or stavudine with other antiretroviral drugs were evaluated for activity against human immunodeficiency virus type 1 (HIV-1) activity in peripheral blood mononuclear cells. Other agents included zidovudine, didanosine, nevirapine, and saquinavir. Paired zidovudine-sensitive and -resistant clinical HIV-1 isolates were used. Additive or synergistic interactions were observed against the zidovudine-sensitive isolate with the following combinations: lamivudine-zidovudine, lamivudine-stavudine, lamivudine-saquinavir, lamivudine-nevirapine, stavudine-zidovudine, stavudine-didanosine, stavudine-saquinavir, stavudine-nevirapine, lamivudine-zidovudine-saquinavir, lamivudine-zidovudine-stavudine, stavudine-zidovudine-nevirapine, lamivudine-zidovudine-nevirapine, and stavudine-zidovudine-saquinavir. Against the zidovudine-resistant isolate, additive or synergistic interactions were seen with most two- and three-drug combinations, but the combination of stavudine-zidovudine was antagonistic. The clinical implications of these in vitro observations should be explored.

The evolution of drug-resistant viruses is a significant issue in the development of antiretroviral agents. Monotherapy with selective human immunodeficiency virus type 1 (HIV-1) inhibitors has been limited by the appearance of drug-resistant variants during therapy [1–3]. This was first observed in persons receiving zidovudine long term [4]. Viruses with reduced sensitivity have also emerged during therapy with didanosine (ddl) [5], zalcitabine [6, 7], nonnucleoside reverse transcriptase inhibitors [8, 9], and protease inhibitors [10].

Because of the rapid turnover of HIV and the consequent emergence of resistant mutants following monotherapy, combination chemotherapy has been actively investigated [11, 12]. Advantages of combination chemotherapy may include additive or synergistic interactions among combined agents, which may allow lower doses of individual drugs with reduced toxicity. Combinations may also target different cell or tissue reservoirs (e.g., lymphocytes vs. macrophages, lymphoid system vs. nervous system), or cells at different stages of activation [11–14].

Stavudine (2'-3'-didehydro-3'-deoxythymidine [d4T]) and lamivudine (2'-3'-dideoxy-3'-thiacytidine [3TC]) are second-generation nucleoside analogs being studied in HIV-1–infected persons [15–17]. The triphosphate derivatives of these agents inhibit HIV-1 reverse transcriptase, which accounts for their antiviral activity [18, 19]. However, rapid selection of resistant virus has been observed with lamivudine in vitro [20] and in vivo [21–23]. A cohort of lamivudine-treated patients, a sharp decline in virus load (1–2 logs) was seen within a week, followed by a rise that remained below baseline [24]. Lamivudine resistance appears to be associated with a mutation at reverse transcriptase codon 184, which changes the amino acids from methionine to valine or isoleucine [25]. When this mutation was introduced into a recombinant zidovudine-resistant virus, a decreased resistance to zidovudine was observed [20]. Early clinical trials suggested a benefit of combining lamivudine with zidovudine, in either zidovudine-experienced or -naive patients with HIV-1 infection [21–23].

Stavudine is a potent inhibitor of HIV-1 replication in vitro [26, 27] and has been approved in the United States for use in patients with advanced HIV-1 infection who either failed to respond to or were intolerant of therapy with standard drugs or had contraindications to such therapy. In a recent study, drug susceptibility assays on matched pairs of clinical isolates derived from patients undergoing stavudine therapy showed that 9 of 11 patients remained stavudine-susceptible after 18–22 months of therapy [28].

We and others have demonstrated synergistic interactions in vitro between zidovudine and agents that disrupt the HIV-1 replicative cycle by similar or different mechanisms [29–32]. Early clinical trials also suggest virologic and immunologic benefit for patients using these regimens [11, 12, 33, 34]. Several other clinical trials of combination therapy are in progress.

In view of the therapeutic potential of stavudine and lamivudine, we evaluated several two- and three-drug combinations of these inhibitors with each other and with other antiretroviral
compounds, including zidovudine, ddi, nevirapine, and saquinavir, in blocking the spread of HIV-1 infection in peripheral blood mononuclear cell (PBMC) cultures. We used zidovudine-sensitive and zidovudine-resistant clinical HIV-1 isolates from the same person.

Materials and Methods

Cells. PBMC from HIV-1-seronegative donors were obtained by Ficoll-Hypaque density gradient centrifugation of heparinized venous blood. PBMC were treated with phytohemagglutinin (PHA-P, 2 μg/mL; Difco, Detroit), propagated in R-20 medium (RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum [Sigma, St. Louis], 50 U penicillin/mL, 50 μg streptomycin/mL, 2 mM l-glutamine, and 10 mM HEPES buffer), supplemented with 10% interleukin-2 (Pharmacia Diagnostics, Silver Spring, MD), and incubated at 37°C in a humidified atmosphere in 5% CO₂.

Virus. The HIV-1 isolates, which were derived from an HIV-1-seropositive person before (14a-pre) or after (14a-post) 26 months of uninterrupted zidovudine monotherapy, were propa-

![Figure 1](image)

Figure 1. Inhibition of HIV-1 p24 antigen production in acutely infected peripheral blood mononuclear cells treated with lamivudine (3TC) and zidovudine (AZT; A), stavudine (d4T; B), saquinavir (Ro31-8959 and Ro31; C), or nevirapine (Nev; D) alone or in 2-drug combinations (COMBO). AZT-sensitive clinical isolate 14a-pre was used. Data are for day 4 in culture. Untreated infected control (INF CTRL) is also shown. Similar data were obtained when adjusted for viable cell numbers.
Lamivudine or Stavudine against HIV-1

Trandolirine or Stavudine against HIV-1

Each drug or combination of drugs was tested in duplicate, and each experiment was repeated at least once. In addition, uninfected drug-treated toxicity controls were maintained at the highest concentration of each agent studied (either alone or in two- or three-drug combination regimens).

Cell-free culture supernatant fluids were harvested for HIV-1 p24 antigen production on day 3 or 4 of culture and again on day 7. Cell proliferation and viability were assessed by the trypan blue dye exclusion method.

Results

Both lamivudine and stavudine inhibited p24 antigen production by HIV-1 in PBMC, with ED50 ranging from 0.07 to 0.2 μM for lamivudine and 0.04 to 0.2 μM for stavudine. There was no toxicity in uninfected PBMC with concentrations of lamivudine up to 10 μM or stavudine up to 1.0 μM over a 10-day culture period, as assessed by cell viability and proliferation. There was no combination toxicity in uninfected PBMC at the highest doses of each combination tested over 7 days.

Lamivudine and stavudine were used either alone or in combination with various other agents over a wide range of concentrations against the zidovudine-sensitive isolate, 14a-pre. Representative experiments of lamivudine in combination with other agents are shown in figure 1A-D. Lamivudine was more effective in suppressing p24 antigen when in combination than as a single agent, particularly when drug concentrations were increased to ED50 or greater.

Additive or synergistic interactions between lamivudine (0.05–0.4 μM), zidovudine (0.0025–0.02 μM), stavudine (0.05–0.4 μM), saquinavir (0.00375–0.06 μM), and nevirapine (0.03–0.12 μM) occurred in two-drug regimens under conditions of high virus infection with 14a-pre (table 1). The CIs were < 1.0 at ED75–ED50 for all lamivudine combination drug regimens.

Figure 2A–D shows results from experiments with stavudine in two-drug combinations with zidovudine (0.0025–0.02 μM), ddI (0.2–1.6 μM), saquinavir (0.0075–0.06 μM), and nevira-

Table 1: Representative combination index values (CIs) for two-drug combination regimens against HIV-1 replication in peripheral blood mononuclear cells, as assessed using zidovudine-sensitive clinical isolate 14a-pre derived from an HIV-1-seropositive person prior to therapy.

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>Day in culture</th>
<th>CIs at % of HIV-1 inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ED50</td>
</tr>
<tr>
<td>3TC/AZT</td>
<td>4</td>
<td>0.73, 1.00</td>
</tr>
<tr>
<td>3TC/Ro-31-8959</td>
<td>4, 7</td>
<td>0.69, 0.82</td>
</tr>
<tr>
<td>3TC/Nev</td>
<td>4</td>
<td>1.30, 1.09</td>
</tr>
<tr>
<td>3TC/ddT</td>
<td>4</td>
<td>0.73, 0.98</td>
</tr>
</tbody>
</table>

NOTE: CIs of <1, 1, and >1 indicate synergism, additive effects, and antagonism, respectively. CIs were determined from median effect plot parameters m (slope) and Dm (ED50) of each drug and each combination based on classic isobologram equation as described previously [36–38]. Data were corrected for viable cell numbers. 3TC, lamivudine; AZT, zidovudine; Ro-31-8959, saquinavir; Nev, nevirapine; ddT, stavudine.
Figure 2. Inhibition of HIV-1 p24 antigen production in acutely infected peripheral blood mononuclear cells treated with stavudine (d4T) and zidovudine (AZT; A), didanosine (ddI; B), saquinavir (Ro31-8959 and Ro31; C), or nevirapine (Nev; D) alone or in 2-drug combinations (COMBO). AZT-sensitive clinical isolate 14a-pre was used. Data are for day 3 or 4 in culture. Untreated infected control (INF CTRL) is also shown. Similar data were obtained when adjusted for viable cell numbers.

Lamivudine (0.015–0.12 μM) against 14a-pre. More complete viral suppression was achieved by each of the two-drug regimens than with each of the agents used singly. Additive or synergistic interactions occurred between stavudine and the other drugs tested (i.e., zidovudine, ddI, saquinavir, and nevirapine), as illustrated by the CIs at ED50–ED95 (<1.0) for all combination regimens tested (table 2). Similar trends were observed when lamivudine and stavudine were used in three-drug regimens against 14a-pre (table 3, figure 3). The combinations of lamivudine-zidovudine-saquinavir, lamivudine-zidovudine-stavudine, lamivudine-zidovudine-nevirapine, stavudine-zidovudine-nevirapine, and stavudine-zidovudine-saquinavir were additive or synergistic (CIs < 1.0) at ED50 and ED95.

To extend these findings, we also evaluated combination therapies against the replication of the paired zidovudine-resistant isolate 14a-post. The interaction of lamivudine and zidovudine was synergistic against 14a-post (CIs < 0.4 at ED50 and ED95, respectively) (table 4, figure 4). In contrast, the interaction between
stavudine and zidovudine was antagonistic against 14a-post (CIs >1.0 at ED$_{50}$–ED$_{95}$) (table 4, figure 4). Additive and synergistic interactions were observed for the other two-drug combinations tested, lamivudine-stavudine, stavudine-nevirapine, lamivudine-nevirapine, lamivudine-saquinavir, and stavudine-saquinavir against 14a-post (CIs <1.0 at ED$_{50}$–ED$_{95}$).

Lamivudine and stavudine were also tested in three-drug regimens against the zidovudine-resistant isolate 14a-post (table 4). The triple-drug combination of lamivudine-saquinavir-nevirapine was synergistic (CIs <0.9 at ED$_{50}$ and ED$_{95}$), whereas the combination of lamivudine-saquinavir-stavudine was only additive to synergistic at high-effect levels (ED$_{50}$ and ED$_{95}$; CIs, 0.76–1.05; table 4).

### Discussion

In the present study, we demonstrated additive or synergistic interactions between lamivudine or stavudine in combination with zidovudine, saquinavir, ddI, and nevirapine in vitro as either two- or three-drug regimens against a zidovudine-sensitive HIV-1 clinical isolate. Interactions were more complex against a paired zidovudine-resistant HIV-1 clinical isolate, ranging from synergy to antagonism.

Synergistic interactions were observed between stavudine-ddI, stavudine-saquinavir, and stavudine-nevirapine against the zidovudine-sensitive HIV-1 clinical isolate. Of particular interest is the synergism seen between the combination of stavudine and saquinavir, since protease inhibitors are showing increased promise as anti-HIV-1 agents in vivo as well as in vitro [40, 41]. In our studies, the concentrations of the drugs tested (i.e., lamivudine, 0.05–0.4 μM; stavudine, 0.05–0.4 μM; zidovudine, 0.0025–1.0 μM; ddI, 0.625–5.0 μM; saquinavir, 0.00375–0.06 μM; and nevirapine, 0.03–0.24 μM) are easily achievable in patients [15, 27, 42, 43]. The concentration of each agent required to inhibit HIV-1 replication varied depending upon the kinetics of infection, ability of the clinical isolate to infect PBMC from a particular donor, and duration of the experiment.

Of some concern is the in vitro antagonism between stavudine and zidovudine against the zidovudine-resistant isolate,
Figure 3. Inhibition of HIV-1 p24 antigen production in untreated acutely infected peripheral blood mononuclear cells treated with (A) lamivudine (3TC), zidovudine (AZT), and nevirapine (Nev) or (B) stavudine (d4T), AZT, and saquinavir (Ro31-8959 and Ro31) alone or in 3-drug combinations (COMBO). AZT-sensitive clinical isolate 14a-pre was used. Data are for day 4 in culture. Uninfected control (INF CTRL) is also shown. Similar data were obtained when adjusted for viable cell numbers.
14a-post. The mechanisms involved in these less favorable interactions are unclear but may involve decreased phosphorylation of stavudine in the presence of the higher concentrations of zidovudine required in experiments with zidovudine-resistant HIV-1. Other studies have shown that zidovudine inhibits phosphorylation of stavudine [18]. If this is a dose-dependent process, then the low concentrations of zidovudine required for sensitive virus may be insufficient to yield this competition, whereas higher concentrations may inhibit stavudine phosphorylation. Experiments to address these points are underway. In any case, the variability in susceptibility among isolates may partly explain differences reported by different laboratories when investigating zidovudine-stavudine interactions [19, 44]. Studies of zidovudine-stavudine in the clinic are underway, although both analogs compete for cellular thymidine kinase, and zidovudine is preferentially phosphorylated over stavudine [19, 45]. The clinical implications of these observations may be decreased intracellular concentrations of stavudine-triphosphate. Thus, the concurrent use of stavudine and zidovudine in patients with extensive zidovudine experience should be explored cautiously.

Lamivudine has anti-HIV-1 activity against a wide spectrum of isolates, including zidovudine-resistant strains in several cell lines with IC₅₀ ranging from 0.003 to 1.14 μM [46, 47]. Recent clinical results have shown sustained improvement in immunologic and virologic markers after zidovudine-lamivudine combination therapy [21, 23]. Since almost all viruses in the combination group had developed the lamivudine resistance mutation at codon 184 by 24 weeks, the sustained virus load difference may reflect the lack of zidovudine resistance in this arm [22].

When lamivudine was tested in combination with various agents (i.e., stavudine, zidovudine, nevirapine, and saquinavir), synergistic interactions occurred at ED₉₀ and ED₉₅ against the zidovudine-sensitive isolate 14a-pre. In vitro synergism between lamivudine and stavudine against this isolate suggests a possible clinical use of this combination in vivo. Whether resistance to this combination develops in vivo is unknown, although clinical trials are underway. Strong synergy (CIs < 0.5) was also observed between lamivudine and zidovudine against the zidovudine-resistant isolate 14a-post. Although lamivudine-induced mutations at codon 184 emerge rapidly in vitro and in vivo and can suppress zidovudine resistance [20], it is unlikely that this was a significant factor in our short-term experiments. The combination of lamivudine-saquinarvir-nevirapine was also synergistic against the zidovudine-resistant isolate, and this combination deserves clinical evaluation in patients who have received prolonged zidovudine therapy. On the basis of our studies, other three-drug combinations deserving clinical evaluation include lamivudine-zidovudine-stavudine, lamivudine-zidovudine-nevirapine, lamivudine-zidovudine-saquinarvir, and stavudine-zidovudine-nevirapine. In support of our in vitro observations, early clinical trials have suggested that during HIV-1 infection, two-drug combination regimens are more effective than single drugs, and three drugs are better than two in suppressing HIV-1 replication and maintaining increases in CD4 cell counts [11, 33, 34].

Recent studies by Ho et al. [48] and Wei et al. [49] demonstrate that HIV-1 infection is an extremely dynamic process involving continuous rounds of replication and rapid cell turnover. As a consequence of the high turnover of HIV-1, emergence of drug-resistant mutants may be augmented during therapy [50, 51]. In addition, the complete replacement of wild type virus by drug-resistant mutants in plasma after only 2–4 weeks of drug therapy with certain agents suggests the enormous potential of HIV-1 to evolve under selection pressure [48, 49, 50]. Results of these studies suggest that early interven-

### Table 4. Representative combination index values (CIs) for two- and three-drug combination regimens against HIV-1 replication in peripheral blood mononuclear cells, as assessed using zidovudine-resistant clinical isolate 14a-post derived from an HIV-1-seropositive person after therapy.

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>Day in culture</th>
<th>ED₉₀ (%)</th>
<th>ED₅₀ (%)</th>
<th>ED₉₅ (%)</th>
<th>ED₅₅ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3TC/AZT</td>
<td>4</td>
<td>0.59</td>
<td>0.30</td>
<td>0.69</td>
<td>0.60</td>
</tr>
<tr>
<td>3TC/d4T</td>
<td>4</td>
<td>0.72</td>
<td>0.59</td>
<td>0.69</td>
<td>0.60</td>
</tr>
<tr>
<td>d4T/AZT</td>
<td>4</td>
<td>1.33</td>
<td>1.08</td>
<td>1.29</td>
<td>1.22</td>
</tr>
<tr>
<td>d4T/Nev</td>
<td>4</td>
<td>1.30</td>
<td>1.08</td>
<td>1.29</td>
<td>1.22</td>
</tr>
<tr>
<td>3TC/Nev</td>
<td>4</td>
<td>1.00</td>
<td>0.75</td>
<td>1.04</td>
<td>1.01</td>
</tr>
<tr>
<td>3TC/Ro-31-8959</td>
<td>7</td>
<td>1.07</td>
<td>0.81</td>
<td>1.05</td>
<td>1.02</td>
</tr>
<tr>
<td>d4T/Ro-31-8959</td>
<td>4</td>
<td>0.84</td>
<td>0.75</td>
<td>0.96</td>
<td>0.93</td>
</tr>
<tr>
<td>3TC/Ro-31-8959/d4T</td>
<td>3, 4</td>
<td>1.12</td>
<td>1.08</td>
<td>1.04</td>
<td>1.02</td>
</tr>
<tr>
<td>3TC/Ro-31-8959/Nev</td>
<td>4</td>
<td>0.51</td>
<td>0.46</td>
<td>0.69</td>
<td>0.73</td>
</tr>
</tbody>
</table>

NOTE. CIs of <1, 1, and >1 indicate synergism, additive effects, and antagonism, respectively. CIs were determined from median effect plot parameters m (slope) and Dm (ED₉₀) of each drug and each combination based on classic isobologram equation as described previously [36–38]. Data were corrected for viable cell numbers. 3TC, lamivudine; AZT, zidovudine; d4T, stavudine; Nev, nevirapine; Ro-31-8959, saquinavir.
Figure 4. Inhibition of HIV-1 p24 antigen production in acutely infected peripheral blood mononuclear cells treated with (A) lamivudine (3TC) or stavudine (d4T), (B) 3TC and zidovudine (AZT), or (C) d4T and AZT. AZT-resistant clinical isolate 14a-post was used. Untreated infected control (INF CTRL) is also shown. Data are for day 4 or 7 in culture. Similar data were obtained when adjusted for viable cell numbers. COMBO = drug combinations.

In summary, our results suggest a good rationale for combined antiviral therapy with either stavudine or lamivudine in suppressing HIV-1 infection. These preliminary results underscore the need for further in vitro studies and subsequent clinical trials of promising and less toxic combination therapies.

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