

Synergistic anti-human immunodeficiency virus type 1 effect of hydroxamate compounds with 2',3'-dideoxyinosine in infected resting human lymphocytes

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Communicated by D. H. R. Barton, August 4, 1994

ABSTRACT The cellular models generally used in the *in vitro* evaluation of anti-human immunodeficiency virus compounds are dividing cells. A model constituted by resting lymphocytes may more accurately reflect a drug's future efficacy in humans, since viral DNA synthesis is known to take place in quiescent cells, creating a reservoir of infected cells awaiting activation to complete their viral replication cycle and to produce infectious virions. We report here the activity of 3'-azido-3'-deoxythymidine, 2',3'-dideoxyinosine, 2',3'-dideoxycytidine, and two hydroxamates, D-aspartic acid β -hydroxamate and hydroxycarbamide (hydroxyurea), alone and in various combinations, in an *in vitro* model based on resting lymphocytes. In our model, resting peripheral blood lymphocytes were infected with human immunodeficiency virus type 1 and treated with drugs for 7 days, at which time drugs were removed and the cells were activated by phytohemagglutinin. We show that under these conditions 3'-azido-3'-deoxythymidine, 2',3'-dideoxyinosine, and 2',3'-dideoxycytidine, alone or in combination, neither fully inhibit viral production nor protect lymphocytes from the cytopathic effect of viral replication, at concentrations corresponding to the peak plasma levels observed in a typical treatment schedule in humans. In contrast, we report the synergistic effect of treatment by each hydroxamate with 2',3'-dideoxyinosine of infected resting lymphocytes, resulting in the total suppression of viral production, total protection against the cytopathic effect induced by viral replication, and no effect on the ability of the cells to replicate in this cell culture system.

Over the past several years, significant effort has been expended in developing compounds that interfere with replication of the human immunodeficiency virus (HIV) (1, 2), the causative agent of acquired immunodeficiency syndrome (AIDS) (3).

Development of an effective treatment will necessitate the suppression of viral replication in virus-producing cells. Established cultured cell lines and mitogen-stimulated human peripheral blood mononuclear cells have been used as models to study the antiviral activity of various anti-HIV drugs (4–9). However, it is far from clear that the sensitivity of HIV to drugs as assessed *in vitro* in long-term dividing cell lines and phytohemagglutinin (PHA)-stimulated mononuclear cells accurately reflects the virus–drug response in patients with HIV infection (10).

The currently available therapeutic agents, 3'-azido-3'-deoxythymidine (AZT), 2',3'-dideoxyinosine (ddI), and 2',3'-dideoxycytidine (ddC), which are active against HIV replication in the *in vitro* models referred to above, are only partially effective in suppressing viral replication in patients with AIDS (11, 12), and the benefits of early AZT interven-

tion yields no statistically significant or clinically important benefit in terms of survival or disease progression (13).

The fact that these compounds have been tested and selected on the basis of models that do not reflect the current state of knowledge of the physiopathology of HIV infection may help to explain their inadequacy in treatment. In general, HIV replicates more abundantly and rapidly in a population of dividing activated cells than in a population of nondividing resting cells. However, HIV proviral DNA synthesis in nondividing resting lymphocytes is initiated as efficiently as in activated lymphocytes, although the production of progeny virion requires further activation (14–17). Infected resting lymphocytes can be considered as transient viral reservoirs in infected individuals (18), and it is, therefore, of prime importance that these cells be targeted for antiretroviral therapy.

We report here the anti-HIV-1 activity of the dideoxynucleoside analogs (ddNs) AZT, ddI, and ddC and two hydroxamates (R-CONHOH), D-aspartic acid β -hydroxamate (DAH) and hydroxycarbamide [hydroxyurea (HU)], in infected resting peripheral blood lymphocytes (R-PBLs) *in vitro*. DAH has been described to be active against HIV-1 in PHA-activated PBLs (PHA-PBLs).^{§¶} More recently, HU has been shown (19) to inhibit HIV-1 DNA synthesis in PHA-PBLs. DAH has also been shown (20, ||) to have antiretroviral activity in Friend disease. One of the reasons for the antiviral activity of HU and DAH may be linked to the known inhibitory effect of HU (21) and of DAH (M. Lepoivre and M. Fontecave, personal communication) on ribonucleotide reductase. This enzyme is the key to the transformation of the

Abbreviations: AZT, 3'-azido-3'-deoxythymidine; DAH, D-aspartic acid β -hydroxamate; ddC, 2',3'-dideoxycytidine; ddI, 2',3'-dideoxyinosine; ddN, dideoxynucleoside analog; HU, hydroxyurea (hydroxycarbamide); HIV-1, human immunodeficiency virus type 1; IL-2, interleukin 2; PBL, peripheral blood lymphocyte; PHA, phytohemagglutinin; PHA-PBL, phytohemagglutinin-stimulated peripheral blood lymphocyte; R-PBL, resting peripheral blood lymphocyte; TCID₅₀, tissue culture 50% infective dose.

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four ribonucleotides to deoxyribonucleotides essential for viral DNA synthesis (21), and deoxyribonucleotides are natural competitors of ddNs for reverse transcription (22, 23). However, it should be kept in mind that hydroxamates are also inhibitors of cellular DNA synthesis occurring preferentially in the S phase of the cell cycle (24). Indeed, hydroxamates have been used as inhibitors of tumor cell growth (25–27).

For these reasons, we chose to test the hydroxamates alone and in various combinations with AZT, ddI, and ddC in HIV-1-infected R-PBLs, at hydroxamate doses at which neither cell viability nor cellular DNA synthesis after PHA activation is impaired. The concentration of 1 mM DAH is easily attainable in plasma, and chronic administration in the mouse is well tolerated (20). HU is a well-studied compound that has been widely used in clinical therapy. A concentration of 0.1 mM HU is well below that seen in cancer chemotherapy, and it is expected that at this concentration the known hematological side effects of higher doses may be avoided (25). The doses used for the ddNs correspond to peak plasma levels observed after oral administration in a typical treatment schedule (28).

MATERIALS AND METHODS

Cells. R-PBLs were purified from blood of healthy HIV-seronegative individuals by centrifugation over Ficoll/Hypaque. Monocyte/macrophages were depleted by adherence to plastic dishes overnight; nonadherent cells were recovered and incubated at 37°C and 5% CO₂/95% air in RPMI medium 1640 supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml). The presence of monocytes/macrophages and activated lymphocytes [bearing the interleukin 2 (IL-2) receptor] was monitored by fluorescence-activated cell sorting with anti-CD14 and anti-CD25 antibodies and indicated that the proportion of each was <1%.

Virus. The viral isolate used in our experiments was HIV-1 IIIB, obtained from 24-hr culture supernatants from a clone of chronically infected H9 cells. Infectivity of viral stocks was assayed by limiting dilution on cultures of PHA-PBLs. The number of tissue culture 50% infective doses (TCID₅₀) per ml of stock was calculated from the maximum dilution, according to the method of Reed and Muench (47), giving a HIV p24 gag-positive supernatant after 3 weeks of culture.

The amount of virus produced by the cells in our experiments was determined by p24 gag protein ELISA assay (DuPont). The quantitation of the p24 gag protein was compared to a positive p24 standard and gave a background of <2 pg of p24 per ml, which corresponds to <1 HIV TCID₅₀ per ml (1 TCID₅₀ HIV-1 IIIB = 5 pg of p24, based on limiting dilution experiments; S.D.M., unpublished data).

Experimental Procedure. R-PBLs were incubated with HIV-1 for 2 hr at 37°C with a multiplicity of infection of 10⁴ TCID₅₀ per 10⁶ cells. Unbound virus was then eliminated by two successive washes with culture medium, and the cells were seeded at a density of 10⁶ cells per ml, in the presence of the various drugs. On day 4, two-thirds of the medium was renewed, and fresh drugs were added to maintain the initial concentration. On day 7, cells were collected and sedimented by centrifugation, and the supernatant was kept for p24 assay. After two washes to remove traces of drug, cells of each culture were put in fresh medium containing PHA (PHA 16, Wellcome; 1 µg/ml) and recombinant IL-2 (Eurocetus, Amsterdam; 20 units/ml). These cultures were maintained for 3 weeks (days 7–28); on days 14, 21, and 28 half of the medium was taken for p24 assay and replaced by fresh medium containing recombinant IL-2 (20 units/ml).

The viability of cells at different time points was quantified by the MTT [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based colorimetric assay (29).

The determination of cellular DNA synthesis at different time points was performed by sampling and further culturing 2 × 10⁵ cells for 4 hr in fresh medium containing [³H]thymidine (5 µCi/ml; 1 Ci = 37 GBq). Incorporated radioactivity was precipitated with 5% (wt/vol) trichloroacetic acid, recovered onto glass-fiber filters, and measured in a scintillation spectrometer.

Drugs. DAH was synthesized and characterized in our laboratory as described (26). HU, AZT, ddI, and ddC were purchased from Sigma.

RESULTS

R-PBLs maintained in culture for 7 days showed a low level of cellular DNA synthesis. After PHA activation, a marked increase in [³H]thymidine uptake was observed on day 10 in infected and noninfected controls (Fig. 1). The p24 measured in the infected control on day 7, prior to PHA activation, showed a low level of viral production (see Fig. 3A), possibly due to the presence of a small proportion of activated lymphocytes in our culture (<1%). After activation, viral

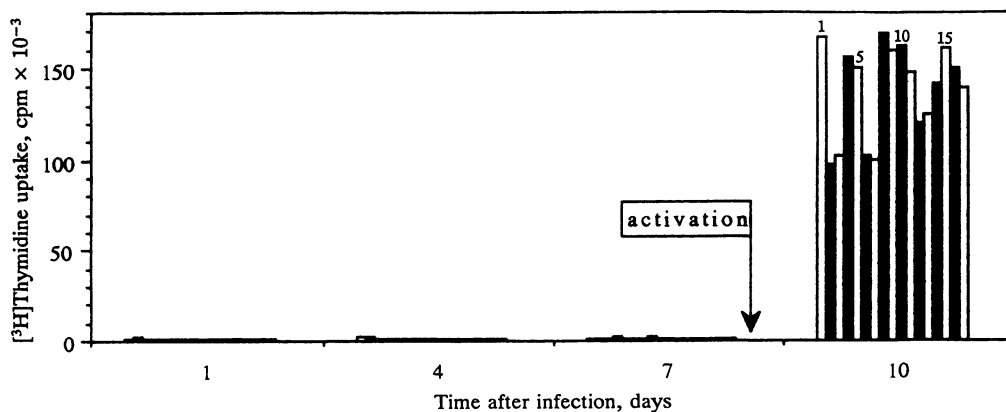


FIG. 1. Cellular DNA synthesis after activation of resting lymphocytes. Resting lymphocytes were infected with HIV-1 and then treated with various drugs at day 0. On day 7, drugs were removed, and the cells were activated with PHA in IL-2-containing medium. Cells were harvested, and DNA synthesis was assayed by [³H]thymidine incorporation, on days 1, 4, and 7 (i.e., before activation), and day 10 (i.e., 3 days after activation). Results are the average of triplicate determinations. Bars: 1, uninfected drug-free control cells; 2–17, HIV-1-infected cells. HIV-1-infected cells were treated with drugs as follows. Bars: 2, no drug; 3, AZT; 4, ddI; 5, ddC; 6, DAH; 7, HU; 8, AZT/ddI; 9, AZT/ddC; 10, ddI/ddC; 11, AZT/ddI/ddC; 12, AZT/DAH; 13, AZT/HU; 14, ddI/DAH; 15, ddI/HU; 16, ddC/DAH; 17, ddC/HU.

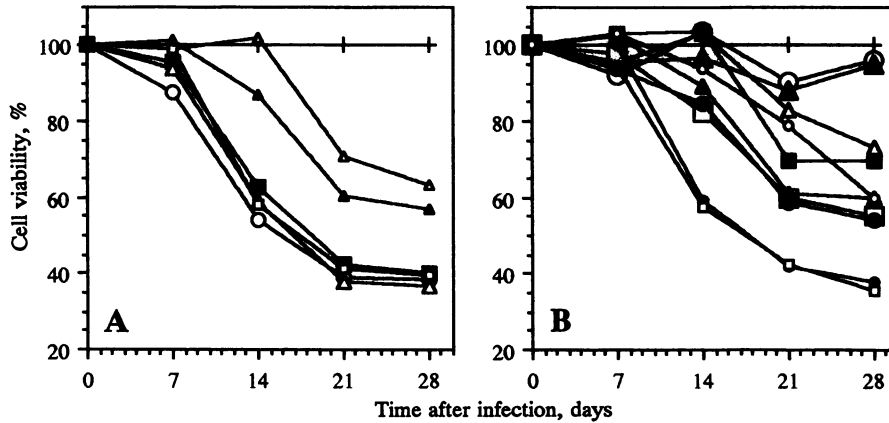


FIG. 2. Cell viability of HIV-1-infected resting lymphocytes after PHA activation. Resting lymphocytes were infected with HIV-1 and then treated with various drugs on day 0. On day 7, drugs were removed, and the cells were activated with PHA and then cultured up to day 28 in drug-free IL-2-containing growth medium. Cell viability was determined by the MTT method. Results are expressed as a percentage of the drug-free uninfected cells at each time point and are the average of duplicate determinations. (A) +, Uninfected drug-free control cells. HIV-1-infected cells were treated with drugs as follows: ■, no drug; □, AZT; △, ddI; ▲, ddC; ○, DAH; △, HU. (B) +, Uninfected drug-free control cells. HIV-1-infected cells were treated with drugs as follows: ■, AZT/ddI; □, AZT/ddC; ○, ddI/ddC; △, AZT/ddI/ddC; □, AZT/DAH; ●, AZT/HU; ▲, ddI/DAH; ○, ddI/HU; ●, ddC/DAH; ▲, ddC/HU.

production increased markedly, peaking at day 14 concomitant with a 37% loss of cell viability, which rose to >59% on day 28 (Fig. 2A). Virus infectivity was confirmed on day 14 yielding 2.7×10^4 TCID₅₀ per ml (1 TCID₅₀ = 7.3 pg of p24). These data show that the virus was efficiently produced from quiescent lymphocytes when stimulated 7 days subsequent to infection.

Each of the three ddNs at concentrations (4 μM AZT, 10 μM ddI, and 0.2 μM ddC) that correspond to the peak plasma levels observed in humans (28) was active in inhibiting HIV-1 in stimulated PBLs: AZT resulted in 99% reduction of viral production, ddI resulted in a 92% reduction, and ddC resulted in a 94% reduction (S.D.M., unpublished data). However, they were found to have contrasting effects in R-PBLs. On day 7, prior to activation, the virus measured in these three groups corresponded to the residual virus input (Fig. 3A), but after activation, viral production was initiated showing a slower rate of increase for ddI and ddC than for the control, reaching the control level on days 28 and 21, respectively, whereas AZT was the least active, reaching the control level by day 14.

A combination of two ddNs showed no additive effect: ddI/ddC and ddI/AZT (Fig. 3B) showed inhibition profiles similar to ddI alone (Fig. 3A). Viral production in the AZT/ddC group was equivalent to that observed with ddC alone.

Combining the three ddNs induced a slight reduction in viral production compared with the combination of any two at days 14, 21, and 28 (Fig. 3B).

With regard to cell viability, the temporary viral inhibition observed with ddNs alone or in combination was accompanied by a partial protection against the cytopathic effect induced by viral replication (Fig. 2).

A 7-day incubation of the hydroxamates (0.1 mM HU and 1 mM DAH) with R-PBLs did not interfere with cellular DNA synthesis, after stimulation by PHA (Fig. 1). However, at these concentrations, no inhibition of viral production was observed.

The combination treatments of DAH/ddI and of HU/ddI on R-PBLs resulted in a remarkable synergistic effect with total suppression of viral production after PHA activation as early as day 14 (Fig. 3B). This total viral suppression was observed with no effect on the capacity of the cells to replicate (Fig. 1) and total protection against the cytopathic effect induced by viral replication (Fig. 2B). In contrast, no synergistic effect was observed with combinations of AZT or ddC with DAH or HU (Fig. 3B); viral production after activation showed a profile similar to that with AZT or ddC alone, respectively.

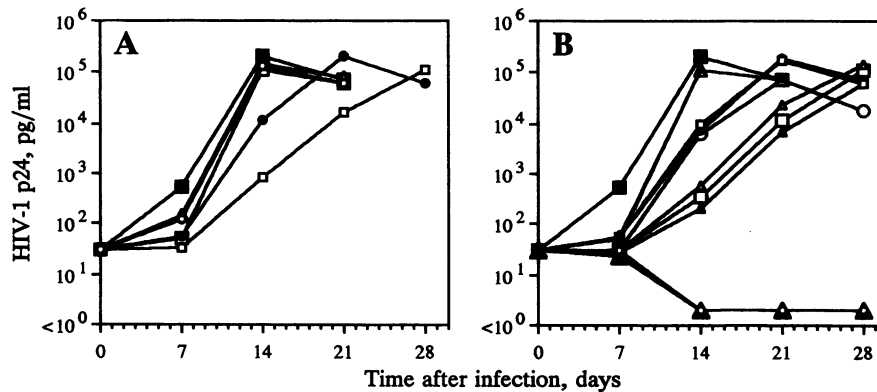


FIG. 3. Drug treatment of HIV-1-infected resting lymphocytes: effect on viral production after PHA activation. Resting lymphocytes were infected with HIV-1 and then treated with various drugs on day 0. On day 7, drugs were removed, and the cells were activated with PHA and then cultured up to day 28 in drug-free IL-2-containing growth medium. Culture supernatants collected at different time points were assayed for HIV-1 p24 gag protein production by ELISA. Results are the average of triplicate determinations. (A) HIV-1-infected cells were treated with drugs as follows: ■, no drug; □, AZT; □, ddI; ●, ddC; △, DAH; ○, HU. (B) HIV-1-infected cells were treated with drugs as follows: ■, no drug; □ AZT/ddI; ○ AZT/ddC; △, ddI/ddC; ▲, AZT/ddI/ddC; △, AZT/DAH; ●, AZT/HU; ▲, ddI/DAH; ○, ddI/HU; ●, ddC/DAH; □, ddC/HU.

DISCUSSION

Our results confirm that R-PBLs can be infected with HIV-1 as described (14–18), giving rise to a latent infection over 7 days. Cell activation by PHA on day 7 resulted in a considerable production of viral particles, as measured by p24 gag assay (Fig. 3). Zack *et al.* (15) report that viral production by infected resting cells is efficient if PHA activation occurs up to 2 days after infection and that the production of progeny virion is less if activation occurs later. Our findings that viral production is efficient when activation occurs on day 7 may be due to the greater infectious dose used in our experiments as compared to that in Zack *et al.* (15).

Under our experimental conditions, an inhibitory effect on viral production was observed 7 days after PHA activation with the ddNs, but this effect was temporary, AZT showing the weakest activity (Fig. 3A). This lack of activity of AZT may be explained by the low level of phosphorylation of the drug by nonactivated cells (30). Furthermore, triphosphate ddNs are known to compete with normal deoxynucleoside 5'-triphosphate (dNTP) for binding to reverse transcriptase (22, 23). As shown by Gao *et al.* (10), the concentration of endogenous competitor dNTP significantly influences the antiviral activity of triphosphate ddNs, and the proportion of AZT triphosphate/deoxythymidine triphosphate is at least 10-fold greater, and the proportion of dideoxyadenosine triphosphate/deoxyadenosine triphosphate is at least 14-fold less in PHA-PBLs than in R-PBLs (10). This may explain why, in our experiments, ddI showed greater anti-HIV-1 activity than AZT in resting lymphocytes.

The most significant result of our experiments was the synergistic effect of 10 μ M ddI with the two hydroxamates, 0.1 mM HU or 1 mM DAH, resulting in the suppression of viral production after activation of resting cells (Fig. 3B). An equivalent synergistic effect was observed with 10 μ M ddC, and each of the two hydroxamates at the same concentrations (S.D.M., unpublished data). However, a ddC dose of 0.06 mg/kg (which corresponds to a plasma concentration in humans of approximately double the concentration of 0.2 μ M used in our experiments) has been reported to induce toxic effects, which become the dose-limiting factor in humans (31, 32). Indeed, there are multiple factors that may intervene in combination therapy as described by Hirsch and d'Aquila (11); one of the interesting features of the synergistic combination reported here is that the side effects of ddI differ from those known for hydroxamates.

The mechanisms responsible for this synergistic effect are not yet fully understood. We believe that the role of these hydroxamates in the synergistic effect observed, at the doses used, may not be fully explained by their known inhibitory effect on ribonucleotide reductase because of the following reasons. (i) Ribonucleotide reductase activity varies greatly during the cell cycle, is at its peak during the S phase, and is barely detectable in resting cells (33–35). (ii) As shown by Reichard (36), ribonucleotide reductase inhibitors induce an imbalance in dNTP pools affecting both DNA synthesis in S phase and DNA repair in resting lymphocytes, resulting in cell death. However, the doses of HU and DAH used in our experiments were sufficiently high to induce the synergistic effect while remaining sufficiently low to permit DNA synthesis after adequate PHA activation (Fig. 1). (iii) Gao *et al.* (19) describe the inhibitory effect of a high concentration of HU on the formation of HIV-1 viral DNA in PHA-PBLs, which they attribute to the depletion of the dNTP pools. However, the level of these pools remains well above those occurring in R-PBLs. We hypothesize that the depletion of the dNTP pools in PHA-PBLs, by treatment with 1 mM HU under the conditions described (19), may induce a cytotoxic effect on these cells, which in turn may be responsible for the

inhibition of the formation of viral DNA observed by Gao *et al.* (19).

A possible explanation for the synergistic effect observed may be linked to the known property of hydroxamates to form a bidentate ligand with zinc in metalloproteins (37, 38). We hypothesize that ddI, acting on the complex involved in retrotranscription as a reverse transcriptase inhibitor, may facilitate the interaction of hydroxamates with certain HIV proteins such as ribonuclease H, where several metal ion binding sites have recently been described (39), and/or with the P7NC nucleocapsid protein, which contains two retroviral zinc-finger sequences (40–42).

With regard to the cellular models used in the *in vitro* evaluation of anti-HIV compounds, our results suggest that any *in vitro* assay system should take into account nondividing cells, bearing in mind the limitations in extrapolating *in vivo* effectiveness from *in vitro* data. It is thought that the majority of circulating lymphocytes are nondividing quiescent cells. As with animal retroviruses (43, 44), HIV proviral DNA synthesis takes place in quiescent lymphocytes. Bukrinsky *et al.* (18) have demonstrated that a large proportion of HIV-1 genome in asymptomatic individuals exists as full-length extra-chromosomal DNA that retains its ability to integrate upon activation of the host cells. Recent studies of HIV-infected individuals indicate that there is a high HIV burden in the lymphoid tissues, as intracellular virus in latent form (45) and extracellular virus trapped in the follicular dendritic cells (45, 46). Our results suggest that a treatment schedule incorporating the simultaneous administration of ddI and a hydroxamate may induce the suppression of HIV infection in resting lymphocytes, which constitute an important reservoir of virus in its latent form. Clinical trials are under way to evaluate whether the effect of this synergistic combination of drugs and treatment schedule is reproducible *in vivo*.

We gratefully acknowledge Gregoire Barrowcliff for his helpful discussions and critical suggestions during the preparation of this manuscript. This work was supported in part by the Agence Nationale pour la Valorisation de la Recherche (ANVAR, France).

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