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Inhibition of ribonucleotide reductase by 2′-substituted deoxycytidine analogs: Possible application in AIDS treatment

(deoxyribunucleotide pools/3′-azidothymidine/cell cycle)

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ABSTRACT **After phosphorylation to the corresponding diphosphates, 2′-azido-2′-deoxycytidine and 2′-difluorocytidine act as powerful inhibitors of ribonucleotide reductase. Phosphorylation requires deoxycytidine kinase, an enzyme with particularly high activity in lymphoid cells. Therefore, the deoxycytidine analogs can be expected to inhibit the reductase with some specificity for the lymphoid system. Pretreatment of human CEM lymphoblasts with the analogs considerably increased the phosphorylation of 3′-deoxy-3′-azidothymidine (AzT). The increased phosphorylation of AzT is caused by a prolongation of the S phase of the cell cycle. Our results suggest the possibility of a combination of 2′-substituted deoxycytidine analogs with AzT in the treatment of AIDS. Gao *et al.* [Gao, W.-Y., Cara, A., Gallo, R. C. & Lori, F. (1993) *Proc. Natl. Acad. Sci. USA 90,* 8925–8928] have suggested the use of the ribonucleotide reductase inhibitor hydroxyurea for this purpose, since the resulting decrease in the size of deoxyribonucleotide pools decreases the processivity of the HIV reverse transcriptase. From our results it would appear that the 2′-substituted deoxycytidine analogs might be preferable to hydroxyurea.**

Transcription of the single-stranded RNA of the human immunodeficiency virus type 1 (HIV-1) into double-stranded DNA is important for the maintenance of viral infection (1). This process involves the polymerization of the four deoxyribonucleoside triphosphates (dNTPs) by the viral reverse transcriptase. Certain deoxynucleotide analogs inhibit this reaction rather specifically, and among them the 5′-triphosphate of 3′-deoxy-3′-azidothymidine (AzTTP) has found clinical application (2). The analog is administered as the nonphosphorylated nucleoside and, to become an inhibitor of the reverse transcriptase, must be phosphorylated by the cellular kinases normally involved in the phosphorylation of thymidine and thymidine phosphates (3). In cultured human CEM lymphoblasts, the phosphorylation of AzT was increased considerably by low concentrations of hydroxyurea, a known inhibitor of the enzyme ribonucleotide reductase (4). This host enzyme provides the dNTPs required for both host and viral DNA synthesis. The mechanism for the accelerated phosphorylation of AzT was not clarified, but it was suggested that the observed effect might be of clinical interest for combination therapy of HIV infection with AzT and hydroxyurea.

Interest in the potential use of hydroxyurea in AIDS therapy obtained a renewed impetus from the recent work of Gao *et al.* (5). From studies of viral DNA replication in infected resting lymphocytes, these authors concluded that the activity of the reverse transcriptase critically depends on dNTP pool sizes. Indeed, resting lymphocytes harbor a pool of incomplete HIV DNA possibly of importance for HIV-1 pathogenesis (6). In stimulated lymphocytes suboptimal dNTP concentrations, induced by inhibition of ribonucleotide reductase with hydroxyurea, reduced the processivity of reverse transcriptase and produced incomplete DNA molecules (5). Thus, it was suggested that low doses of hydroxyurea, alone or in combination with nucleotide analogs, could be employed in clinical trials. Also other recent work (7) suggests that inhibition of dNTP synthesis *per se* might be of benefit in AIDS therapy.

In this context it seemed likely that the diphosphates of various 2′-substituted deoxycytidine analogs might have a theoretical advantage over hydroxyurea as inhibitors of ribonucleotide reductase. The phosphorylation of the nucleoside analog depends on the activity of deoxycytidine kinase, an enzyme highly expressed in lymphoid cells (8). Therefore, inhibition of the reductase should mainly occur in such cells. Here we compare the effects of two such analogs, 2′-deoxy-2′-azidocytidine (AzC) and 2′-deoxy-2′-difluorocytidine (F_2C), with that of hydroxyurea on the phosphorylation of AzT in cultured CEM cells. All three drugs strongly stimulate AzT phosphorylation, apparently by a delay of the cell's progression through the cell cycle.

MATERIALS AND METHODS

Materials. [^{3}H]AzT was from Moravec Biochemicals (La Brea, CA). The drug was diluted with unlabeled AzT (Sigma) to a final specific activity between 810 and 11,000 cpm/pmol. AzC was obtained from F. Eckstein (Göttingen, Germany) and was purified by HPLC (9) before use. F_2C (10) was a gift from J. Liliemark (Stockholm).

Cell Culture and Isotope Experiments. CEM cells were cultured as described (11). Experiments were started by explanting cells (300,000 per 1.5 ml) in the exponential growth phase into separate wells of a 24-well microtiter plate. After 12 to 24 h of incubation at 37°C, inhibitors of ribonucleotide reductase were added, and incubation was continued for the indicated time periods before addition of [^{3}H]AzT.

Determination of AzT Phosphorylation. Parallel cultures of cells were harvested (11) and used (*i*) to determine cell growth by counting in a hemocytometer, (*ii*) to analyze their DNA content by flow cytometry (12), and (*iii*) to extract with 60% (vol/vol) methanol for the determination of dNTP pools (13) and AzT phosphorylation. The total radioactivity of the methanolic extract gives the total phosphorylation of AzT. To obtain the amounts of AzTTP and AzTDP, a portion of the extract was separated by HPLC on a Nucleosil 100-5 C_{18} (250 × 4.6 mm) column by isocratic elution with 0.1 M ammonium phosphate, pH 3.5/20% methanol at a flow rate of 1 ml/min. This separated AzTTP (3.5–4.5 min) from AzTDP (4.5–6 min) and AzTMP (7.5–9.5 min). The relative amounts of each

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Abbreviations: AzC, 2′-deoxy-2′-azidocytidine; AzT, 3′-deoxy-3′-azidothymidine; F_2C, 2′-deoxy-2′-difluorocytidine; HIV, human immunodeficiency virus.

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Table 1. Ribonucleotide reductase inhibitors increase the phosphorylation of AzT

Inhibitor	Conc., μM	Growth inhibition,* %	AzT nucleotides: Total, pmol per 10^6 cells	AzTTP, % of total	AzTDP, % of total
Control	—	0	47	1.4	4.5
HU	100	53	124	1.5	4.2
	200	87	232	1.2	4.4
AzC	1	42	59	1.8	4.7
	3	45	126	2.0	6.1
	5	66	167	2.0	4.6
F_2C	0.010	45	70	1.9	4.9
	0.020	45	99	1.7	4.7
	0.030	82	174	1.8	4.0

Inhibitors were added at the indicated concentrations 24 h from seeding CEM cells in individual wells. After an additional 24 hr the cells were incubated with [^{3}H]AzT (final concentration, 0.3 μM) for 5 h, removed from the wells, counted, and analyzed for AzT phosphorylation. HU, hydroxyurea.
*Growth inhibition was calculated from the difference in increase in cell number between the treated and control culture during the 29 h of the experiment.

nucleotide were calculated from the total radioactivity in the relevant fractions.

In some experiments the medium after removal of cells by centrifugation was deproteinized (11) and analyzed by HPLC. To separate AzT from thymine, an aliquot of deproteinized medium was separated by HPLC as described above by using 20% methanol in water as eluent. Thymine appeared between 4.5 and 6 min, and AzT appeared between 16 and 18 min. To separate AzT from AzT phosphates, a portion of the medium was added to a 1-ml column of Dowex-1-Cl^-, and AzT was removed by 10 ml of 25 mM KCl. AzT phosphates were subsequently recovered from the column by elution with 3 ml of 0.3 M HCl/25 mM KCl and quantitated from the radioactivity in this fraction.

Flow Cytometric Analyses. Cells (1×10^6) were centrifuged, washed and fixed with 2 ml of cold 70% ethanol, and stored at 4°C until processing (2–4 days). Fixed cells were washed with phosphate-buffered saline and then stained at 37°C for 30 min with 0.12% sodium citrate containing 0.05 mg of propidium iodide (Sigma) and 1 mg of pancreatic ribonuclease (Sigma) per ml. After overnight incubation in the dark at 4°C, flow-cytometric analysis of DNA (12) was performed by using an Epics-Elite flow cytometer (Coulter) equipped with a 488-nm argon ion laser running at 15 mW. Propidium iodide fluorescence of at least 10^4 cells was analyzed for each sample.

RESULTS

Comparison of the Effects of Hydroxyurea, AzC, and F_2C. Parallel cultures of exponentially growing CEM cells were pretreated for 24 h with various concentrations of the three drugs. [^{3}H]AzT was added to a low concentration (0.3 μM), and after 5 h the amounts of mono-, di-, and triphosphates of AzT were determined. Table 1 shows that all three drugs inhibited cell growth and stimulated the phosphorylation of AzT. The inhibition of cell growth was not a general toxic effect that resulted in cell death, as shown by the flow cytometric analyses described below. The stimulation of AzT phosphorylation increased when the growth inhibition became more severe. F_2C gave clear effects in the nanomolar range, whereas a 100-fold higher concentration of AzC or a 10,000-fold higher concentration of hydroxyurea was required. In all cases, AzTMP accounted for almost 95% of the total AzT nucleotides, and AzTTP, the nucleotide responsible for the inhibition of reverse transcriptase, accounted only for between 1.2% and 2.0%. Thus, the three drugs stimulated not only the phosphorylation of AzT but also that of AzTMP and AzTDP. The effect of the two deoxycytidine analogs was identical to that of hydroxyurea, aside from the differences in drug concentration.

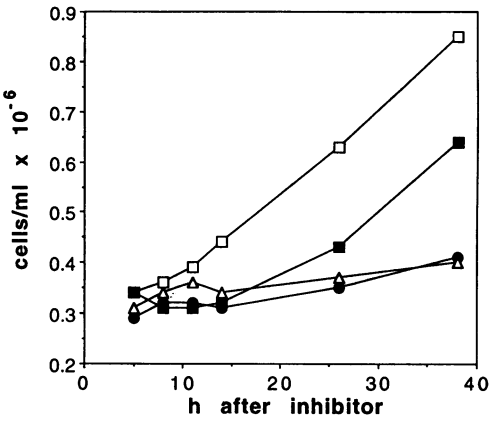

FIG. 1. Effects of ribonucleotide reductase inhibitors on the growth of CEM cells. Hydroxyurea (final concentration, 150 μM) (●), AzC (3 μM) (△) or F_2C (30 nM) (■) was added to CEM cells 14 h after seeding. At the indicated time intervals, duplicate samples of the treated and control (□) cells were collected and counted in a hemocytometer.

We next investigated how the concentration of AzT in the medium affected the results by comparing the stimulatory effect of the inhibitors in the presence of 0.1 and 1.0 μM AzT (Table 2). The total amount of AzT nucleotides was increased 10-fold when AzT was at 1 μM compared with 0.1 μM, showing that in this concentration range the phosphorylation of AzT was directly proportional to its concentration. However, this did not apply to the next two phosphorylation steps, since AzTDP and AzTTP did not increase in proportion to AzT concentration. At 0.1 μM AzT the sum of AzTDP + AzTTP represented approximately 10% of the total AzT nucleotides, whereas at 1 μM AzT it amounted to only about 5%. Cells treated with the deoxycytidine analogs did not differ from the controls in this respect.

Table 2. Correlation between AzT phosphorylation and AzT concentration

Inhibitor	Conc., μM	Growth inhibition, %	AzT nucleotides, pmol per 10^6 cells: 0.1 μM AzT Total	0.1 μM AzT AzTTP	0.1 μM AzT AzTDP	1 μM AzT Total	1 μM AzT AzTTP	1 μM AzT AzTDP
Control	—	0	9	0.1	0.6	78	1.2	2.0
AzC	2	88	19	ND	ND	180	ND	ND
	3.3	65	17	0.4	1.7	165	Lost	6.5
F_2C	0.020	65	14	ND	ND	140	ND	ND
	0.030	100	23	0.5	2.0	254	4.3	6.6

The inhibitors were added 24 h after seeding. After an additional 12 h, [^{3}H]AzT (either 0.1 or 1 μM) was added and incubation was continued for 5 h. At the end of this time period, the amount of intracellular AzT nucleotides was determined, and cells from parallel cultures (without AzT) were counted to determine the inhibition of growth. ND, not determined.

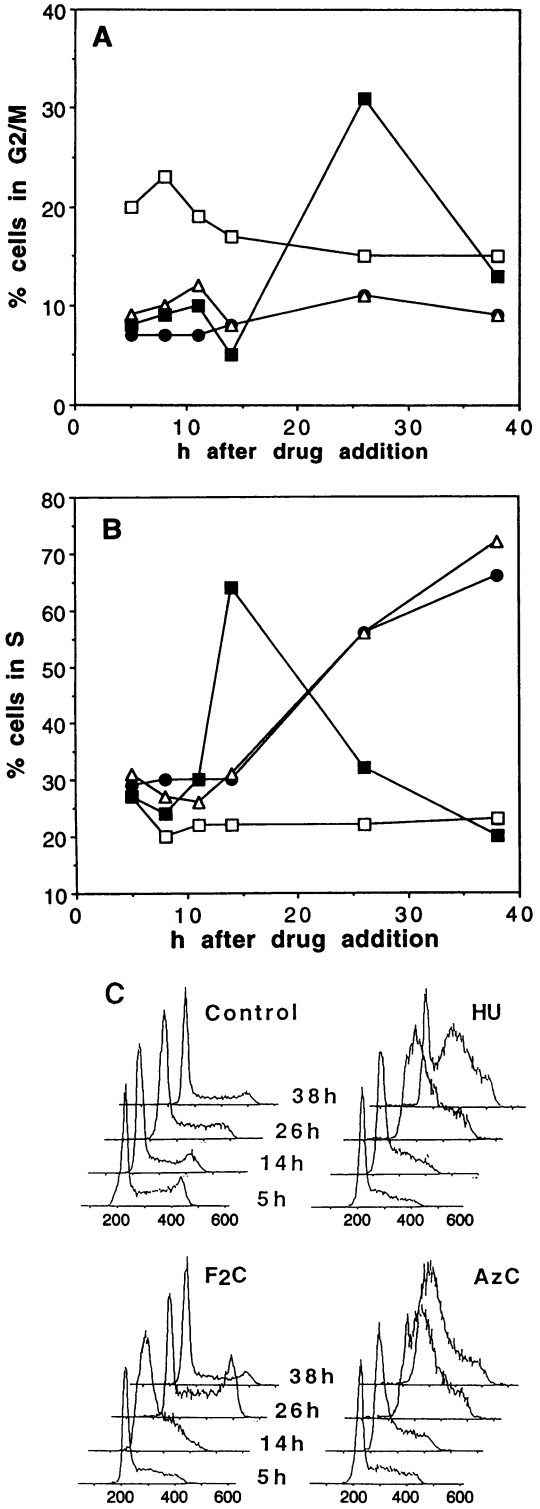

FIG. 2. Cell cycle analyses by flow cytometric DNA determinations during treatment with ribonucleotide reductase inhibitors. Parallel samples of the cells described in Fig. 1 were analyzed. Cells were treated with hydroxyurea (●), AzC (△), or F_2C (■). □, Controls. (*A*) Percentage of cells in G_2 and M phases. (*B*) Percentage of cells in S phase. The values in *A* and *B* were calculated from DNA fluorescence patterns shown in *C* in which the second and third time points are not included. The ordinate in *C* indicates the relative number of cells, and the abscissa indicates the mean fluorescence channels. HU, hydroxyurea.

Stimulation of AzT Phosphorylation Requires Preincubation with Drugs. In the experiments described above, AzT phosphorylation was determined after pretreatment with reductase inhibitors for 12 or 24 h. To assess the effect of pretreatment more closely, we determined AzT phosphorylation at various time points in cells pretreated with the inhibitors from 3 to 36 h. We also determined the effect of the drugs on cell growth and progression through the cell cycle.

During the first 14 h after addition of the three drugs, cell replication was almost completely blocked, as shown by the growth curves in Fig. 1. After that time, cells started to divide again, slowly in the hydroxyurea- and AzC-inhibited cultures, with a 30% increase in cell number between 14 and 38 h. With F_2C, the recovery was more rapid and resulted in a doubling of cell number during the same time period. The nontreated control cultures grew exponentially with a 24-hr generation time during the whole course of the experiment.

Cell cycle analyses by flow cytometry shown in Fig. 2 demonstrate that the drugs profoundly affected the length of the cell cycle and the distribution of cells in the different growth phases. Five hours after drug addition, the percentage of cells in G_2/M had decreased to almost one-third (Fig. 2*A*), suggesting that drug treatment had severely diminished the recruitment of cells from S phase without affecting the exit from G_2/M. Already at this time the number of cells in early S phase was slightly increased, and this increase became still more apparent at later time points (Fig. 2*B*). From the DNA fluorescence patterns (Fig. 2*C*), it is apparent that the distribution of cells within the S phase changed with time in the drug-treated cultures. At first, early S-phase cells dominated and later were replaced by cells in middle and late S phase, indicating a slower progression through S phase. Note that in the F_2C-treated cultures, the delay in S phase lasted for a shorter time period and that the DNA pattern after 38 h had reverted to that of the control.

AzT phosphorylation (Fig. 3) was stimulated to some extent at all time points by the drugs, but the magnitude of the effect was time dependent. Between 3 and 5 h, AzT phosphorylation was on the average augmented only 1.2-fold, whereas after 12–14 h the stimulation was 2.6-fold. With AzC and hydroxyurea the stimulation remained high to the end of the experiment, whereas the effect of F_2C declined rapidly. A comparison of these results with the cell cycle data (Fig. 2) correlates the increase in phosphorylation with the effects of the drugs on the cell cycle. AzC and hydroxyurea maintained their effect to the very end of the experiment, with a large portion of the cells in S phase, whereas the effect of F_2C was more short-lived and disappeared after 38 h.

Accumulation of AzT Nucleotides During Drug Treatment. To determine the extent of AzT phosphorylation, labeled

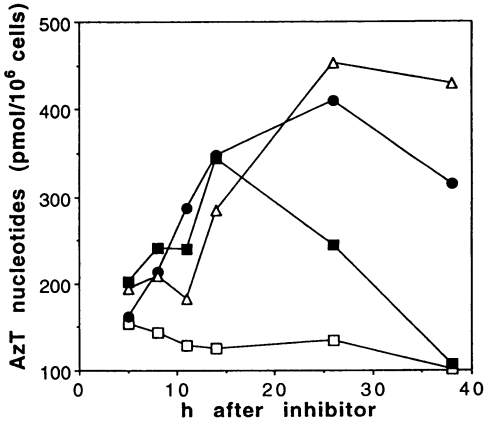

FIG. 3. Time course of AzT phosphorylation. Cells described in Fig. 1 were pulsed with 1 μM [^{3}H]AzT for 2 h at different times after addition of inhibitor: hydroxyurea (●), AzC (△), or F_2C (■). The time on the abscissa denotes the end of the pulse. The phosphorylation of AzT was calculated from the total radioactivity present in the 60% methanolic extract of cells. □, Controls.

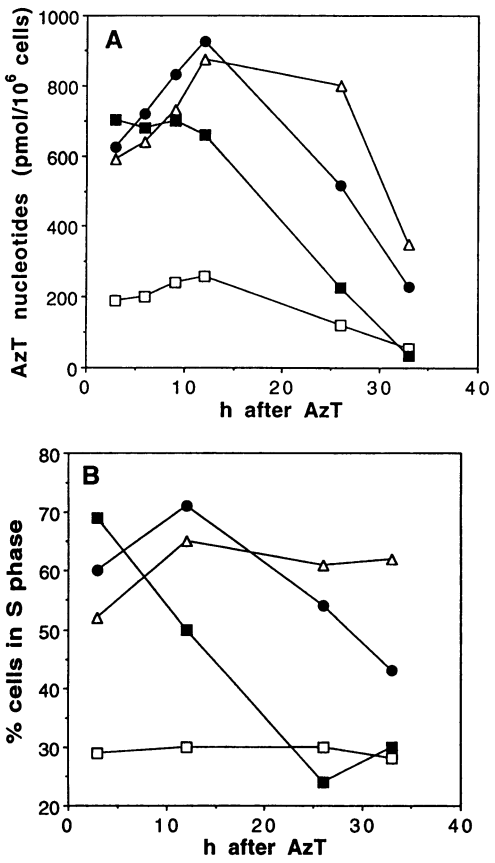

FIG. 4. Accumulation of AzT nucleotides during inhibition. The inhibitor hydroxyurea (●), AzC (△), or F_2C (■) was added to cells 14 h after seeding; 10 h after their addition, 1 μM [^{3}H]AzT was added and incubations were continued. At the indicated times (with the addition of AzT as time zero), samples were removed and analyzed. (*A*) Amount of AzT phosphorylated. (*B*) Percentage of cells in S phase calculated from the flow cytometric DNA determinations. □, Controls.

AzT was added to untreated control cultures and to cultures pretreated with the three drugs for 10 h. Parallel samples were removed at time intervals between 3 and 33 h after AzT addition and analyzed for AzT phosphorylation (Fig. 4*A*) and for cell cycle distribution (Fig. 4*B*).

In the control cultures, AzT nucleotides increased slowly by 50% between 3 and 12 h, but then declined to 30% of the original value after 33 h. Hydroxyurea, AzC, and F_2C had all increased AzT phosphorylation 3- to 4-fold after 3 h. Thereafter the effects of the three drugs ran different time courses. In the hydroxyurea- and AzC-treated cultures, the amount of AzT nucleotides showed an increase of about 50% between 3 and 12 h, which was then followed by a decrease. Note, however, that the decrease occurred later with AzC. Compared with the controls, AzT phosphorylation was 3 to 4 times greater at all time points. The stimulation by F_2C was already maximal after 3 h and then decreased continuously, with the 26- and 33-h values not differing from the corresponding control values.

A comparison of these results with the cell cycle analyses (Fig. 4*B*) is illuminating. In the control cultures, approximately 30% of the cells were in S phase at all times. In the cultures pretreated with drugs, twice as many cells were already in S phase at the first time point, 3 h after addition of AzT (but 13 h after drug addition). In the AzC-treated cultures, this value remained constant at all times; also the hydroxyurea-treated cells maintained a large percentage of cells in S phase, even though there was a clear decline after 33 h. The situation was different with F_2C: between 3 and 12 h, the number of S-phase cells decreased from 68% to 50% and after 26 h did not differ from the control.

The DNA fluorescence pattern in this experiment was similar to results shown in Fig. 2*C* (data not shown). In the controls no redistribution of cells between cycle phases had occurred during the time of the experiment. In the drug-treated cultures, early S-phase cells dominated at the 3-h time point, to be replaced in time first by middle and then by late S-phase cells. The passage of cells through S phase was fastest in the F_2C-treated cultures and slowest during AzC treatment. These results parallel the effects of the drugs on AzT phosphorylation.

One final aspect of this experiment concerns the decline of AzT nucleotides at later time points. One possibility was that AzT was exhausted because of metabolism of the drug. However, the medium analyzed at different time points only contained small amounts of AzTMP (14) and no thymine; at the end of the experiment close to 90% of the original AzT was still present (data not shown).

Changes in dATP and dTTP Pools Caused by Inhibitors. Inhibition of ribonucleotide reductase in cultured cells primarily decreases the size of the dATP pool (9). Addition of the reductase inhibitors at the concentrations used in the experiment of Fig. 2 after 5 h decreased the dATP pool of CEM cells from a value of 76 pmol per 10^6 cells in the controls to values of 53 (hydroxyurea), 55 (AzC), and 54 (F_2C). This demonstrates that F_2C acted as a reductase inhibitor, similar to hydroxyurea and AzC. The decrease in pool size may appear small. However, the inhibitors affect primarily cells in S phase, which constitute only between 20% and 30% of the total cell population. In S-phase cells, the decrease was probably larger.

The inhibitors had a complicated effect on the dTTP pool. In the experiment shown in Fig. 2, the inhibitors increased the pool by 30% during the first 5 h, but later decreased it to 70–80% of the control. The comparison is obscured by the fact that the inhibited cells differed from the controls in their distribution in the cell cycle. Thus, at later times a majority of the inhibited cells, but only a little more than 20% of the controls, were in S phase. The low dTTP pool of the inhibited cells conceivably contributed to the stimulation of AzT phosphorylation, since dTTP inhibits thymidine kinase, the enzyme responsible for AzT phosphorylation.

DISCUSSION

Similar to hydroxyurea, the two deoxycytidine analogs AzC and F_2C increase the phosphorylation of AzT by human lymphoblastoid CEM cells. Hydroxyurea and the diphosphate of AzC scavenge the tyrosyl radical of ribonucleotide reductase (15) and thereby inactivate the enzyme. In cultured cells the resulting decrease in the production of dNTPs primarily gives a diminished dATP and dGTP pool, with concomitant inhibition of DNA synthesis (9, 16). Also F_2C inhibits ribonucleotide reductase (17) but is, in addition, incorporated into DNA (18), and this gives an essentially irreversible inhibition of chain elongation. At the low concentrations of the analog used in the present experiments, inhibition was reversible and therefore probably due to an effect of the drug on the reductase.

Our flow cytometric DNA analyses demonstrate that the three drugs delayed the passage of cells through the S phase of the cell cycle. This is indicated by the large drop in the percentage of cells in the G_2 and M phases at early times after addition of drugs (Fig. 2*A*). Thereafter, cells accumulated in late G_1 and early S phases and finally shifted progressively to middle and late S phase (Fig. 2 *B* and *C*). It was shown earlier that hydroxyurea can block the cell cycle at the G_1/S boundary and be used to synchronize cultured cells (19). In our experiments the inhibition of DNA synthesis was incomplete and resulted in a slowdown of strand elongation, with

partial synchronization, and gradual accumulation of the cells in S phase.

The increase in phosphorylation of AzT closely followed the changes in the frequency distribution. This does by itself not prove a causal relationship but strongly suggests one. Phosphorylation of AzT is mainly catalyzed by the cytoplasmatic thymidine kinase (20), a cell cycle-regulated enzyme with highest activity in late G_1 and S phases. Therefore, one can expect that an accumulation of cells in these growth phases will increase the activity of the enzyme in the cell population and the phosphorylation of AzT.

At the chosen concentration of the inhibitors, each had the same effect during the first 14 h. After that time the effects of F_2C declined rapidly, compared with those of hydroxyurea and AzC. This might be explained by degradation of F_2C nucleotides by dCMP deaminase (21). The "normalization" of the cell cycle (Fig. 2*C*) and the drop in AzT phosphorylation (Fig. 3) were parallel phenomena, strengthening our concept that the increased phosphorylation of AzT is caused by the effect of the drug on the cell cycle.

In connection with AIDS therapy, our results have two main implications. The first concerns the possibility to achieve a more selective effect in lymphoid cells by choosing deoxycytidine analogs as inhibitors of ribonucleotide reductase rather than hydroxyurea. The second implication is that the use of any inhibitor of the reductase will interfere with the normal passage of cells through the cell cycle. This effect will potentiate the activity of AzT and other thymidine analogs, if they are combined with the reductase inhibitors. In addition to the direct inhibition of reverse transcriptase they will, however, also interfere with cell replication. By using a low dosage of deoxycytidine analogs, it might be possible to minimize the toxic effects and limit them to the lymphoid system. On balance, it does not seem unreasonable to test such an approach in an appropriate animal system.

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1. Vaishnav, Y. & Wong-Staal, F. (1992) *Annu. Rev. Biochem.* **60,** 578–630.
2. Mitsuya, H., Yarchoan, R., Kageyama, S. & Broder, S. (1991) *FASEB J.* **5,** 2369–2381.
3. Mitsuya, H., Weinhold, K. J., Furman, P. A., St. Clair, M. H., Nusinoff Lehrman, S., Gallo, R. C., Bolognesi, D., Barry, D. W. & Broder, S. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 7096–7100.
4. Karlsson, A., Reichard, P. & Eckstein, F. (1989) *Eur. J. Biochem.* **186,** 689–694.
5. Gao, W.-Y., Cara, A., Gallo, R. C. & Lori, F. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8925–8928.
6. Zack, J. A., Haislip, A. M., Krogstad, P. & Chen, I. S. Y. (1992) *J. Virol.* **66,** 1717–1725.
7. Meyerhans, A., Vartanian, J. P., Hultgren, C., Plikat, U., Karlsson, A., Wang, L., Eriksson, S. & Wain-Hobson, S. (1994) *J. Virol.* **68,** 535–540.
8. Durham, J. P. & Ives, D. H. (1969) *Mol. Pharmacol.* **5,** 358–375.
9. Åkerblom, L. & Reichard, P. (1985) *J. Biol. Chem.* **260,** 9197–9202.
10. Hertel, L. W., Kroin, J. S., Misner, J. W. & Tustin, J. M. (1988) *J. Org. Chem.* **53,** 2406–2409.
11. Bianchi, V., Ferraro, P., Borella, S., Bonvini, P. & Reichard, P. (1994) *J. Biol. Chem.* **269,** 16677–16683.
12. Gray, J. W. & Coffino, P. (1979) *Methods Enzymol.* **58,** 233–248.
13. Lindberg, U. & Skoog, L. (1970) *Anal. Biochem.* **34,** 152–160.
14. Fridland, A., Connelly, M. C. & Ashmun, R. (1990) *Mol. Pharmacol.* **37,** 665–670.
15. Thelander, L., Larsson, B., Hobbs, J. & Eckstein, F. (1976) *J. Biol. Chem.* **251,** 1398–1405.
16. Bianchi, V., Pontis, E. & Reichard, P. (1986) *J. Biol. Chem.* **261,** 16037–16042.
17. Heinemann, V., Xu, Y. Z., Chubb, S., Sen, A., Hertel, L. W., Grindey, G. B. & Plunkett, W. (1990) *Mol. Pharmacol.* **38,** 567–572.
18. Huang, P., Chubb, S., Hertel, L. W., Grindey, G. B. & Plunkett, W. (1991) *Cancer Res.* **51,** 6110–6117.
19. Hashihara, T. & Baserga, R. (1979) *Methods Enzymol.* **58,** 248–262.
20. Munch-Petersen, B., Cloos, L., Tyrsted, G. & Eriksson, S. (1991) *J. Biol. Chem.* **266,** 9032–9038.
21. Xu, Y. Z. & Plunkett, W. (1992) *Biochem. Pharmacol.* **44,** 1819–1827.