0026-895X/90/020237-07$02.00/0

MOLECULAR PHARMACOLOGY, 38:237-243



# 2′,3′-Dideoxycytidine Toxicity in Cultured Human CEM T Lymphoblasts: Effects of Combination with 3′-Azido-3′-deoxythymidine and Thymidine

YVONNE TÖRNEVIK and STAFFAN ERIKSSON

*Medical Nobel Institute, Department of Biochemistry I, Karolinska Institute, Stockholm, Sweden*

Received January 2, 1990; Accepted May 16, 1990

**SUMMARY**

2′,3′-Dideoxycytidine (ddCyd), a potent inhibitor of human immunodeficiency virus DNA replication, requires phosphorylation by cellular nucleoside kinases for antiviral activity. Deoxycytidine kinase (NTP:deoxycytidine 5′-phosphotransferase, EC 2.7.1.74) is responsible for the formation of dideoxycytidine monophosphate and this enzyme is controlled by feedback regulation by the natural endproduct, dCTP. We have examined whether a decrease in intracellular dCTP levels affects the growth inhibition caused by ddCyd, as well as the capacity to accumulate dideoxycytidine triphosphate (ddCTP), using human T lymphoblast (CEM) cells in culture. Subtoxic concentrations of thymidine were used to decrease the dCTP pool. The effects of 3′-azido-3′-deoxythymidine (AZT), alone or in combination with ddCyd, on cell growth, DNA precursor pools, and accumulation of ddCTP were also studied. The combination of ddCyd and thymidine led to growth inhibition of CEM cells that was twice what would be expected from addition, whereas the combination of AZT and ddCyd showed an additive effect. CEM cells accumulated ddCTP efficiently, so that with 10 μM ddCyd (corresponding to the $EC_{50}$ value) and a 6-hr incubation the ddCTP pool was 3-fold higher than the dCTP pool. Simultaneous addition of thymidine (10 μM) increased the dTTP pool 2-fold and gave a 50% reduction in the dCTP level but only a 10% increase in ddCTP accumulation. The presence of AZT (300 μM, corresponding to the $EC_{50}$ value) led, in contrast, to an elevation of dCTP and no significant change in the other DNA precursor pools. With this high concentration of AZT, the accumulation of ddCTP decreased 42%. It was also found that ddCyd is metabolized into two additional compounds, besides the dideoxycytidine mono-, di-, and triphosphate, i.e., the liponucleotides dideoxycytidine diphosphate-ethanolamine and dideoxycytidine diphosphate-choline, constituting 45 and 6% of the total phosphorylated ddCyd metabolites, respectively, whereas the mono-, di-, and triphosphate corresponded to 3, 21, and 25% of the phosphorylated dideoxynucleotides. These results indicate that the formation of dideoxycytidine monophosphate is not rate limiting in the synthesis of ddCTP in human lymphoblasts, which clearly differs from what was observed earlier in mouse cells (*Mol Pharmacol* **32**:798–806 1988). Furthermore, growth inhibition by ddCyd seems to be related to the ratio between dCTP and ddCTP. There was no direct interference between ddCyd and AZT metabolism in clinically relevant concentrations, which may encourage the use of combination of these compounds for anti-human immunodeficiency virus treatment.

A number of nucleoside analogs have been reported to inhibit the replication of HIV, the causative agent of acquired immunodeficiency syndrome, *in vitro* (1–5). One of these, AZT, has shown definite clinical effects by reducing morbidity and mortality in patients with acquired immunodeficiency syndrome (4, 6). Of the dideoxynucleosides studied to date, ddCyd has been shown to be the most potent antiviral agent (2, 4).

The mode of action of these two analogs is dependent on their phosphorylation by cellular enzymes in the cytoplasm to yield the corresponding 5′-triphosphates, which can compete with normal nucleotides for the retroviral reverse transcriptase enzyme. Incorporation of analogs with modified 3′-positions leads to termination of the growing viral DNA chain, and the selectivity is based on the fact that cellular α-polymerase binds these analogs with much lower affinity than reverse transcriptase (7–9).

Initial clinical trials with ddCyd have been performed and significant negative side effects in the form of peripheral neuropathy for both AZT and ddCyd have been observed (4, 10). The major toxicity of AZT is significant bone marrow suppression, and recently the emergence of AZT-resistant variants of HIV have limited the usefulness of this drug (10, 11). For this reason, combinations of antiretroviral drugs are of great inter-

This work was supported by grants from the Swedish Medical Research Council (MFR), the Swedish Cancer Society, the Medical Faculty of the Karolinska Institute, and the Swedish Board for Technical Development (STU).

**ABBREVIATIONS:** HIV, human immunodeficiency virus; ddCyd, 2′,3′-dideoxycytidine; ddCMP, 2′,3′-dideoxycytidine-5′-monophosphate; AZT, 3′-azidothymidine; ddCTP, 2′,3′-dideoxycytidine-5′-triphosphate; ddCDP, 2′,3′-dideoxycytidine-5′-diphosphate; HPLC, high performance liquid chromatography; FIC, fractional inhibitory concentration.

est, not only to increase their efficiency and reduce their toxicity but also to diminish the risk of developing drug-resistant viruses.

The combination of acyclovir and AZT has shown a higher anti-HIV-1 effect in ATH8 cell cultures than either of the drugs alone (3). Also, combinations of 2′,3′-dideoxynucleosides have been shown to give additive to synergistic effects in the inhibition of HIV-1 replication in MT-4 cells (12). Unexpectedly, antagonism between the anti-HIV activity of ribavarin and AZT (12, 13), as well as ribavarin and pyrimidine-2′,3′-dideoxynucleosides, has been reported (12).

Deoxycytidine kinase is responsible for the metabolic activation of ddCyd (7, 8, 14, 15) but the $K_m$ for this analog is approximately 60-fold greater than for the natural substrate dCyd. We have recently purified deoxycytidine kinase to homogeneity from human leukemic spleen and the enzyme (a dimer of two $M_r$ 30,000 subunits) showed a broad substrate specificity and efficiently phosphorylates cytosine and purine deoxyribonucleosides (16). The enzyme is controlled by feedback inhibition by dCTP (16, 17).

The object of this investigation was to examine whether a decrease in the intracellular concentration of dCTP alters the capacity of human T lymphoblasts to accumulate ddCTP from extracellular ddCyd, i.e., whether the activity of deoxycytidine kinase is rate limiting for the formation of ddCTP *in vivo*. We used subtoxic concentrations of thymidine, which is known to decrease the dCTP pool by allosteric inhibition of ribonucleotide reductase (18, 19). The capacity of AZT to mimic the effects of thymidine was also investigated, with regard to both growth inhibition and intracellular DNA precursor pool changes, using the well studied, HIV-infectable, human T lymphoblast cell line CEM as a model system.

Similar studies have been performed with mouse L1210 cells and in part also with ATH8 human T cells (14). However, our results differ in several, important aspects from these earlier studies. It appears that in CEM cells the activity of deoxycytidine kinase is not rate limiting in the accumulation of ddCTP from ddCyd.

## Materials and Methods

**Chemicals.** AZT and thymidine were bought from Sigma Chemical Company. Tritiated ddCyd was a gift from Drs. D. Johns and S. Broder, National Cancer Institute (Bethesda, MD) and was also bought from Moravek Biochemical Company. Tritiated 2′-deoxycytidine was bought from Amersham and ddCTP was purchased from Pharmacia.

**Cell culture.** The CCRF-CEM wild-type line, a malignant human T lymphoblastoid cell line, was kindly provided by B. Ullman, University of Oregon (Portland, OR). CEM cells were routinely propagated at 37° in Dulbecco's modified Eagle's medium with 10% heat-inactivated (56°, 30 min) horse serum, in a humidified 7% $CO_2$ atmosphere. Karyotype analysis of the cell line demonstrated that they were of human origin[1] and they were also routinely checked for *Mycoplasma* infection.

**Growth rate experiments.** The ability of the CEM cell line to survive and multiply in cell culture medium containing growth-inhibitory and cytotoxic agents was compared as follows. One-milliliter aliquots of cell culture medium containing $10^5$ cells were placed in Linbro multiwell (24-well) plates. Small aliquots (10–50 μl) of the respective growth-inhibitory agents were added. After approximately three divisions (72 hr) of exponential growth in control wells, cells were counted by using a cell counter (Analysis Instrument VDA 140). The difference between the final and the initial cell density was plotted as a percentage of the number of cells in control wells without drug added.

**Measurement of intracellular deoxynucleotide concentrations.** To ensure exponential and asynchronous growth, CEM cells were grown overnight. At a density of 0.6–0.8 × $10^6$ cells/ml in a 50-ml flask, the cells were incubated for different time periods (2–10 hr) with the different agents at the concentrations indicated. Cells were harvested by centrifugation at 200 × *g* for 10 min at room temperature, followed by two washes in cold phosphate-buffered saline. The nucleotides were extracted with 0.6 M trichloroacetic acid (250 μl), and the acid was removed according to the method of Khym (20).

When cells were incubated with tritiated deoxycytidine or ddCyd, 2 × $10^6$ cells in 2 ml of medium were incubated for 6 hr with 0.8 μM deyxocytidine or 1.0 μM ddCyd (specific activities, 6.5 and 2 Ci/mmol, respectively), and nucleotides were extracted as described above.

Nucleotides were separated by HPLC, using a Whatman Partisil 10-SAX anion exchange column and a Waters Associates absorbance detector (model 440). After oxidation of the ribonucleotides with sodium periodate in the presence of methylamine (21), the deoxynucleoside triphosphates were eluted isocratically with 0.4 M ammonium phosphate/2.5% acetonitrile, pH 3.4, at a flow rate of 1.5 ml/min. When mono-, di-, and triphosphates were measured, the column was eluted with a gradient (mainly as described in Ref. 22) from 10 mM ammonium phosphate, pH 3.8/7% methanol (buffer A) to 0.5 M ammonium phosphate, pH 3.8/7% methanol (buffer B). The gradient ran isocratically for 10 min at 0.5 ml/min using buffer A. From 10–11 min, flow was changed to 1 ml/min using buffer A. From 11 to 16 min, a linear gradient simultaneously changed the solvent from 100% buffer A to 100% buffer B and flow from 1.0 ml/min to 1.5 ml/min. Buffer B ran isocratically from 16 to 32 min. The column was reequilibrated by running a linear gradient from 32 to 37 min, changing buffer from 100% B to 100% A, and then was run isocratically using buffer A from 37 to 52 min at a flow rate of 2 ml/min. All deoxynucleotides were detected by their relative absorbances at 254 and 280 nm and quantified by comparison with known standards or, for the labeled extracts, by their specific activities. Radioactivity measurements on the eluent from the HPLC was done with an on-line radioactivity detector (FLO ONE/Beta Series A-200; Radiomatic Instruments & Chemical Co.) with a liquid scintillation cell (size 2.5 ml), using Insta-gel (Packard Instruments) as scintillation liquid.

Samples were treated with alkaline phosphatase (Sigma) and phosphodiesterase (from *Crotalus durissus*; Boehringer Mannheim) (in a reaction mixture containing 7 μM $MgCl_2$, 5 μM Tris·HCl, pH 9.3, 90 units alkaline phosphatase or phosphodiesterase, plus cell extract from 1 × $10^6$ cells, incubated at room temperature overnight) in order to identify which peaks correspond to dideoxynucleotides. The protein was precipitated with 5% trichloroacetic acid and neutralized according to the method of Khym (20). The metabolites were separated by chromatography both on Partisil 10-SAX, described above, and on Nucleosil C-18 (Scandinavian GeneTec AB) in 20 mM ammonium acetate, pH 5.0, where the ddCMP is separated from liponucleotide peaks.

## Results

**Growth rate determinations.** The sensitivity of CCRF-CEM cells to increasing concentrations of ddCyd was studied with or without simultaneous addition of thymidine or AZT. The effective concentration of ddCyd that inhibits cell growth by 50% (the $EC_{50}$ value) is 10 μM. When thymidine (1 and 3 μM, which were not growth inhibitory on their own) was added simultaneously with ddCyd, the inhibition curve was shifted downwards, i.e., the cells became more sensitive to ddCyd. The $EC_{50}$ values decreased from 10 μM ddCyd without thymidine to 7 and 5 μM with 1 and 3 μM thymidine added, respectively. Also, the effect of increasing concentrations of AZT was studied, with or without simultaneous additions of ddCyd. The $EC_{50}$

[1] L. Zech, personal communication.

value for AZT alone was 300 μM. When combined with ddCyd (2 and 4 μM, which led to 20 and 30% growth inhibition, respectively), the $EC_{50}$ values of AZT were decreased to 150 and 45 μM, respectively. Data from several experiments like these were evaluated by the isobologram method (23, 24), in which the $EC_{50}$ value was used for calculations of the FIC. The combination of thymidine and ddCyd was demonstrated to be synergistic, with a minimum FIC index of 0.4, whereas the combination of AZT and ddCyd was found to be additive to subsynergistic, with the minimum FIC index being 0.6 (Fig. 1).

**Intracellular deoxyribonucleotide concentrations.** The deoxyribonucleoside triphosphates were analyzed and quantified by HPLC after various additions. In these experiments, the concentration of ddCyd that caused 50% inhibition of cell growth (i.e., 10 μM) was used, alone or in combination with the $EC_{50}$ concentration of thymidine (10 μM), in order to make comparisons with the growth inhibition data relevant, and the time of incubation was varied from 2 to 10 hr. We found, by comparison with a known standard, that the ddCTP peak eluted between dATP and dGTP, with a retention time of 25.2 min (Fig. 2), in the isocratic HPLC system described above and, thus, could be determined directly without the use of radiolabeled compound. The results in Fig. 3A show that the ddCTP pool increases almost linearly with time for 6 hr. The dCTP pool, on the other hand, decreased throughout this time period (Fig. 3B). The ddCTP pool also increased almost linearly with increasing extracellular ddCyd up to 20 μM (Fig. 4). With 10 μM ddCyd and 6 hr of incubation, the ddCTP was 3-fold higher than the dCTP pool (Fig. 4 and Table 1).

When thymidine was added, the intracellular dCTP concentration decreased significantly (Figs. 3B and 4B and Table 1) as a consequence of feedback inhibition of CDP reduction because of the high dTTP level. Addition of ddCyd did not alter the effects produced by thymidine alone but, surprisingly, the accumulation of ddCTP was not enhanced significantly by the simultaneous presence of thymidine (10 μM) (Fig. 3 and Table 1). The effect of increasing concentrations of ddCyd was also studied, alone or in combination with 10 μM thymidine (Fig. 4), using 6 hr of incubation. The effect of thymidine addition on the ddCTP level was minimal.

The intracellular concentrations of the other deoxynucleotides, i.e., dTTP, dATP, and dGTP, were not significantly affected by addition of ddCyd (Table 1).

In contrast, when 300 μM AZT was added, the dCTP level increased more than 200% and the dTTP pool also increased, whereas dATP and dGTP were at normal levels (Table 1). A 40% decreased accumulation of ddCTP was observed when 300 μM AZT was added together with ddCyd (Table 1), whereas the other pools were not changed, as compared with 300 μM AZT alone. Addition of clinically relevant concentrations of AZT (10 μM) gave no effect on the accumulation of ddCTP from ddCyd (10 μM) during a 6-hr incubation (data not shown).

When radioactive ddCyd was used to label the cells, we could follow the intracellular phosphorylation of the analog. To determine the identity of the various radioactive peaks in the chromatogram, a portion was treated with alkaline phosphatase (7) to hydrolyze the nucleotides. We found that the peak at 19 min (Fig. 5) was totally resistant to this treatment, whereas the peak at 12 min was partially resistant (suggesting that it contains more than one compound). Both peaks were sensitive to phosphodiesterase cleavage. This result indicates that the compounds represent liponucleotides, presumably ddCDP-choline (19 min) and ddCDP-ethanolamine (12 min), and that ddCDP-ethanolamine coelutes with ddCMP on the Partisil 10-SAX column. To separate ddCMP from the liponucleotide ddCDP-ethanolamine, we used a reverse phase C-18 column where ddCMP and the presumed ddCDP-ethanolamine and ddCDP-choline elute at 27, 13.5, and 17 min, respectively (assuming that the dideoxyliponucleotides are eluting in the same order as the natural deoxynucleotides, i.e., dCDP-ethanolamine at 9 min, followed by dCDP-choline at 11 min) (Fig. 6). The peak at 27 min totally disappeared after treatment with alkaline phosphatase, demonstrating that it was ddCMP.

It was found (Fig. 5 and Table 2) that the intracellular pools of ddCDP and ddCTP are about equal and 8-fold higher than the ddCMP level. Addition of 30 μM AZT gave a slight reduction of the ddCDP level, whereas the ddCTP level was reduced 23% (Table 1). Preincubation for 12 hr with AZT did not give any additional effects, as was observed earlier with mouse L1210 cells by Balzarini *et al.* (14). With 30 μM thymidine added, the respective ddCyd nucleotide pools remained approximately the same (Table 2).

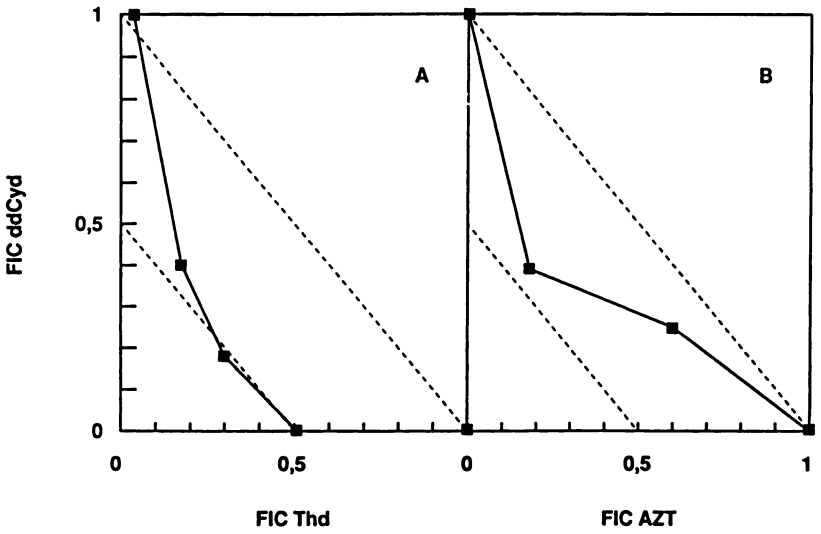


**Fig. 1.** Isobologram representation of the inhibitory effects of combinations of ddCyd and thymidine or AZT on the growth of CEM cells. The combined inhibitory effects of ddCyd and thymidine (*Thd*) (A) or AZT (B) on CEM cell growth were examined by checkerboard combinations of various concentrations of the respective agents. The 50% effective concentration ($EC_{50}$) was used for calculation of the FIC. When the minimum FIC index, which corresponds to the FIC of compounds combined (e.g., $FIC_x + FIC_y$), is equal to 1.0, the combination is additive. When it is between 1.0 and 0.5 the combination is subsynergistic, and when it is less then 0.5, it is synergistic. – – –, Unity lines for FIC equal to 1 and 0.5, respectively. Representative results from two to three experiments are shown.

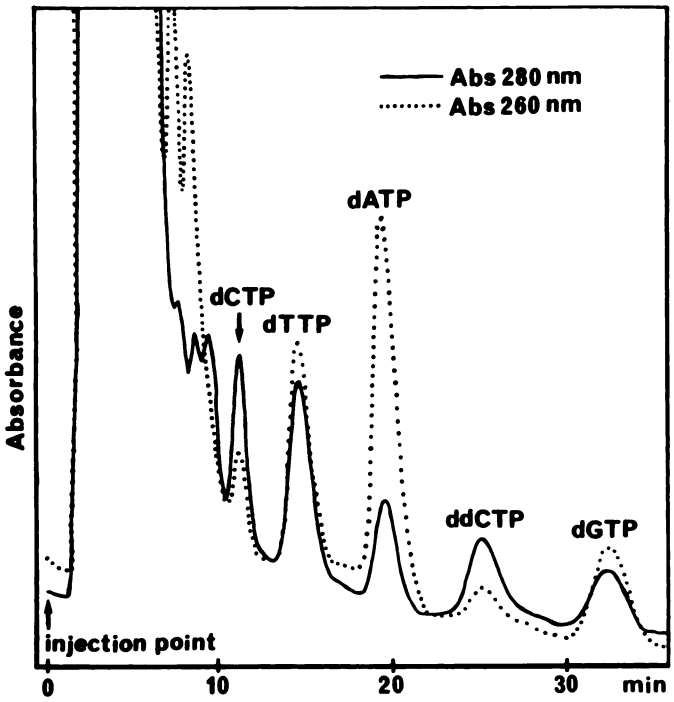


**Fig. 2.** Ion exchange (Partisil 10-SAX) HPLC elution profile of deoxynucleoside triphosphates in CEM cell extracts incubated with ddCyd. CEM cells were incubated for 6 hr with 10 μM ddCyd, the intracellular nucleotides were extracted with trichloroacetic acid, and the ribonucleotides were oxidized with periodate, as described in Materials and Methods. Extract from $10 \times 10^6$ cells was separated on a Partisil 10-SAX column in 0.4 M ammonium phosphate/2.5% acetonitrile, pH 3.4, and quantified by UV absorbance at 260 and 280 nm and comparison with known standards.

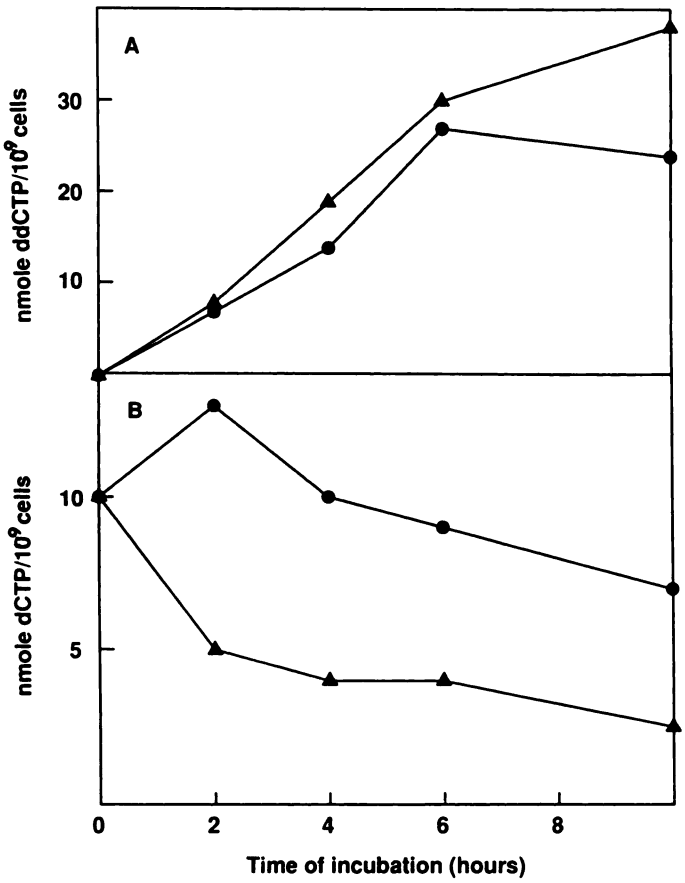


**Fig. 3.** Intracellular accumulation of ddCTP and dCTP in CEM cells incubated with ddCyd, alone or in combination with thymidine ddCTP (A) and dCTP (B) levels were determined by HPLC after 2–10 hr of incubation with 10 μM ddCyd alone (●) or in combination with 10 μM thymidine (▲), after prior periodate oxidation of the ribonucleotides, as described in Materials and Methods. The levels represent those of one typical experiment that has been repeated at least twice with the same results.

The labeling pattern with the natural deoxynucleoside [$^3$H] dCyd was different, in that about 21% of the intracellular nucleotides were present as dCTP, 4 and 1% as dCMP and dCDP, respectively, and 64 and 11% as the liponucleotides dCDP-choline and dCDP-ethanolamine (Table 2 and Fig. 5). These results are very similar to those reported by Spyrou and Reichard (22).

## Discussion

In an attempt to investigate the mechanism of toxicity of antiviral nucleoside analogs in human cells and specifically the role of the enzyme deoxycytidine kinase in the metabolism of ddCyd, we have used human CEM lymphoblasts as a model system. These cells are very sensitive to added deoxynucleosides, due to high anabolism and low catabolism of deoxyribonucleotides, as was recently demonstrated in the anabolism of dideoxyadenosine (25). They can be infected by HIV (26) and a large number of mutant cells with altered nucleotide metabolism have been selected and used in studies of the mechanism of immunodeficiency diseases (27).

We found, as reported by Ullman *et al.* (28), that CEM cells were as sensitive to ddCyd as many other human lymphocytic cell lines (14, 15, 29). However, their high capacity to accumulate ddCTP from ddCyd enabled determination of this metabolite directly in cell extracts using HPLC, without the use of radioactive ddCyd.

Some of the [$^3$H]ddCyd was metabolized into two compounds that were resistant to hydrolysis by alkaline phosphatase but not by phosphodiesterase, suggesting they are not deoxynucleotides. By comparison with retention times for the normal liponucleotides dCDP-ethanolamine and dCDP-choline, we suggest that the metabolites are ddCDP-choline and ddCDP-ethanolamine. ddCDP-choline makes up only 6% of the total phosphorylated metabolites, which is lower than what Cooney *et al.* (7) found in ATH8 cells, whereas ddCDP-ethanolamine constitutes 45% and ddCMP only 3%. Balzarini *et al.* (15) have studied the metabolism of ddCyd in L1210, ATH8, and Molt/4F cells but do not report the presence of liponucleotides, probably because they coelute with ddCMP and ddCyd in anion exchange HPLC analysis. The significance of these ddCDP liponucleotides for the biological effects of ddCyd is not known, but it is possible that they could play a role in the development of the peripheral neuropathy.

Earlier studies have shown that deoxycytidine kinase is the enzyme responsible for the phosphorylation of ddCyd (7, 8, 14, 15). However, Starnes and Cheng (8) found that ddCyd was a poor substrate for their preparations of both cytoplasmic and mitochondrial deoxycytidine kinases. Recent work in this laboratory with pure cytoplasmic human deoxycytidine kinase showed that this enzyme phosphorylates ddCyd efficiently, with a 40-fold higher $K_m$ and 80% the $V_{max}$ of the natural substrate dCyd (16).[2]

[2] B. Kierdaszuk, C. Bohman, B. Ullman, and S. Eriksson. Substrate specificity of purified human deoxycytidine kinase toward antiviral 2′,3′-dideoxynucleoside analogs. Submitted for publication.

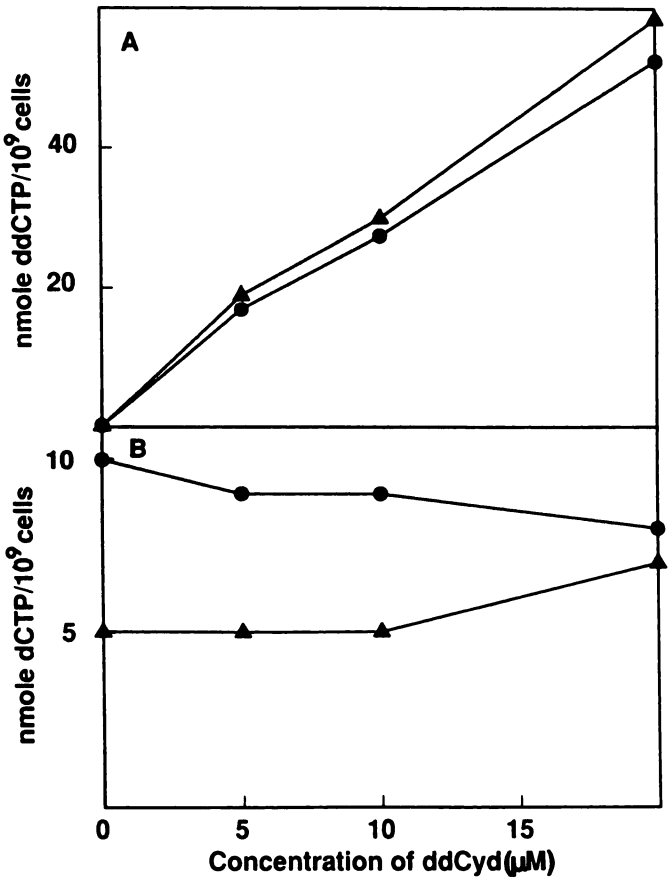


**Fig. 4.** Intracellular ddCTP and dCTP levels in CEM cells incubated with increasing concentrations of ddCyd, with or without simultaneous addition of thymidine ddCTP (A) and dCTP (B) levels were determined by HPLC after 6 hr of incubation with various concentrations of ddCyd alone (●) or together with 10 μM thymidine (▲), after oxidation of the ribonucleotides, as described in Materials and Methods. The values represent the mean from two experiments.

TABLE 1

**Summary of deoxynucleotide triphosphate pool effects in CEM cells after addition of various deoxynucleosides**

Deoxyribonucleoside triphosphates (including ddCTP) were measured by HPLC after 6 hr of incubation with the different agents indicated. The levels represent the mean from two to six experiments, with the standard deviation shown after each value.

| Addition | Deoxynucleoside triphosphate | | | | |
|---|---|---|---|---|---|
| | dCTP | dTTP | dATP | dGTP | ddCTP |
| | *nmol/10⁹ cells* | | | | |
| None (control) | 10 ± 1 | 30 ± 4 | 37 ± 5 | 24 ± 5 | |
| 10 μM Thymidine | 5 ± 0 | 57 ± 4 | 38 ± 1 | 38 ± 4 | |
| 10 μM ddCyd | 9 ± 3 | 29 ± 6 | 36 ± 11 | 24 ± 6 | 27 ± 1 |
| 300 μM AZT | 31 ± 9 | 35 ± 5 | 38 ± 5 | 23 ± 1 | |
| 10 μM Thymidine plus 10 μM ddCyd | 5 ± 1 | 66 ± 8 | 41 ± 3 | 38 ± 1 | 30 ± 2 |
| 300 μM AZT plus 10 μM ddCyd | 40 ± 14 | 36 ± 8 | 38 ± 4 | 25 ± 2 | 16 ± 1 |

Thymidine was used in this study to lower the intracellular dCTP level. Our hypothesis was that an increase in the *in vivo* activity of deoxycytidine kinase would lead to increased accumulation of ddCTP and possibly enhanced toxicity due to inhibition of DNA polymerization. Balzarini *et al.* (14) have performed a very careful study with a similar approach, using

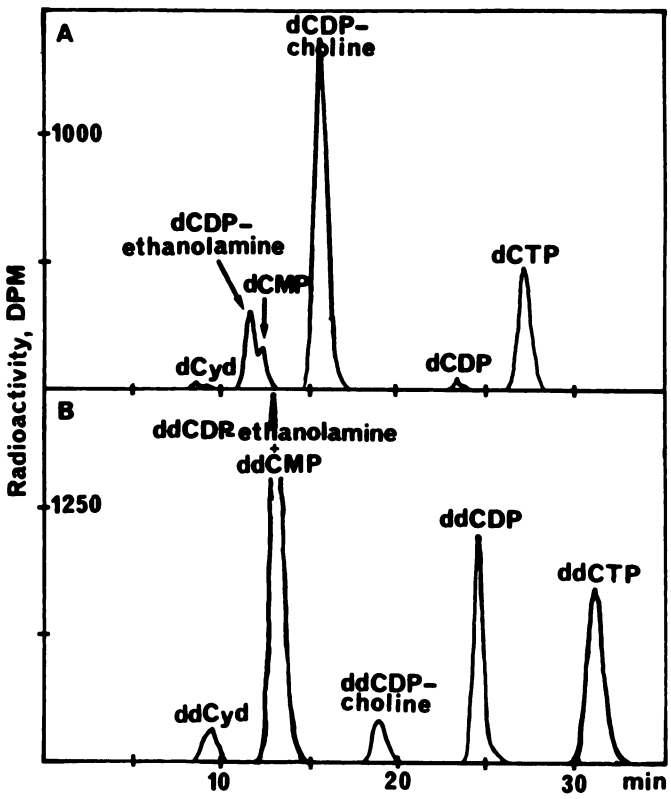


**Fig. 5.** Ion exchange (Partisil 10-SAX) HPLC elution profile of extracts of CEM cells incubated for 6 hr with [$^3$H]deoxycytidine or [$^3$H]ddCyd. CEM cells were incubated with 0.8 μM [$^3$H]deoxycytidine (A) or 1 μM [$^3$H] ddCyd (B). After harvest, the trichloroacetic acid-extracted nucleotides were separated by HPLC on a Partisil 10-SAX column eluted with a gradient, as described in Materials and Methods. *dCyd*, deoxycytidine.

L1210 cells. They found that addition of thymidine stimulates the formation of phosphorylated metabolites of ddCyd and they also showed that this stimulation was dependent on preincubation time and thymidine concentration. However, these cells are sensitive to growth inhibition by ddCyd at a 30-fold higher concentration than the CEM cells and their total capacity to convert ddCyd to ddCTP is also much lower (14, 15).

Preliminary experiments in this laboratory have shown that deoxycytidine kinase from mouse spleen has a much lower capacity to phosphorylate ddCyd, as compared with the human deoxycytidine kinase.[3] Because of this dependence on the cell species with regard to ddCyd metabolism, it was essential to characterize the regulation of this step in human cells. Balzarini *et al.* (15) have also earlier reported important species differences between murine (L1210) and human (ATH8 and Molt/4F) cell lines.

We found no increase in the accumulation of ddCTP as a consequence of a 50% decrease in the dCTP pool, with or without preincubation of CEM cells with thymidine for 12 hr. Thus, there seems to be no direct relation between the size of the dCTP pool and the phosphorylation capacity of deoxycytidine kinase in human CEM cells. We conclude that the activity of deoxycytidine kinase is probably not rate limiting in the accumulation of ddCTP *in vivo*. Clearly, with murine cells or other human cell lines, large variation in the metabolic phosphorylation of dideoxynucleosides is observed (14, 15) and,

[3] A. Habteyesus, A. Nordenskold, C. Bohman, and S. Eriksson. Manuscript in preparation.

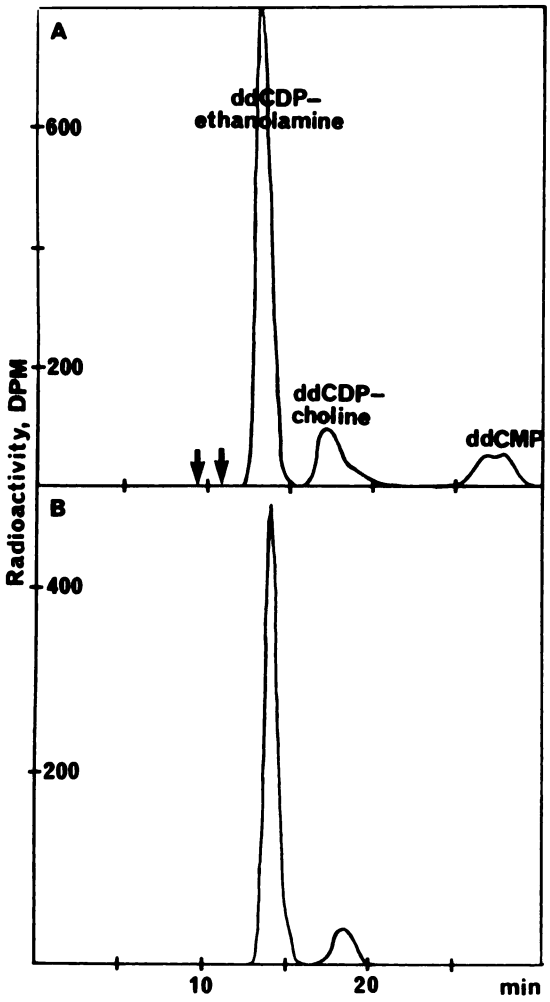


**Fig. 6.** Separation of dideoxyliponucleotides and ddCMP in CEM cell extracts by C-18 HPLC, before and after alkaline phosphatase treatment. CEM cells were incubated for 6 hr with 1 μM [$^3$H]ddCyd, and the intracellular nucleotides were extracted as described in Materials and Methods. The extracts were separated on a reverse phase C-18 column in 20 mM ammonium acetate, pH 5.0, before (A) and after (B) treatment with alkaline phosphatase. *Arrows*, positions where the natural liponucleotides dCDP-ethanolamine (9 min) and dCDP-choline (11 min) elute.

therefore, generalizations about these pathways from one cell line to others should not be made.

No synergistic effect of the combination of ddCyd and thymidine was observed in ATH8 human T cells by Balzarini *et al.* (14). This result, together with other observations, led these investigators to suggest that the absolute intracellular level of ddCTP was not directly involved in the cytostatic effect of the compound. However, we observe a synergistic effect of thymidine in combination with ddCyd on growth inhibition of CEM cells, which is most likely linked to the change in the ratio of ddCTP to dCTP, i.e., from 3:1 with only ddCyd to 6:1 when combined with thymidine. Thus, our results indicate that the cytotoxicity is related to the ratio of dCTP to ddCTP in the cells, a conclusion that has been drawn about the toxicity of dideoxynucleosides by others (30) as well. The direct effect of these pool alterations on cellular DNA synthesis is not known, but a high incidence of chain terminations is to be expected. It is interesting to note that the isobologram shown here for the growth-inhibitory effect of combinations of AZT and ddCyd is very similar to the isobologram obtained by Baba *et al.* (12) measuring the anti-HIV-1 activity of combinations of these two drugs.

Growth inhibition of CEM cells by the thymidine analog AZT occurred at 3 to 10 times lower concentrations than reported for other human lymphocytes (H9 cells and peripheral blood lymphocytes) and fibroblasts (MRHF and MRC5 cells), i.e., 1000 μM (5), and similar to that in one study (31) with CEM cells (i.e., 690 μM) but at 80-fold higher concentrations than those in another study with the same type of cells (i.e., 4 μM) (32). Furthermore, the effect of AZT on DNA precursor pools was clearly different than that reported initially for H9 and Molt/4F cells (5), because we found no decrease in either the dTTP or dCTP pools. On the contrary, we found a 2-fold higher dCTP level in AZT-treated CEM cells. Frick *et al.* (33) have reinvestigated the effect of AZT on the DNA precursor pools in several human cell lines (HL-60, H-9, and K-562) and their results with HL-60 cells are essentially similar to what we find in CEM cells. The very pronounced decline of all ribonucleoside triphosphate pools reported by Avramis *et al.* (32) as an effect of AZT in CEM cells was not observed in these experiments (data not shown). The reason for these large discrepancies in the response of CEM cells to AZT reported in the literature is at present not known. The mechanism of dCTP elevation by AZT is also not known but the high AZT monophosphate pool might inhibit intracellular nucleotidase activities, leading to a decreased turnover of the dCTP pool.

AZT did not mimic thymidine with regard to ddCyd metabolism in CEM cells. The effects of combinations were considerably less in terms of growth inhibition and the DNA precursor pool changes were different. We may conclude from these data that azido-dTTP does not serve as a feedback inhibitor for human ribonucleotide reductase. This is in agreement with *in vitro* studies, with partially purified enzyme, by Harrington *et al.* (34). The lack of metabolic interference and synergistic growth-inhibitory properties of ddCyd and AZT in a human cell culture model system may encourage the use of these compounds in anti-HIV combination therapy.

## References

1. Mitsuya, H., K. J. Weinhold, P. A. Furman, M. H. St. Clair, S. Nusinoff-Lehrman, R. C. Gallo, D. Bolognesi, D. W. Barry, and S. Broder. 3′-Azido-3′-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus *in vitro*. *Proc. Natl. Acad. Sci. USA* **82**:7096–7100 (1985).
2. Mitsuya, H., and S. Broder. Inhibition of the *in vitro* infectivity and cytopathic effect of human T-lymphotrophic virus type III lymphadenopathy-associated virus (HTLV-III/LAV) by 2′,3′-dideoxynucleosides. *Proc. Natl. Acad. Sci. USA* **83**:1911–1915 (1986).
3. Mitsuya, H., and S. Broder. Strategies for antiviral therapy in AIDS. *Nature* (Lond.) **325**:773–778 (1987).
4. Yarchoan, R., R. V. Thomas, J.-P. Allain, N. McAtee, R. Dubinsky, H. Mitsuya, T. J. Lawley, B. Safai, C. E. Myers, C. F. Perno, R. W. Klecker, R. J. Wills, M. A. Fischl, M. C. McNeely, J. M. Pluda, M. Leuther, J. M. Collins, and S. Broder. Phase I studies of 2′,3′-dideoxycytidine in severe human immunodeficiency virus infection as a single agent and alternating with zidovudine (AZT). *Lancet* **1**:76–81 (1988).
5. Furman, P. A., J. A. Fyfe, M. H. St Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. Nusinoff-Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **83**:8333–8337 (1986).
6. Yarchoan, R., R. W. Klecker, K. J. Weinhold, P. D. Markham, H. K. Lyerly, D. T. Durack, E. Gelmann, S. Nusinoff-Lehrman, R. M. Blum, D. W. Barry, G. M. Shearer, M. A. Fischl, H. Mitsuya, R. C. Gallo, J. M. Collins, D. P. Bolognesi, C. E. Meyers, and S. Broder. Administration of 3′-azido-3′-deoxythymidine, an inhibitor of HTLV-III/LAV replication, to patients with AIDS or AIDS-related complex. *Lancet* **1**:575–580 (1986).
7. Cooney, D. A., M. Dalal, M. Mitsuya, J. B. McMahon, M. Nadkarni, J. Balzarini, S. Broder, and D. G. Johns. Initial studies on the cellular phar-

TABLE 2

**Intracellular phosphorylation of [$^3$H]ddCyd, in combination with thymidine and AZT, and [$^3$H]dCyd in human lymphoblast CEM cells**

CEM cells were incubated with the respective additions for 6 hr and harvested, and the intracellular nucleotides were extracted with trichloroacetic acid. After neutralization, the nucleotides were analyzed by HPLC, as described in Materials and Methods. The experiment was repeated three times with very similar results.

| Addition | Metabolite | | | | |
|---|---|---|---|---|---|
| | ddCMP[a] | ddCDP-choline[a] | ddCDP | ddCTP | Sum |
| | *nmol/10$^9$ cells* | | | | |
| 1.0 μM [$^3$H]ddCyd | 6.8 | 0.8 | 2.9 | 3.5 | 14.0 |
| + 30 μM AZT | 6.0 | 0.7 | 2.4 | 2.7 | 11.8 |
| + 30 μM AZT with 12-hr preincubation | 5.6 | 0.7 | 2.3 | 2.5 | 11.1 |
| + 30 μM Thymidine | 7.0 | 0.8 | 3.0 | 3.6 | 14.4 |
| + 30 μM Thymidine with 12-hr preincubation | 6.9 | 0.7 | 2.8 | 3.8 | 14.2 |

| Addition | Metabolite | | | | | |
|---|---|---|---|---|---|---|
| | dCMP | dCDP-ethanolamine | dCDP-choline | dCDP | dCTP | Sum |
| | | | *nmol/10$^9$ cells* | | | |
| 0.8 μM [$^3$H]-Deoxycytidine | 0.6 | 1.8 | 10.9 | 0.2 | 3.6 | 17.1 |

[a] When labeling with ddCyd, we could identify two metabolites that were resistant to alkaline phosphatase treatment, suggesting that they were liponucleotides, presumably ddCDP-ethanolamine and ddCDP-choline as judged from their retention times in comparison with those of dCDP-ethanolamine and dCDP-choline. One of these peaks coeluted with ddCMP on the Partisil 10-SAX column but separated on a C-18 column (see Figs. 7 and 8), indicating that only 7% of the ddCMP/ddCDP-ethanolamine peak consisted of ddCMP.


macology of 2′,3′-dideoxycytidine, an inhibitor of HTLV-III infectivity. *Biochem. Pharmacol.* **35**:2065–2068 (1986).

8. Starnes, M. C., and Y. Cheng. Cellular metabolism of 2′,3′-dideoxycytidine, a compound active against human immunodeficiency virus *in vitro*. *J. Biol. Chem.* **262**:988–991 (1987).
9. Mitsuya, H., R. F. Jarett, M. Matsukura, F. Di Marzo Veronese, A. L. DeVicio, M. G. Sarangadharan, D. G. Johns, M. S. Reitz, and S. Broder. Long-term inhibition of human T-lymphotropic virus type III/lymphadenopathy-associated virus (human immunodeficiency virus) DNA synthesis and RNA expression in T cells protected by 2′,3′-dideoxynucleosides *in vitro*. *Proc. Natl. Acad. Sci. USA* **84**:2033–2037 (1987).
10. Yarchoan, R., H. Mitsuya, C. E. Myers, and S. Broder. Clinical pharmacology of 3′-azido-2′,3′-dideoxythymidine (zidovudine) and related dideoxynucleosides. *N. Engl. J. Med.* **321**:726–738 (1989).
11. Larder, B. A., G. Darby, and D. D. Richman. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. *Science (Washington, D. C.)* **243**:1731–1734 (1989).
12. Baba, M., R. Pauwels, J. Balzarini, P. Herdewijn, E. de Clerq, and J. Desmyter. Ribavirin antagonizes the inhibitory effects of pyrimidine 2′,3′-dideoxynucleosides but enhances the inhibitory effects of purine 2′,3′-dideoxynucleosides on the replication of human immunodeficiency virus *in vitro*. *Antimicrob. Agents Chemother.* **31**:1613–1617 (1987).
13. Vogt, M. W., K. L. Hartshorn, P. A. Furman, T.-C. Chou, J. A. Fyfe, L. A. Coleman, C. Crupacker, R. T. Schooley, and M. S. Hirsch. Ribavirin antagonizes the effect of azidothymidine on HIV replication. *Science* (Washington, D. C.) **235**:1376–1379 (1987).
14. Balzarini, J., D. A. Cooney, M. Dalal, G.-J. Kang, J. E. Cupp, E. De Clercq, S. Broder, and D. G. Johns. 2′,3′-Dideoxycytidine: regulation of its metabolism and antiretroviral potency by natural pyrimidine nucleosides and by inhibitors of pyrimidine nucleotide synthesis. *Mol. Pharmacol.* **32**:798–806 (1988).
15. Balzarini, J., R. Pauwels, M. Baba, P. Herewijn, E. De Clerq, S. Broder, and D. G. Johns. The *in vitro* and *in vivo* activity and intracellular metabolism of 3′-azido-3′-deoxythymidine and 2′,3′-dideoxycytidine are highly dependent on the cell species. *Biochem. Pharmacol.* **37**:897–903 (1988).
16. Bohman, C., and S. Eriksson. Deoxycytidine kinase from human leukemic spleen: preparation and characterization of the homogeneous enzyme. *Biochemistry* **27**:4258–4265 (1988).
17. Ives, D. H., and J. P. Durham. Deoxycytidine kinase. III. Kinetics and allosteric regulation of the calf thymus enzyme. *J. Biol. Chem.* **245**:2285–2294 (1970).
18. Bjursell, G., and P. Reichard. Effects of thymidine on deoxyribonucleoside triphosphate pool and deoxyribonucleic acid synthesis in Chinese hamster ovary cells. *J. Biol. Chem.* **248**:3904–3909 (1973).
19. Dahbo, Y., and S. Eriksson. On the mechanism of deoxyribonucleoside toxicity in human T-lymphoblastoid cells: reversal of growth inhibition by addition of cytidine. *Eur. J. Biochem.* **150**:429–434 (1985).
20. Khym, J. X. An analytical system for rapid separation of tissue nucleotides at low pressure on conventional anion exchangers. *Clin. Chem.* **21**:1245–1252 (1975).
21. Garrett, C., and D. V. Santi. A rapid and sensitive high pressure liquid chromatography assay for deoxyribonucleoside triphosphates in cell extracts. *Anal. Biochem.* **99**:268–273 (1979).
22. Spyrou, G., and P. Reichard. Compartmentation of dCTP pools; evidence from deoxylipophosphate synthesis. *J. Biol. Chem.* **262**: 16425–16432 (1987).
23. Loewe, S. The problem of synergism and antagonism of combined drugs. *Arzneim. Forsch.* **3**:285–320 (1953).
24. Elion, G. B., S. Singer, and G. H. Hitchings. Antagonists of nucleic acid derivates. VIII. Synergism in combinations of biochemically related antimetabolites. *J. Biol. Chem.* **208**:477–488 (1954).
25. Carson, D. A., T. Haertle, D. B. Wasson, and D. D. Richman. Biochemical genetic analysis of 2′,3′-dideoxyadenosine metabolism in human T lymphocytes. *Biochem. Biophys. Res. Commun.* **151**:788–793 (1988).
26. Dagleish, A. G., P. C. L. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature (Lond.)* **312**:763–767 (1984).
27. Martin, D. W., Jr., and E. W. Gelfand. Biochemistry of diseases of immunodevelopment. *Annu. Rev. Biochem.* **50**:845–877 (1981).
28. Ullman, B., T. Coons, S. Rockwell, and K. McCartan. Genetic analysis of 2′,3′-dideoxyctidine incorporation into cultured human T lymphoblasts. *J. Biol. Chem.* **263**:12391–12396 (1988).
29. Balzarini, J., and S. Broder. in *Clinical Use of Antiviral Drugs* (E. De Clercq, ed.). Martinus Nijhoff Publishing, Kluwer Academic Publisher, Norwell, MA, 361–385 (1988).
30. Perno, C.-F., R. Yarchoan, D. A. Cooney, N. R. Hartman, S. Gartner, M. Popovic, Z. Hao, T. L. Gerrard, Y. A. Wilson, D. G. Johns, and S. Broder. Inhibition of human immunodeficiency virus (HIV-1/HTLV-III$_{Ba-L}$) replication in fresh and cultured human peripheral blood monocytes/macrophages by azidothymidine and related 2′,3′-dideoxynucleosides. *J. Exp. Med.* **160**:1111–1125 (1988).
31. Vince, R., M. Hua, J. Brownell, S. Daluge, F. Lee, W. M. Shannon, G. C. Lavelle, J. Qualls, O. S. Weislow, R. Kiser, P. G. Canonico, R. H. Schultz, V. L. Narayanan, J. G. Mayo, R. H. Shoemaker, and M. R. Boyd. Potent and selective activity of a new carbocylic nucleoside analog (Carbovir: NSC 614846) against human immunodeficiency virus *in vitro*. *Biochem. Biophys. Res. Commun.* **156**:1046–1053 (1988).
32. Avramis, V. I., W. Marson, R. L. Jackson, and E. Gomperts. Biochemical pharmacology of zidovudine in human T-lymphoblastoid cells (CEM). *AIDS* **3**:417–422 (1989).
33. Frick, L. W., D. J. Nelson, M. H. St Clair, P. A. Furman, and T. A. Krenitsky. Effects of 3′-azido-3′-deoxythymidine on deoxynucleotide pools of cultured human cells. *Biochem. Biophys. Res. Commun.* **154**:124–129 (1988).
34. Harrington, J., W. Miller, and T. Spector. Effector studies of 3′-azidothymidine nucleotides with human ribonucleotide reductase. *Biochem. Pharmacol.* **36**:3757–3761 (1987).


**Send reprint requests to:** Yvonne Törnevik, Medical Nobel Institute, Department of Biochemistry I, Karolinska Institute, Box 60 400, S-104 01 Stockholm, Sweden.