Divergent Anti-human Immunodeficiency Virus Activity and Anabolic Phosphorylation of 2',3'-Dideoxynucleoside Analogs in Resting and Activated Human Cells*

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The mechanism of divergent anti-human immunodeficiency virus type 1 (HIV-1) activity of various 2',3'-dideoxynucleoside analogs (ddNs) in peripheral blood mononuclear cells (PBM) was studied. We demonstrate that the in vitro anti-HIV-1 activity of various ddNs varies substantially and that the divergent antiviral activity is related to the extent of anabolic phosphorylation of each ddN and its counterpart 2'-deoxynucleoside (dN). We also show that certain ddNs cause a reduction of their counterpart dNTP formation in PBM in the following order: 2',3'-dideoxycytidine (ddC) > 2',3'-dideoxycytidine (ddT), 3'-thio-2',3'-dideoxycytidine (3TC), 2',3'-dideoxyinosine (ddI), 2',3'-dideoxynucleosides (ddG) > 3'-azido-2',3'-dideoxythymidine (AZT) > 2'-fluoro-2',3'-dideoxyadenosine (F-ara-ddA). Based on the phosphorylation profiles, anti-HIV-1 ddNs can be classified into two groups: (i) cell-activation-dependent ddNs such as AZT and ddT that are preferentially phosphorylated, yield higher ratios of ddNTP/dNTP, and exert more potent anti-HIV-1 activity in activated cells than in resting cells; and (ii) cell-activation-independent ddNs including ddI and ddC that produce higher ratios of ddNTP/dNTP and exert more potent anti-HIV-1 activity in resting cells. These data should provide a basis for the elucidation of the mechanism of the divergent antiretroviral activity of ddNs.

The proviral DNA synthesis catalyzed by human immunodeficiency virus type 1 (HIV-1) reverse transcriptase represents one of the critical steps in the initial phase of HIV-1 infection. As in the case of avian and murine retroviruses (1, 2), this step occurs not only in activated cells but also in resting, nondividing (unstimulated) cells (3-5). It has recently been demonstrated that HIV-1 proviral DNA synthesis can be completed in resting peripheral blood mononuclear cells (R-PBM), although this synthesis occurs extremely slowly and inefficiently (6). Bukrinsky and his co-workers (7) have also demonstrated that a large proportion of the HIV-1 genome in asymptomatic individuals exists as full-length, extrachromosomal DNA, which retains the ability to be integrated upon activation (stimulation for division) of the host cell. In addition, proviral DNA can be detected in a relatively large percentage of PBM from HIV-1-infected individuals, the majority of which are resting cells, and the proviral DNA level seems to correlate with the progression of HIV-1 diseases (8). Thus, HIV-1 infection of resting cells appears to play an important role in both the viral replication cycle and the pathogenesis of acquired immunodeficiency syndrome (AIDS).

A group of nucleosides, 2',3'-dideoxynucleoside analogs (ddNs), has been shown to be active against HIV-1 replication both in vitro and in vivo (9-11). ddNs are successively phosphorylated in the cytoplasm of a target cell to produce ddN-5'-triphosphates (TP) and become analogs of the 2'-deoxynucleoside (dN)-TP, the natural substrates for cellular DNA polymerases and the viral reverse transcriptase. It is thought that the ratio of 2',3'-dideoxynucleoside-5'-triphosphate to 2'-deoxynucleoside-5'-triphosphate (ddNTP/dNTP) is one of the most important determinants of ddN antiviral activity against HIV-1 (9). We have recently reported that when compared with 2',3'-dideoxycytidine (ddC), 3'-azido-2',3'-dideoxythymidine (AZT or zidovudine) achieved higher ratios of AZTTP/dTTP intracellularly and exerted much greater activity against HIV-1 replication in phytohemagglutinin-stimulated PBM (PHA-PBM) (12). In contrast, ddI produced higher ratios of ddATP (the putative active form of ddI) to dATP relative to AZTTP/dTTP in R-PBM (12). These results suggest that the anti-HIV-1 activity of AZT is superior in activated cells, whereas ddI is more effective in resting cells. These data appear to be in agreement with a recent clinical observation that a simultaneous regimen of AZT and ddI had more profound and persistent anti-HIV-1 activity in patients with advanced HIV-1 infection than an alternating regimen of the two drugs (13).

In the present study, we demonstrate that the in vitro activity of various ddNs against HIV-1 differs substantially in PBM and that such divergent antiviral activity is related to the differential anabolic phosphorylation of ddNs and dNs. We propose that ddNs can be classified into two groups: (i) cell activation-dependent ddNs and (ii) cell activation-independent ddNs.

EXPERIMENTAL PROCEDURES

Chemicals—AZT was purchased from Sigma, 2',3'-Dideoxy-2',3'-dideoxycytidine (ddATP or stavudine), 2',3'-dideoxythymidine (ddC or zalcitabine), ddI, 2'-fluoro-2',3'-dideoxyadenosine (F-ara-ddA), and 2',3'-
Dideoxynucleotides and deoxynucleotides in resting and PHA-stimulated peripheral blood mononuclear cells

Resting PBM (R-PBM) and PHA-stimulated PBM (PHA-PBM) (10^7 cells) were exposed to 10 μM 3H-labeled 3TC, ddC, d4T, ddG, or F-ara-ddA for 12 h. Cell pellets were washed with cold phosphate-buffered saline and extracted with 60% methanol. Cellular dideoxynucleotide metabolites were quantified by HPLC. Cellular deoxynucleotide pool sizes were determined by enzymatic assay.

### Table I

Dideoxynucleotides and deoxynucleotides in resting and PHA-stimulated peripheral blood mononuclear cells

| Nucleotides | Pool size | R-PBM | PHA-PBM |
|-------------|-----------|-------|---------|
| d4T         | ddTTP     | 0.02 ± 0.01 | 0.49 ± 0.11 |
| ddC         | dCTP      | 0.34 ± 0.04 | 3.80 ± 1.35 |
| ddG         | ddCTP     | 0.06      | 0.13     |
| ddA         | AZTTP     | 0.02 ± 0.01 | 4.15 ± 0.98 |
| ddCTP       | dGTP      | 0.48 ± 0.10 | 6.79 ± 0.62 |
| ddAP4TP     | dGTP      | 0.04      | 0.61     |

### Differential anti-HIV-1 activities of 2',3'-dideoxynucleoside analogs in PHA-activated PBM

The relative anti-HIV-1 potency of various 2',3'-dideoxynucleoside analogs (ddNs) was evaluated in a system using PHA-activated PBM as target cells and an inhibition of p24 Gag protein production as an end point, as described under "Experimental Procedures." PHA-PBM isolated from five different HIV-seronegative individuals were exposed to a primary HIV-1 isolate at a 2500 TCID₅₀ concentration in the presence or absence of ddNs. The culture supernatants were harvested on day 7 of culture and assayed for HIV-1 p24 Gag protein production by radioimmunoassay. Data are presented as drug concentrations that suppressed p24 Gag protein production by 50% (IC₅₀). All cultures were performed in quadruplicate. Each data point represents the mean IC₅₀ value of a test drug in each PBM population.

dideoxyguanosine (ddG) were supplied by K. Flora, Developmental Therapeutics Program, National Cancer Institute. 3'-Thia-2',3'-dideoxyadenosine (3TC; sometimes abbreviated as SddC) was provided by R. F. Schinazi, Emory University, Decatur, GA. L3H1AZT (specific activity: 14 Ci/mmol), L3H1F-ara-ddA for 12 h. Cell pellets were then counted in a liquid scintillation counter. The radioactivity on the filters was measured with a scintillation counter.

**Determination of Anti-HIV-1 Activity of ddNs in PHA-PBM** — The HIV-1 strain used was isolated from a patient with advanced HIV-1 infection (HIV-1_Zm95/90) prior to antiviral therapy, as described previously (14). Briefly, PBM obtained from HIV-1-seronegative donors were stimulated with PHA (10 μg/ml) (PHA-PBM) for 72 h in recombinant interleukin-2-containing RPMI 1640-based culture media, seeded in 24-well tissue culture plates at a density of 1 x 10⁷ cells/ml, and exposed to 2500 TCID₅₀ of HIV-1 preparation in the presence or absence of various concentrations of ddNs. The antiviral potency of ddNs was defined as the drug concentration that inhibited HIV-1 p24 Gag protein production by 50% (IC₅₀). All experiments were performed in quadruplicate.

**Metabolism Studies** — Freshly isolated, resting, nondividing PBM (R-PBM) and activated, dividing PBM (PHA-PBM) were incubated with 10 μM [3H]ddN (1 Ci/mmol) for 12 h. Cells were then harvested, centrifuged, and washed twice with cold phosphate-buffered saline. After centrifugation, the cell pellets were subjected to extraction of nucleosides and nucleotides with 60% methanol. The extracts were then heated for 2 min at 95 °C and analyzed by HPLC using an ion-exchange column (Partisil 10 SAX; Whatman Inc., Hillboro, NJ) as previously described (12, 15).

**Quantification of dNTP Pools in PBM Exposed to ddN** — An enzymatic assay using a DNA polymerase, previously described by Sherman and Fyfe (16), was used with modifications (12) to quantify dNTP in PBM. In the modified assay, a dNTP, present in excess, was radiolabeled. The amount of radioactivity incorporated into DNA, catalyzed by the Sequenase enzyme (version 2.0, United States Biochemical Corp., Cleveland, OH), was proportional to the dNTP quantified. The reaction mixture contained 0.05 units of Sequenase enzyme, 50 μM Tris-HCl, pH 7.5, 10 μM MgCl₂, 5 μM dithiothreitol, 0.25 μM template-primer (the sequences of the oligodeoxynucleotides have been described elsewhere (16)), 2.5 μM [3H]ddATP (30 Ci/mmol), for dCTP (ddCTP, and ddG dNTPs) or 2.5 μM [3H]ddTPP (15 Ci/mmol), for ddTP (ddCTP, ddATP, and ddT dNTPs) as substrate. The reaction was terminated by spotting 0.5 μl of reaction mixture onto a Whatman DE81 filter. The filters were dried, extensively washed with 5% Na₂HPO₄, briefly rinsed with distilled water and with 95% ethanol, and dried. The radioactivity on the filters was then counted in a liquid scintillation counter. The radioactivity (dissintegrations/minute) was plotted as a function of the quantity of dNTPs. The dNTP quantities in test samples were determined from these standard curves. In determining dNTP quantity in samples extracted from ddN-treated cells, an extract (5 μl) from -10° cells cultured in the absence of ddN was mixed with an extract from ddN-exposed cells in various ratios, and the mixtures were subjected to the polymerase assay. From thus obtained linear curves for correction, the reduction by ddNTP was determined and the dNTP quantities were adjusted to represent the values otherwise obtained in the absence of ddNTP.

**Nucleoside Kinase Assays** — Nucleoside kinase assays were performed as previously described (12). One unit of TK and dCK activities is defined as 1 nmol of dTTP or dCTP formed per hour. The observed enzymatic activities were normalized to the unit of enzymatic activity/mg of protein using the Bio-Rad protein assay reagent (Bio-Rad).

**RESULTS**

Anti-HIV-1 Activities of ddNs Differ Substantially in Vitro in PHA-activated PBM — We evaluated the relative antiviral activities of AZT, d4T, ddI, ddG, F-ara-ddA, and 3TC against HIV-1 in cell cultures using PHA-PBM as target cells and employing ddN inhibition of p24 Gag protein production as an end point. As shown in Fig. 1, the ddNs exhibited quite different degrees of antiviral potency. The mean IC₅₀ values of the drugs tested were, in the order of potency, AZT (with an IC₅₀ value of 0.07 μM) > 3TC (0.51 μM) > d4T (0.62 μM) > ddI (3.5 μM) > ddG (7.2 μM) > F-ara-ddA (34 μM).
Classification of Anti-HIV ddN

**ddNs Are Differentially Phosphorylated in Resting and Activated PBM**—In an attempt to elucidate the mechanism responsible for the substantially different anti-HIV-1 potencies of ddNs in PHA-PBM, we studied the anabolic phosphorylation of ddNs in R-PBM and PHA-PBM. After the cells were exposed to 10 μM [3H]ddN for 12 h, cellular ddN nucleotide metabolites were extracted with 60% methanol and quantitated using the anion-exchange HPLC method (12). Based on anabolic phosphorylation patterns and the cell activation state, the phosphorylation profiles were categorized into three groups: (i) anabolic phosphorylation of thymidine analogs such as AZT and d4T, which were most affected by PHA stimulation (cell activation) and were efficiently phosphorylated in PHA-PBM. The triphosphate levels of AZT and d4T were greater by 208- and 25-30-fold, respectively, in PHA-PBM than those in R-PBM (Table I and Fig. 2). It is noteworthy that both AZT and d4T underwent little triphosphorylation in resting cells. (ii) Anabolic phosphorylation of purine analogs including ddi, F-ara-ddA, and ddG, which did not show a response to PHA stimulation and, with the exception of F-ara-ddA, were poorly phosphorylated in both resting and activated cells (Table I and Fig. 2). (iii) Anabolic phosphorylation of cytidine analogs such as ddC and 3TC, which were more efficiently phosphorylated in both resting and activated cells than the purine analogs, although their phosphorylation was not significantly potentiated by PHA-stimulation (Table I and Fig. 2).

**Inhibition of Cellular dNTP Formation by ddN**—Since the intracellular dNTP pools correlate with the antiretroviral activity of ddN, we asked whether the level of dNTP pools was affected by ddN treatment *per se*. As shown in Table II, when PBM were exposed to AZT for 72 h, substantial alterations of the dNTP pools were observed. First, the dTTP pool was decreased by an average of 40% in R-PBM, although variable results were observed in PHA-PBM (three cases of increase versus one case of decrease). Second, AZT inhibited dGTP formation by 21 and 14% for resting and activated cells, respectively. Finally, AZT stimulated dCTP formation in both R-PBM and PHA-PBM by 38 and 25%, respectively. Exposure to d4T decreased the dTTP pool of R-PBM and PHA-PBM slightly to moderately, whereas it failed to stimulate dCTP formation. Exposure to ddC resulted in a profound depletion of the cellular dCTP pool, especially in R-PBM. This depletion was concentration dependent with more potent inhibition of dCTP formation at higher ddC concentrations in R-PBM (data not shown). Exposure to 3TC caused a slight inhibition of dCTP formation in both R-PBM and PHA-PBM. Reductions of the dATP and dCTP pools in R-PBM were found to be 19- and 8.5-fold, respectively, more profound than those in PHA-PBM (40 versus 20% and 44 versus 17% for ddI and ddG treatment, respectively, Table II). Exposure to F-ara-ddA did not cause an appreciable decrease of dATP pools in either R-PBM or PHA-PBM.

**Ratios of dNTP to dNTP in Resting and Activated PBM**—One of the most critical factors that determine the antiviral potency of ddN against HIV is the ratio of intracellular ddNTP to its competing (or natural) dNTP (9). We, therefore, evaluated the ratio of each ddNTP and its counterpart dNTP in PBM. It was found that d4T, like AZT, was more efficiently phosphorylated in PHA-PBM than in R-PBM, with higher ratios of d4TTP to dTTP seen in PHA-PBM (Table I). The level of d4TTP increased 25-fold upon PHA stimulation (0.02-0.49 pmol/10⁶ cells). At the same time, the level of dTTP increased 11-fold, from 0.34 to 3.80 pmol/10⁶ cells. As a result, the ratio of d4TTP/dTTP was ~2-fold higher in PHA-PBM compared with that in R-PBM. The level of AZTTP in PHA-PBM was found to be 208-fold higher than in R-PBM, resulting in an AZTTP/dTTP value that was increased ~15-fold after cell activation.

Unlike AZT and d4T, the cytidine analogs, ddC and 3TC, as well as the purine analogs, ddi, F-ara-ddA, and ddG, produced higher ddNTP/dNTP ratios in R-PBM than in PHA-PBM (Table I). The ddC triphosphate increased by 1.7-fold upon PHA activation, whereas the dCTP pool underwent a 7.4-fold increase. These disproportionate changes resulted in a 4.4-fold higher ddCTP/dCTP ratio in R-PBM (Table I). The triphosphate form of 3TC was increased by 3.8-fold upon PHA stimulation, whereas dCTP was increased by 7-fold, yielding an ~2-fold higher ratio of 3TCTP/dCTP in R-PBM compared with PHA-PBM. As for ddi and F-ara-ddA, the triphosphate levels in R-PBM and PHA-PBM were similar, while levels of dATP increased by 19- and 8.5-fold upon PHA activation, resulting in a decrease in the ddNTP/dNTP values by 15.8- and 8.5-fold, re-
Effects of dideoxynucleosides on dNTP pool sizes of resting and PHA-activated PBM

Table II

| dNTP analogs | R-PBM             | PHA-PBM            |
|--------------|-------------------|--------------------|
|              | dTP               | dCTP               | dATP               | dGTP               | dTP               | dCTP               | dATP               | dGTP               |
| No dddN      | 0.41 ± 0.02       | 0.37 ± 0.04        | 0.11 ± 0.01        | 0.16 ± 0.01        | 5.85 ± 0.13       | 2.35 ± 0.12        | 1.35 ± 0.06        | 0.96 ± 0.07        |
| Thymidine analogs |                |                    |                    |                    |                   |                    |                    |                    |
| dT          | 0.34 ± 0.03       | 0.36 ± 0.03        | 0.13 ± 0.01        | 0.15 ± 0.01        | 3.80 ± 0.14       | 1.78 ± 0.16        | 1.34 ± 0.06        | 0.85 ± 0.04        |
| AZT*        | 0.61 ± 0.26       | 1.59 ± 0.76        | 0.16 ± 0.04        | 0.11 ± 0.04        | 6.89 ± 0.54       | 5.29 ± 0.97        | 2.42 ± 0.31        | 1.28 ± 0.31        |
| Cytidine analogs |            |                    |                    |                    |                   |                    |                    |                    |
| 3TC         | 0.33 ± 0.02       | 0.28 ± 0.01        | 0.12 ± 0.01        | 0.16 ± 0.01        | 5.60 ± 0.43       | 1.90 ± 0.16        | 1.38 ± 0.04        | 0.93 ± 0.04        |
| ddC*        | 0.73 ± 0.43       | 0.21 ± 0.06        | 0.12 ± 0.04        | 0.10 ± 0.06        | 8.99 ± 1.50       | 1.87 ± 0.73        | 2.94 ± 0.61        | 1.92 ± 0.88        |
| Adenosine analogs |          |                    |                    |                    |                   |                    |                    |                    |
| F-ara-ddA   | 0.41 ± 0.05       | 0.34 ± 0.03        | 0.11 ± 0.02        | 0.16 ± 0.01        | 5.35 ± 0.50       | 2.20 ± 0.22        | 1.24 ± 0.08        | 0.90 ± 0.09        |
| ddI*        | 0.89 ± 0.21       | 0.97 ± 0.35        | 0.09 ± 0.01        | 0.11 ± 0.03        | 5.47 ± 0.42       | 3.60 ± 0.45        | 1.53 ± 0.11        | 1.10 ± 0.43        |
| Guanine analog |                |                    |                    |                    |                   |                    |                    |                    |
| ddG         | 0.42 ± 0.01       | 0.33 ± 0.02        | 0.13 ± 0.01        | 0.09 ± 0.01        | 5.21 ± 0.35       | 1.96 ± 0.24        | 1.28 ± 0.09        | 0.80 ± 0.03        |

* Intracellular dNTP pool sizes for AZT, ddC, and ddI are shown as references.

Effects of Pyrimidine dNds on Thymidine Kinase and Deoxycytidine Kinase Activities in R-PBM and PHA-PBM—The S-phase-specific cytoplasmic thymidine kinase type 1 (TK1) and the constitutively expressed mitochondrial thymidine kinase type 2 (TK2) play major roles in the phosphorylation of thymidine, while the cytoplasmic deoxycytidine kinase (dCK) and mitochondrial deoxycytidine kinase (dPydK) play a major role in the phosphorylation of deoxycytidine (17-21). We therefore asked whether or not 1 mM concentration of the four pyrimidine dNds studied here could affect the phosphorylation of [14C]dT or [14C]ddC catalyzed by these kinases in protein extracts from R-PBM and PHA-PBM using phosphocreatine as the phosphate donor (Table III). The addition of AZT to PHA-PBM protein extract decreased the amount of phosphorylated dT (which represents TK activity) by 88% (2.82–0.35 nmol/h/mg protein), although no decrease was detected using R-PBM protein extract. This result suggests that AZT in PHA-PBM is phosphorylated by TK1, whose activity is enhanced upon PHA activation. AZT also caused a reduction in the phosphorylation of ddC in stimulated cells (Table III), suggesting that AZT could compete with [14C]ddC with respect to the activity of cytoplasmic dCK. Unlike AZT, d4T did not cause a significant decrease in the phosphorylation of dT in PHA-PBM (Table III). This appears to agree with a report that d4T is a poor substrate for TK1, TK2 (19), dCK, and dPydK (20). In contrast, 3TC caused a slight decrease in the phosphorylation of [14C]ddC by PHA-PBM protein extracts (Table III). This appears to support previous data showing that 3TC has a higher affinity for dCK as compared with ddC (21), although further study regarding the substrate specificity of dCK toward 3TC is required.

No Significant Metabolic Interaction Occurs When AZT and ddI Are Used Simultaneously—A recent report shows that a simultaneous regimen of AZT plus ddI has more significant and persistent antiviral activity in patients with AIDS or ARC than the sequential regimen of AZT and ddI (13). We then asked whether the enhanced antiviral activity observed in patients with HIV was due to a metabolic interaction of AZT and ddI. It was found that when PBM were treated with 10 μM [3H]AZT in the presence of 10 μM ddI, the formation of AZTTP was not affected either in R-PBM or PHA-PBM (Table IV). The triphosphorylation profiles of AZT assessed by HPLC in the presence and absence of ddI were virtually identical. Concentrations of intracellular dTTP upon exposure to AZT plus ddI were also...
TABLE IV  
Effects of combinations of AZT and ddI on their triphosphorylation in PBM from two donors  
R-PBM and PHA-PBM were exposed to 10 μM [3H]AZT or [3H]ddI in the presence and absence of 10 μM ddI or AZT, respectively. Cellular nucleotides were extracted and determined as described under “Experimental Procedures.”

| [3H]ddN | TP         | Donor A |        | Donor B |        |
|---------|------------|---------|--------|---------|--------|
|         | R-PBM      | PHA-PBM |        | R-PBM   | PHA-PBM |
| AZT     |            |         | Pool size (pmol/10⁶ cells) |
|         | Alone      | With ddI|        | Alone    | With ddI|
| ddATP   | 0.03±0.01  | 0.02±0.01| 2.10±0.41| 2.23±0.48 |
| dATP    | 0.17±0.02  | 0.13±0.02| 1.66±0.17| 2.11±0.21 |
| AZT/ddI | 0.11       | 0.15    | 1.26    | 1.06    |
|         |            |         |        |         |
| ddATP   | 0.03±0.01  | 0.02±0.01| 0.96±0.02| 0.97±0.02 |
| dATP    | 0.08±0.02  | 0.10±0.03| 1.07±0.11| 0.93±0.10 |
| ddATP/ddATP | 0.38 | 0.30 | 0.06 | 0.08 |

DISCUSSION

The present study has demonstrated that anabolic phosphorylation of ddNs differs substantially depending on the activation state of the target cells. The phosphorylation of ddNs in response to cell activation appears to be primarily determined by the increase in the appropriate kinase and therefore related to the base moiety of ddNs, whereas sugar modification affects the efficiency of anabolic triphosphorylation. Moreover, we demonstrated that the formation of intracellular dNTP is substantially affected by exposure to ddNs, with the degree of inhibition varying as a function of the target cell activation state. The anti-HIV-1 activities of ddN, therefore, must vary disproportionately in resting and activated cells. It is worth noting that HIV-1 infects not only activated, but also resting CD4+ cells (3–5). Thus, for antiretroviral therapy with nucleoside analogs, it is important to define the metabolism of each derivative in regard to the triphosphorylation pattern in terms of the target cell activation state.

Our results suggest that ddNs can be classified into two subfamilies. First, cell activation-dependent ddNs, such as AZT and d4T, that are preferentially phosphorylated and exert more potent anti-HIV-1 activity in activated cells than in resting cells. In this regard, Ferno et al. (22) have reported that the anti-HIV-1 activities of AZT and several thymidine analogs in monocytes/macrophages are potentiated by granulocyte macrophage colony-stimulating factor, suggesting that the phenomenon is not seen only with PHA activation. The second subfamily, cell activation-independent ddNs, including ddI (and ddA), F-ara-ddA, ddG, ddC, and 3TC, that are preferentially phosphorylated and exert more potent anti-HIV activity in resting cells than in activated cells. Regarding the anti-HIV-1 activity of cell activation-independent ddNs in R-PBM, we have recently demonstrated that ddI can, on a molar basis, exert more potent anti-HIV activity than AZT in vitro.

Furthermore, our results show that there are two different subtypes within the subfamily of cell activation-independent ddNs: cytidine analogs (ddC and 3TC) and purine analogs (ddI, F-ara-ddA, and ddG). Cytidine analogs were phosphorylated quite efficiently in resting cells (Table I and Fig. 2), and their triphosphorylation was further enhanced upon cell activation. In contrast, triphosphorylation of purine analogs occurred independent of cell activation (Table I and Fig. 2), presumably because the enzymes responsible for their phosphorylation, such as cytosolic 5'-nucleotidase, are not responsive to cell activation (12). In addition, cellular dCTP formation was found to be differentially inhibited by cytidine analogs. Therefore, the cytidine analog triphosphate to dCTP ratio varies among different analogs. Indeed, ddC caused a more substantial inhibition of dCTP formation in R-PBM than in PHA-PBM, especially at higher concentrations (data not shown), resulting in a higher ratio of ddCTP to dCTP in R-PBM. In the case of 3TC, 3TCdTTP/dCTP values in R-PBM were just 2-fold higher than in PHA-PBM, presumably because 3TC was less inhibitory of dCTP formation than ddC.

The reason for the changes of dTTP, dCTP, and dGTP pools upon exposure to AZT remains unclear. At a high concentration (1 μM), AZT substantially inhibited TK activity in PHA-PBM, but this inhibition was not distinct in R-PBM. In this regard, AZT is a relatively poor substrate for TK2, which is constitutively expressed in both resting and activated cells, as compared with TK1, which is greatly enhanced in activated cells (17). Thus, the kinase activity detected in R-PBM may represent mostly TK2 activity, and, therefore, it is possible that the inhibition of dTTP formation in R-PBM (Table II) may involve a mechanism(s) other than TK inhibition. In contrast, TK1 activity was profoundly suppressed by AZT (Table III), which may also account for the depletion of the dTTP pool in PHA-PBM and the inhibition of thymidylate kinase activity by AZT-MP described by Furman et al. (25). Moreover, in PHA-PBM, the AZT exposure caused an increase in the dCTP pool (Table II), whereas AZT inhibited the dCTP pool (Table III). One possible explanation is that the AZT depletion of dTTP stimulated the activity of ribonucleotide reductase, an enzyme catalyzing the rate-limiting step of de novo dNTP synthesis. TTP is a feedback inhibitor of pyrimidine ribonucleotide reduction and an activator of guanine ribonucleotide reduction (24).

Thus, the observed dGTP pool decrease by AZT exposure could be due to an indirect effect of dTTP depletion and a direct inhibitory effect of AZT on deoxyxycytidine kinase. Cytosolic dCK exhibits a broad substrate specificity and can phosphorylate dG

1 T. Shirasaka and H. Mitsuya, unpublished data.
to dGMP (25) as well as F-ara-ddA to F-ara-ddATP (26).

In terms of the magnitude of triphosphorylation, 3TC presented a different picture than the other ddNs in this study. 3TC was very efficiently converted to its triphosphate, yielding 3TCTP/dCTP values of 8.59 and 4.58, in R-PBM and PHA-PBM, respectively (Table I). This is apparently in agreement with a report that 3TC is a good substrate for cytoplasmic dCK (21) and contrasts with its close ddN analog, ddC, which is a poor substrate for both cytoplasmic and mitochondrial dCKs (20). In fact, a moderate reduction in the phosphorylation of [14C]dC by dCKs was seen in the presence of 3TC in both R-PBM and PHA-PBM, although no reduction was seen with ddC (Table III). Nevertheless, the anti-HIV-1 potency of 3TC is comparable to that of ddC when examined in PHA-PBM system (27). One possible explanation is the higher $K_i$ of 3TCTP for HIV-1 reverse transcriptase compared to that of ddCTP (28).

The triphosphorylation profile of F-ara-ddA (Table I) was particularly intriguing. F-ara-ddA has been reported to be as potent against HIV-1 as its analogs ddA and ddI when evaluated in the cytopathic effect inhibition assay employing CD4+ATH8 target cells (29, 30). F-ara-ddA, however, was substantially less potent than ddA or ddI in the PHA-PBM system using the inhibition of p24 Gag protein production as an end point (Fig. 1). In this regard, considering the observation that the F-ara-ddATP/ddATP ratio is quite high in R-PBM, F-ara-ddA could exert a favorable antiviral activity in vivo where the majority of target cells for HIV-1 infection are thought to be resting cells (3-5). Indeed, as examined in severe combined immunodeficiency mice reconstituted with human peripheral blood leukocytes (hu-PBL-SCID mice), F-ara-ddA has exhibited a potent anti-HIV activity that appeared to be superior to that of AZT (31). The current data on F-ara-ddA suggest further investigation of the agent as a potential anti-HIV-1 drug.

We previously reasoned that the favorable antiviral activity seen in patients receiving the simultaneous regimen of AZT plus ddI as compared with those receiving the alternating regimen of AZT plus ddI (13) was due to the complementary effect of AZT and ddI (12). In the present study, we found that neither AZT nor ddI significantly influenced the metabolism of the other dideoxynucleoside (Table IV). These data further support the possibility that AZT and ddI complement each other by exerting antiviral effects in activated and resting cells, respectively. The present results also suggest that the potential for effective combination chemotherapy might be enhanced if drugs from each of the two categories, the cell activation-dependent ddNs and the cell activation-independent ddNs, are combined. It should be noted, however, that the pharmacokinetics of each drug and the emergence of drug-resistant HIV-1 variants also have to be considered as critical factors in designing effective combination antiretroviral chemotherapy.

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REFERENCES

1. Harel, J., Rassart, E., and Jolicoeur, P. (1981) Virology 110, 202-207
2. Varmus, H. E., Padgett, T., Deasley, S., Simon, G., and Bishop, J. M. (1977) Cell 11, 307-319
3. Stevenson, M., Stanwick, T. L., Dempsey, M. P., and Lamonica, C. A. (1990) EMBO J. 9, 1551-1560
4. Zack, J. A., Arigo, S. J., Witzman, S. R., Go, A. S., Haislip, A., and Chen, I. S. Y. (1990) Cell 61, 213-222
5. Zack, J. A., Haislip, A. M., Krogstad, P., and Chen, I. S. Y. (1993) J. Virol. 67, 1717-1720
6. Gao, W. X., Cao, A., Gallo, R. C., and Lori, F. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8925-8928
7. Bukrinsky, M. I., Stanwick, T. L., Dempsey, M. P., and Stevenson, M. (1992) Science 254, 425-427
8. Bagasra, O., Hauptman, S. P., Lischer, H. W., Sachs, M., and Pomerantz, R. J. (1992) N. Engl. J. Med. 326, 1385-1391
9. Mitsuya, H., Yarchoan, R., and Broder, S. (1990) Science 248, 1533-1544
10. Yarchoan, R., Pluda, J. M., Perne, C. F., Mitsuya, H., and Broder, S. (1991) Blood 78, 859-884
11. Johnston, M. I., and Roth, D. F. (1989) Science 260, 1296-1293
12. Guo, W. Y., Shiranska, T., Johns, D. G., Broder, S., and Mitsuya, H. (1993) J. Clin. Invest. 91, 3262-3263
13. Yarchoan, R., Lietzau, A. J., Nguyen, B.-Y., Brawley, O. W., Pluda, J. M., Wyyll, K. M., Steinberg, S. M., Agarica, R., Mitsuya, H., and Broder, S. (1993) J. Infect. Dis. 168, 810-817
14. Shirasaka, T., Yarchoan, R., O'Brien, M., husson, R., Anderson, B., Kojima, E., Broder, S., and Mitsuya, H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 562-566
15. Hartman, N. R., Ahluwalia, G. S., Cooney, D. A., Mitsuya, H., Kageyama, S., Fridland, A., Broder, S., and Johns, D. G. (1991) Mol. Pharmacol. 40, 118-124
16. Sherman, P. A., and Fye, A. J. (1989) Anal. Biochem. 180, 222-225
17. Munche-Petersen, B., Blos, L., Trysted, G., and Eriksson, S. (1991) J. Biol. Chem. 266, 9092-9098
18. Arnér, E. S. J., Valentin, A., and Eriksson, S. (1992) J. Biol. Chem. 267, 10983-10975
19. Eriksson, S., Kardaszuk, B., Munche-Petersen, B., Oberg, B., and Johansson, N. G. (1991) Biochem. Biophys. Res. Commun. 178, 568-592
20. Starnes, M. C., and Cheng, Y.-C. (1987) J. Biol. Chem. 262, 988-991
21. Chang, C.-N., Skalski, V., Zhou, J. H., and Cheng, Y.-C. (1992) J. Biol. Chem. 267, 29414-29420
22. Perne, C. F., Yarchoan, R., Cooney, D. A., Hartman, N. R., Webb, D. S., Hao, Z., Mitsuya, H., Johns, D. G., and Broder, S. (1989) J. Exp. Med. 169, 933-951
23. Furman, P. A., Fye, A. J., Brown, S., Weinhold, K., Ribeout, J. L., Freeman, G. A., Nussinov Liemahn, S., Bolognesi, D. P., Broder, S., Mitsuya, H., and Barry, D. W. (1990) Proc. Natl. Acad. Sci. U. S. A. 83, 8333-8337
24. Jackson, R. C. (1989) The Role of Ribonucleotide Reductase in Regulation of the Deoxyribonucleoside Triphosphate Pool Composition: Studies with a Kinetic Model, pp. 127-150, Pergamon Press Inc., Elmsford, NY.
25. Johnson, J. A., and Fyfe, P. (1989) Mol. Pharmacol. 36, 291-295
26. Masood, R., Ahluwalia, G. S., Cooney, D. A., Fridland, A., Marquez, V. E., Driscoll, J. S., Hao, Z., Mitsuya, H., Perne, F. F., Broder, S., and Johns, D. G. (1990) Mol. Pharmacol. 37, 590-596
27. Coates, J. A. V., Canning, N., Jenkinson, H. J., Jowett, A. J., Jowett, M. I., Pearson, B. A., Penn, C. R., Figueiredo, H. T., Gray, N. M., Boehme, R., and Cameron, J. M. (1990) Antimicrob. Agents Chemother. 34, 733-739
28. Hart, G. J., Orr, D. C., Penn, C. R., Figueiredo, H. T., Gray, N. M., and Cameron, J. M. (1989) Antimicrob. Agents Chemother. 36, 1688-1694
29. Marquez, V. E., Tseng, C. K.-H., Driscoll, J. S., Mitsuya, H., Broder, S., Roth, J. S., and Kelly, J. A. (1987) Biochem. Pharmacol. 36, 2719-2722
30. Marquez, V. E., Tseng, C. K.-H., Mitsuya, H., Kojima, E., Kelley, J. A., Ford, H. J., Roth, J. S., Broder, S., Johns, D. G., and Driscoll, J. S. (1986) J. Med. Chem. 29, 978-985
31. Ruxrungh, K., Boone, E., Ford, J. J., Driscoll, J. S., Davey, R. T., Jr., and Lane, H. C. (1993) IX International AIDS Conference, Berlin, June 6-11, 1993 (Abstract PO-A18-0354)