Sequential versus Simultaneous Combination Antiretroviral Regimens for the Treatment of Human Immunodeficiency Virus Type 1 Infection In Vitro

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Two-, three-, and four-drug antiretroviral combinations in either simultaneous or sequential regimens were evaluated for their ability to suppress human immunodeficiency virus (HIV) type 1 replication in vitro. Zidovudine, lamivudine, saquinavir, and nevirapine were used at IC_{90}, IC_{99}, or IC_{999} in a CD4-positive human lymphoblastoid cell line (H9 cells) acutely infected with HIV-1. In sequential regimens, drugs were added at weekly intervals. In simultaneous regimens, all drugs were added on day 0. Increasing the number of drugs in a combination regimen both increased the degree of viral inhibition and delayed the time of breakthrough viral replication. Simultaneous regimens provided more profound and earlier viral inhibition than did sequential regimens. However, sequential addition provided relatively more durable viral inhibition than did simultaneous regimens when drug concentrations were low. The relative effectiveness of different HIV-1 therapeutic strategies depends on both the numbers and concentrations of the drugs used.

Materials and Methods

Cells. H9 cells, a CD4-positive human lymphoblastoid cell line derived from peripheral blood of a patient with Sézary syndrome [5], were provided by the National Cancer Institute (NIH, Bethesda, MD) and maintained in R20 medium (RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum [Sigma, St. Louis], 50 U of penicillin/mL, 50 µ g of streptomycin/mL, 2 mM L-glutamine, and 10 mM HEPES) and incubated at 37°C in a humidified atmosphere in the presence of 5% CO₂.

Virus. The HIV-1 isolate used (HIV-1 14a-PRE), derived from an HIV-1 seropositive person before any antiretroviral treatment, was propagated in H9 cells and titrated in C8166 cells to determine the TCID_{50} of HIV-1 per milliliter of virus stock, as previously described [6].

Compounds. Zidovudine and lamivudine were obtained from Glaxo Wellcome (Research Triangle Park, NC); saquinavir was obtained from Roche Products (Welwyn Garden City, UK); and nevirapine was obtained from Boehringer Ingelheim Pharmaceuticals ( Ridgefield, CT). These drugs were used at IC_{90}, IC_{99}, and IC_{999}.

Viral replication assay. Cell-free culture supernatants were assayed by an HIV-1 p24 antigen ELISA (DuPont, Boston).

Experimental design. Uninfected H9 cells (0.8 × 10⁶ cells) were suspended in 2 mL of R20 medium in 24-well tissue culture plates in triplicate. Simultaneously, drugs and a virus inoculum of either 4000 or 40,000 TCID_{50} (MOI = 0.005 or 0.05, respectively) were added to the wells, without a subsequent wash. Wells containing medium plus virus only were also maintained in parallel as virus input controls. In all experiments, sequential regimens were compared with simultaneous regimens. In sequential regimen experiments, the order of addition of new drugs at weekly intervals was zidovudine, lamivudine, saquinavir, and nevirapine (figure 1). All drugs (2, 3, or 4) in simultaneous treatment regimens were added on day 0. In all experiments, culture medium was changed twice weekly so that 0.5 mL of the cell suspension was resuspended in 1.5 mL of fresh medium that contained the original drug concentrations. Uninfected cells were not added at any time once experiments were underway.
Supernatants were harvested and assayed for HIV-1 by a p24 antigen ELISA. Cultures were maintained for up to 143 days. Cell proliferation and viability were assayed by a trypan blue dye exclusion on uninfected, drug-treated H9 cells, maintained in parallel.

**Drug susceptibility assay.** The drug susceptibility of breakthrough viruses was determined as previously described [7]. The infectivity titer of a culture supernatant was determined by using a streamlined end point dilution technique and was analyzed by the Spearman-Karber statistical method [8]. After the titer of virus stock was determined, 5000 TCID<sub>50</sub> of virus stock was used to infect 10<sup>5</sup> H9 cells, which were either drug-free or treated with one of the drugs used. The ranges of drug concentrations used were as follows: zidovudine, 0.16–10.0 μM; lamivudine, 0.5–32.0 μM; saquinavir, 0.01–0.64 μM; nevirapine, 0.06–4.0 μM. The drug concentration required to inhibit p24 antigen production by 50% versus the drug-free cultures was determined by a uniform method and was calculated by use of the median effect equation [9].

**Results**

In all experiments, increasing the number of drugs in a combination regimen both increased the degree of viral inhibition and delayed the time of breakthrough viral replication.

In experiments using drugs at IC<sub>90</sub> concentrations (figure 2A, B), neither sequential nor simultaneous 2-drug combination regimens were effective in inhibiting HIV-1 replication. In 3-drug combinations, the sequential regimen provided minimal but more durable suppression of HIV-1 replication than did the simultaneous regimen. In 4-drug combinations, the simultaneous regimen was able to suppress viral replication to undetectable levels by day 31, but breakthrough replication developed soon after. In contrast, the sequential 4-drug regimen suppressed viral replication up to day 100, although the degree of initial suppression was less than that in the simultaneous regimen. These observations were reproducible in at least 2 experiments (data not shown).

When higher concentrations (IC<sub>99</sub>) of drugs were used (figure 2C, D), 4-drug combinations, added either sequentially or simultaneously, eliminated detectable HIV-1 for up to 110 days in culture. Under these circumstances, simultaneous regimens eliminated viral replication 20 days earlier than did sequential regimens. However, neither 2- nor 3-drug sequential nor simultaneous regimens eliminated viral replication. Although the 3-drug simultaneous regimen resulted in earlier and more profound suppression initially, sequential regimens provided more durable viral inhibition overall (>1 log suppression for >70 days, compared with 30–40 days for simultaneous regimens).

Breakthrough viruses emerging during 2-drug combination therapy developed phenotypic resistance to lamivudine as early as day 21. The breakthrough viruses emerging during simultaneous 3-drug combinations at IC<sub>99</sub> were susceptible to all 3 drugs used, whereas the breakthrough viruses emerging during the other 3-drug combination treatments (i.e., either simultaneous or sequential 3-drug combinations at IC<sub>90</sub> and sequential 3-drug combinations at IC<sub>99</sub>) developed phenotypic resistance to lamivudine. Lamivudine IC<sub>50</sub>s for the breakthrough viruses were ~1.5 log higher than those for untreated control virus. Viruses that showed breakthrough replication against 4-drug combinations, in either simultaneous or sequential regimens, were still susceptible to all of the drugs used.

In experiments with low MOI (0.005) and IC<sub>99</sub> (figure 2E, F), all but the 2-drug sequential regimens were able to eliminate HIV-1 replication for up to 140 days in culture. However, when infections with higher virus input (MOI of 0.05) were treated with the same concentrations of drugs, 2- and 3-drug sequential regimens failed to durably suppress viral replication, whereas 4-drug sequential and all simultaneous regimens were able to completely suppress viral replication (figure 2G, H).

In all of the experimental conditions in which drug combinations eliminated detectable p24 antigen, cultures were maintained in the absence of drugs for an additional 14 days. They remained negative for p24 antigen for the duration of the experiments.

There was no evidence of drug toxicity as measured by cell viability and proliferation of treated, but uninfected, cells maintained in parallel.
Figure 2. Inhibition of HIV-1 p24 antigen production in acutely infected H9 cells treated with combinations of 2 (■), 3 (▲), and 4 (▲) drugs in either sequential (A, C, E, G) or simultaneous (B, D, F, H) regimens. ●, untreated control. H9 cells were infected at MOIs of 0.005 or 0.05. Zidovudine, lamivudine, saquinavir, and nevirapine, at IC₉₀, IC₉₉, or IC₂₅₀, were used to treat infection. In sequential regimen experiments, order of addition of new drugs at weekly intervals was zidovudine, lamivudine, saquinavir, and nevirapine. All (2, 3, or 4) drugs in simultaneous treatment regimens were added on day 0.

Discussion

Laboratory and clinical data increasingly support the concept of multidrug antiretroviral combinations for the treatment of HIV-1 infection. Our results suggest that if favorable interactions occur among individual components of a drug combination regimen, control of HIV-1 replication is more effective as the number of drugs in combination increases. In either sequential or simultaneous regimens, 4-drug combinations provided more profound inhibition of viral replication than did 3-drug combinations, which
resulted in greater suppression than did 2-drug combinations. Increasing the number of drugs in a combination also provided more durable suppression of viral replication. These findings support previous in vitro studies [1, 6, 10]. Recent clinical trials have also shown that 2-drug combination regimens may be superior to single drugs and that 3-drug regimens provide greater and more sustained increases in CD4 cell counts and decreases in virus load than do 2-drug regimens [11].

Simultaneous combinations provided more rapid and complete suppression of HIV-1 replication than did regimens using sequential administration of drugs. When relatively high concentrations (IC90) of drugs were used, 4-drug combinations in either simultaneous or sequential regimens were effective in eliminating HIV-1 replication for up to 110 days in culture (figure 2C, D). In this circumstance, simultaneous regimens eliminated viral replication more rapidly than did sequential regimens. Simultaneous combinations of 3 drugs also caused earlier and more profound initial suppression than did sequential 3-drug regimens, even though neither sequential nor simultaneous 3-drug regimens could completely eliminate viral replication.

Although sequential addition of drugs might reduce acute toxicity and allow easier identification of those drugs causing toxicity, the addition of individual drugs to a regimen over time could result in suboptimal virus suppression and selection of resistant viruses. However, in our study, when relatively low concentrations of individual drugs (IC50) were used, sequential regimens provided more durable virus suppression than did simultaneous regimens. Although the results obtained may be a function of the particular culture system we used, it is possible that stepwise decreases in virus load could be achieved without selecting for resistant viruses by sequential regimens in which study drugs are added at weekly intervals. For example, virus load as detected by p24 antigen measurement was 1 log lower at day 60 in sequential versus simultaneous 4-drug regimens (figure 2A, B). Thus, there may be some rationale for staggering onset of combination components over a short time period prior to the emergence of resistant virus. This, however, may depend on the identity and order of the drugs selected and should not be generalized from our limited studies.

In some cultures, lamivudine resistance was observed as early as day 21. It is of note that a multidrug-resistant HIV-1 isolate did not emerge even though the viruses were treated with 3 or 4 drugs for >100 days. One possible explanation for this favorable outcome may be the suppression of zidovudine resistance by lamivudine-induced mutations at codon 184 [12].

The viruses that broke through despite either simultaneous or sequential 4-drug combinations at IC90 or 3-drug simultaneous regimens at IC50 remained susceptible to all of the drugs used. This observation indicates that mechanisms other than viral resistance may account for virus breakthrough during therapy, as in vitro observation also noted by others [13]. This phenomenon has also been reported in patients treated with zidovudine and other agents [14]. Changes in cellular enzymes that phosphorylate individual nucleoside analogs have been hypothesized as a possible explanation for the rebound [14].

We were able to eliminate detectable HIV-1 replication from cultures by use of several combination regimens. All of the effective drug concentrations in this study can be achieved clinically. However, our studies do not necessarily indicate that HIV-1 infection can be eradicated with these regimens. Virus inocula in our experiments, ranging from 4000 to 40,000 TCID50, were orders of magnitude lower than plasma virus levels and turnover observed in vivo [15]. In addition, there are no isolated drug sanctuaries in our in vitro acute infection model. Moreover, we did not evaluate our “virus-free” cells for the presence of latent genomes by sensitive molecular assays, although they failed to produce p24 antigen even in the absence of drug.

In summary, our results suggest that simultaneous antiretroviral regimens are most advantageous when used at high effective concentrations relative to virus burden and provide more rapid and more profound inhibition than do sequential regimens. However, when drug concentrations are low relative to virus burden, certain sequential regimens, in which new drugs are added at frequent intervals and before emergence of resistant virus, may be useful. Only by carefully controlled clinical trials can the in vivo validity of these hypotheses be established.

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References

Variance of Plasma Human Immunodeficiency Virus Type 1 RNA Levels Measured by Branched DNA within and between Days

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The clinical utility of an assay depends, in large part, on its variability. In a clinically stable patient, plasma human immunodeficiency virus type 1 (HIV-1) RNA levels reflect a steady state, in which rates of virion clearance approximate rates of virion production. In patients with a baseline viral RNA level $>500$ copies/mL, plasma RNA levels have a week-to-week variability of $\sim 0.5 \log$. Factors influencing variation include inherent biologic changes and those introduced by the performance characteristics of the particular assay.

CD4 cell counts demonstrate significant within-patient variability, limiting their usefulness for monitoring a patient’s response to therapy [1, 2]. The cause of this variance is multifactorial but includes predictable diurnal fluctuations. Absolute levels of CD4 cells are lowest in the morning (8 A.M.) and highest in the late evening (10 P.M.). The impact of diurnal variation can be considerable, with counts increasing by as much as 100% during the day. Similar variation has been seen in healthy volunteers and HIV-infected adults [3–5]. Although the cause for this variation is not completely understood, diurnal fluctuations in plasma cortisol and an associated redistribution of CD4 T cells from lymphoid organs to the peripheral circulation or the demargination of CD4 T cells within the vasculature may be contributing factors [6, 7]. However, it remains unclear whether HIV-1 RNA levels exhibit a diurnal pattern. In one 8-patient study using an early polymerase chain reaction assay, levels did not vary significantly when assayed at 8 A.M. and 5 P.M. [8].

To better understand the performance characteristics of the branched DNA (bDNA) assay, and to explore whether HIV-1 RNA levels vary in a predictable manner over a 24-h period and from day to day, we performed repeated HIV-1 RNA testing on 11 HIV-infected adults. Given the rapid rate of clearance of HIV-