Cellular Metabolism of 2',3'-Dideoxycytidine, a Compound Active against Human Immunodeficiency Virus * regulatory effect of human immunodeficiency virus on human OKT4 + lymphocytes in vitro (1). Metabolism of ddCyd by human T-lymphoblastic cells (Molt 4) negative for human immunodeficiency virus and OKT4 was examined. Molt 4 cells accumulated ddCyd and its phosphorylated derivatives into acid-soluble and acid-insoluble material in a dose-dependent manner. For each concentration tested, 2',3'-dideoxycytidine triphosphate represented 40% of the total acid-soluble pool of ddCyd metabolites. Uptake of 5 μM ddCyd was linear for 4 h after addition of drug. Efflux of ddCyd metabolites from cells followed a biphasic course with an initial retention half-life of 2.6 h for 2',3'-dideoxycytidine triphosphate. DNA, but not RNA, of cells incubated with [3H]ddCyd became radioisotopically labeled. Nuclease and phosphatase treatment of DNA followed by reverse-phase high-pressure liquid chromatography showed that the nucleoside was incorporated into DNA in its original form. ddCyd was not susceptible to deamination by human Cyd-dCyd deaminase. It was a poor substrate for human cytoplasmic and mitochondrial dCyd kinases, with K_m values of 180 ± 30 and 120 ± 20 μM, respectively. DNA polymerases α, β, and γ varied in their sensitivity to inhibition by ddCTP with K_i values of 110 ± 40, 2.6 ± 0.3, and 0.016 ± 0.008 μM, respectively; however, inhibition was competitive with dCTP in each case.

Acquired immunodeficiency syndrome (AIDS) has emerged as a major health threat in the last several years. Human immunodeficiency virus (HIV) is now recognized as the etiological agent of this disease (2–8). This virus preferentially infects and destroys OKT4+ (helper inducer) T-lymphocytes (6). In AIDS and its preceding lymphenopathies, there is detectable virus replication as determined by human T-lymphoblastic cells (Molt 4) negative for OKT4 and OKT8 (helper inducer) T-lymphocytes. The etiological agent of this disease, the human immunodeficiency virus, is now recognized as a major health threat in the last several years. The activity of ddCyd against HIV and its low cytotoxicity make it an attractive candidate for clinical trial; therefore, it is important to study its metabolism in human cells. We have chosen a T-lymphoblastic cell line (Molt 4) for these studies and report here on the uptake, efflux, phosphorylation, deamination, and incorporation into DNA of ddCyd.

Materials and Methods

Results

Uptake of ddCyd—Molt 4 cells were incubated with 2, 5, or 10 μM [3H]ddCyd for 6 h, and the uptake into acid-soluble and acid-insoluble fractions was analyzed. As seen in Table 1, there was a concentration-dependent uptake into both fractions, although incorporation of [3H]ddCyd into the acid-insoluble pool was only a small percentage of the total. When the acid-soluble fractions were analyzed by anion exchange HPLC (Fig. 1), there were three major metabolites in addition to a peak which coeluted with a ddCyd marker and an unidentified "solvent breakthrough" peak. The metabolite which eluted in fraction 37 was in a position identical to authentic ddCTP. Metabolites of ddCyd which eluted in fractions 11 and 23 were identified as ddCMP and ddCDP, respectively, on the basis of their elution positions with respect to dCMP and dCDP and by identification of the original nucleoside (ddCyd) after phosphatase treatment (see below). The amount of each metabolite found in cells was dependent on the extracellular concentration of ddCyd, and the proportion of the total acid-soluble radioactive material represented by each metabolite remained the same. In each case, ddCMP, ddCDP, and ddCTP represented approximately 30, 10, and 40%, respectively, of the total acid-soluble radioactive pool.
When the acid-soluble fraction from cells treated with 10 µM ddCyd was treated with alkaline phosphatase and venom phosphodiesterase, the radioactivity associated with each metabolite decreased, and there was a corresponding increase in radioactivity associated with [3H]ddCyd. A portion of this phosphatase-treated extract was analyzed on a reverse-phase HPLC column, and more than 50% of the radioactivity coeluted with authentic ddCyd (data not shown). This system completely separates ddCyd, which elutes at 8.6 min from Cyd and dCyd, which elute at 4.5 and 5.3 min, respectively.

**Uptake and Efflux of ddCyd with Time**—Cells were incubated for 2, 4, or 6 h with 5 µM ddCyd, a concentration approximately equal to the ID₅₀ in this cell line (data not shown). The uptake of ddCyd and formation of its phosphorylated metabolites were linear with time for 4 h after addition of drug (Fig. 2A). Efflux of ddCyd and its metabolites after resuspension of cells in drug-free medium followed a biphasic course, with an initial retention half-life for the triphosphate of 2.6 h (Fig. 2B). When all phosphorylated metabolites of ddCyd were included in this calculation, the initial retention half-life was 5.7 h.

**Incorporation of ddCyd into Nucleic Acids**—Purified nucleic acids from Molt 4 cells treated for 12 h with 5 µM [3H]ddCyd were subjected to ultracentrifugation in a Cs₂SO₄ gradient. There was one major peak of radioactivity which corresponded to the position of DNA in the gradient (Fig. 3). It is uncertain at this time whether or not the small amounts of radioactivity associated with RNA represent true incorporation, and more sensitive techniques must be employed to address this question. Nucleic acids digested with micrococcal nuclease, venom phosphodiesterase, and alkaline phosphatase were analyzed on a reverse-phase HPLC column. The majority of the radioactivity eluted in a position identical to that of authentic ddCyd (Fig. 4). The amount and position of radioactive material which eluted before ddCyd varied in different experiments and were most likely due to incomplete digestion of nucleic acids.

**Susceptibility of ddCyd to Deamination**—Since only three major phosphorylated metabolites of ddCyd were seen on the anion exchange HPLC profiles after incubation of cells with ddCyd, it was expected that ddCyd would not be significantly susceptible to deamination. This was indeed the case (Fig. 5A). When 0.4 µM ddCyd was incubated with 10 units/ml l DNA polymerase and dCyd deaminase for periods up to 90 min, there was no significant catalysis of this compound.

In addition, as seen in Fig. 5B, ddCyd interfered only slightly with deamination of dCyd. Deamination of 100 µM dCyd was 90% of control in the presence of 1 mM ddCyd after 30 min.

**Phosphorylation of ddCyd**—As shown in Table 2, ddCyd was a poor substrate for both cytoplasmic and mitochondrial dCyd kinase activities, with Kₘ values of 180 ± 30 and 120 ± 20 µM, respectively. In addition to a high Kₘ value, the maximum rate of ddCyd phosphorylation was significantly less than that for dCyd with both enzymes.

**Inhibition of Human DNA Polymerases**—The three major human DNA polymerases varied widely in their susceptibility to inhibition by ddCTP; however, inhibition was competitive with dCTP for each enzyme. Polymerase γ was the most sensitive, with a Kᵢ value 19-fold less than the Kₘ value for dCTP. Polymerase α was quite resistant to inhibition, with a Kᵢ value for ddCTP greater than 100 times its Kₘ for dCTP. Polymerase β was intermediate in sensitivity with a Kᵢ/Kₘ ratio of 1:1.7. Kᵢ values for ddCTP against DNA polymerases α, β, and γ were 110 ± 40, 2.6 ± 0.3, and 0.016 ± 0.008 µM, respectively.

**DISCUSSION**

The data presented show that significant amounts of ddCTP are formed in Molt 4 cells, with the uptake and phosphorylation of ddCyd dependent on its extracellular concentration. ddCTP is a substrate for at least one of the mammalian DNA polymerases since it is incorporated into DNA. Whether or not the small amounts of incorporation can account for the cytotoxicity of ddCyd is under investigation. The relative sensitivities of DNA polymerases α, β, and γ to inhibition by ddCTP are similar to those noted by other investigators with ddTTP (14–16) and indicate that polymerases β and/or γ are the most likely targets for inhibition. The observation that polymerase α, the major enzyme involved in cellular DNA replication (15), is quite resistant to inhibition by ddCTP may explain the relatively low cytotoxicity of ddCyd.

Disappearance of ddCyd metabolites from cells followed a biphasic course, with an initial retention half-life for the triphosphate of 2.6 h. This value is less than the 4-h half-life reported previously for 1-β-d-arabinofuranosylcytosine triphosphate and approximately equal to the half-life of 1-β-d-arabinofuranosyl-5-azacytosine triphosphate in Molt 4 cells (10). The biphasic efflux phenomenon may be related to the concentration dependence of enzymatic dephosphorylation of ddCTP, such that when the ddCTP concentration nears the Kᵢ value for this process, a slower rate of degradation occurs. It should be noted that ddCTP accumulation will be determined not only by the rate of dephosphorylation, but also by the rates of phosphorylation and incorporation into nucleic acids; therefore, it is conceivable that different cell types may vary greatly in their ability to accumulate ddCTP and thus in their susceptibility to ddCyd cytotoxicity.

The studies with human Cyd-dCyd deaminase show that ddCyd is not significantly susceptible to deamination, a major pathway for inactivation of 1-β-d-arabinofuranosylcytosine in humans (17).

The greater potency of ddCyd compared to other dideoxynucleosides to protect against the cytopathic effect of HIV (1) could be due to differences in phosphorylation of these compounds if inhibition of reverse transcriptase by the triphosphate is their mechanism of action as postulated. However, the amounts of phosphorylated ddCyd derivatives formed in cells is small compared to formation of phosphorylated metabolites of other ddCyd analogs (10). Indeed, as shown here, ddCyd is a very poor substrate for both kinases responsible for monophosphorylation of ddCyd. It is not certain at this time which dCyd kinase, cytoplasmic or mitochondrial, is responsible for the majority of ddCyd monophosphorylation in Molt 4 cells. There is no evidence at this time that HIV induces any unique nucleoside-metabolizing enzymes; therefore, it is likely that one of these kinases is responsible for phosphorylation of ddCyd in HIV-infected cells.

Unpublished results from this laboratory using homopolymer primer/template combinations and purified HIV reverse transcriptase indicate that ddTTP and ddCTP are potent inhibitors of this enzyme with Kᵢ values in the nanomolar range. It is unfortunate that inhibition by ddCTP cannot be examined using this system since HIV reverse transcriptase will not use a poly(dG)-oligo(dC) template; however, it is likely that the enzyme has a low Kᵢ value for ddCTP as well. The small amounts of ddCTP formed in cells may be sufficient to inhibit the viral reverse transcriptase without much effect on cellular DNA polymerases, thus explaining the selective antiviral effect.

---

3 Cheng, Y.-C., Dutschman, G. E., Bastow, K. F., Sarraghadharan, M. G., and Ting, R. Y. C. (1987) J. Biol. Chem., in press.
Several compounds in addition to ddCyd have been shown to be active against HIV in vitro (18-22). The clinical experience with these compounds is too limited to allow conclusions concerning their potential usefulness in slowing the progression of AIDS; however, there has been some cause to be optimistic (23, 24), especially when treatment is instituted early in the course of this disease. This study on the metabolism of ddCyd represents a contribution to the body of knowledge needed about any compound with potential usefulness in the clinic. A detailed study of the mechanism of ddCyd cytotoxicity is now in progress.

Note Added in Proof—During preparation of this manuscript, a paper which described phosphorylation of ddCyd by various cell types appeared in Biochem. Pharmacol. (Cooney, D. A., Dalai, M., Mitsuya, H., McMahon, M. B., Nadkarni, M., Salzarini, J., Broder, S., and Johns, D. G. (1986) Biochem. Pharmacol. 35, 2065-2068).

REFERENCES

1. Mitsuya, H., and Broder, S. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1911-1915
2. Popovic, M., Sarnghadaran, M. G., Read, E., and Gallo, R. C. (1984) Science 224, 497-500
3. Gallo, R. C., Salahuddin, S. Z., Popovic, M., Shearer, G. M., Kaplan, M., Haynes, B. F., Falke, T. J., Redfield, R., Olesi, J., Safai, B., White, G., Foster, P., and Markham, P. D. (1984) Science 224, 500-503
4. Schubach, J., Popovic, M., Gilden, R. V., Gonda, M. A., Sarnghadaran, M. G., and Gallo, R. C. (1984) Science 224, 503-505
5. Sarnghadaran, M. G., Popovic, M., Broder, S., and Schubach, J., and Gallo, R. C. (1984) Science 224, 506-508
6. Klatzmann, D., Barre-Sinoussi, F., Nugeyre, M. T., Dauguet, C., Mitsuva, H., and Broder, S. (1984) Science 224, 509-511
7. Feorino, P. M., Kalyanaraman, V. H., Cabral, C. D., Warfield, D. T., Jaffe, H. W., Harrison, A. K., Gottlieb, M. D., Goldfinger, D., Chermann, J.-C., and Montagnier, L. (1984) Science 225, 69-72
8. Brun-Vezinet, F., Rouzioux, C., Montagnier, L., Charnet, S., Gruest, J., Barre-Sinoussi, F., Geroldi, D., Chermann, J. C., McMurrick, J., Mitchell, S., Piot, P., Taellin, H., Mirlangu, K. B., Wobin, M. N. O., Mazzeo, P. K., Britas, C., Besnier, J., Weisnud, F. M., and Quin, T. C. (1984) Science 226, 453-456
9. Cheng, Y., Grill, S. P., Dutschman, G. E., Nakatsuka, K., and Bastow, K. F. (1983) J. Biol. Chem. 258, 12460-12464
10. Townsend, A., Leclerc, J. M., Dutschman, G., Gooney, D., and Cheng, Y. C. (1985) Cell 46, 3529-3538
11. Maritaias, T., Frisch, E. P., and Sunnck, J. (1982) in Molecular Cloning, A Laboratory Manual, pp. 468-469, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. Wentworth, D. F., and Woldenberg, R. (1975) Biochemistry 14, 5095-5010
13. Chen, Y.-C., Domin, B., and Lee, L. S.-S. (1977) Biochim. Biophys. Acta Suppl. 481, 481-492
14. Edenberg, H. J., Anderson, S., and DePamphilis, M. L. (1978) J. Biol. Chem. 253, 3273-3280
15. Waque, M. A., Evans, M. J., Manly, K. F., Hughes, R. G., and Huberman, J. A. (1984) J. Cell. Physiol. 121, 402-408
16. Van der Vliet, P. C., and Kwan, M. T. (1978) Nature 276, 552-554
17. Cammer, G. W., and Smith, C. G. (1966) Biochem. Pharmacol. 14, 1405-1416
18. Mitsuya, H., Popovic, M., Yarchoan, R., Matsushita, S., Gallo, R. C., and Broder, S. (1984) Science 226, 172-174
19. McCormick, J. B., Getchell, J. P., Mitchell, S. W., and Hicks, D. R. (1984) Lancet 2, 1367-1369
20. Ho, D. D., Harshorn, K. L., Rota, T. R., Andrews, C. A., Kaplan, J. C., Schooley, R. T., and Hirsch, M. S. (1985) Lancet 1, 602-604
21. Sandstrom, E. G., Kaplan, J. C., Byington, R. E., and Hirsch, M. S. (1985) Lancet 1, 1480-1484
22. Mitsuya, H., Weinhold, K., Furman, P. A., St. Clair, M. H., Lehman, S. N., Gallo, R. C., Bolognesi, D., Barry, D. W., and Broder, S. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7096-7100
23. Yarchoan, R., Klecker, R. W., Weinhold, K. J., Markham, P. D., Laury, H. K., Durack, D. T., Gelmann, E. L., Lehman, S. N., Blum, R. M., Barry, D. W., Shearer, G. M., Fischl, M. A., Mitsuya, H., Gallo, R. C., Collins, J. M., Bolognesi, D. P., Myers, C. E., and Broder, S. (1986) Lancet 1, 575-580
24. Rozenbaum, W., Dormont, D., Spire, B., Vilmer, E., Gentilini, M., Griscelli, C., Montagnier, L., Barre-Sinoussi, F., and Chermann, J. C. (1986) Lancet 1, 450-461

Supplemental Material for
Cellular Metabolism of 2',3'-Dideoxyctydine, a Component Active Against Human Immunodeficiency Virus (HIV)

MATERIALS AND METHODS

Chemicals and Enzymes—ddCyd was purchased from Fluka, Milwaukwe, Wis., and all other reagents and nucleosides were from Sigma. [3H]Thymidine and [3H]cytidine nucleotides were from New England Nuclear. [3H]Thymidine labeled phosphatase and [3H]cytosine deaminase phosphorylase were from Sigma. Enzymes for [3H]nucleoside analogues deacetylation were purchased from Boehringer Mannheim Biochemicals.

Cell Culture—Hut 10 cells, obtained from the American Type Culture Collection (Rockville, Md.), were maintained at 37°C in RPMI 1640 medium (Gibco Island Biologicals, Grand Island, N.Y.), supplemented with 10% fetal calf serum and 1% dialyzed fetal calf serum.

HPLC Analysis—Aliquots (50 μl) of the enzymatic reaction mixture were mixed with 50 μl of 10 M sulfuric acid and centrifuged at 14,000 × g for 10 min. The supernatant was analyzed by reversed phase HPLC (Shimadzu Model LC-3A, Kyoto, Japan) equipped with a Shimadzu CR-20A chromatography computer. A reverse phase C18 column (4.6-mm internal diameter × 25-cm long, 5-μm particle size, Shimadzu) was equilibrated with 0.1% trifluoroacetic acid. The mobile phase was 0.1% trifluoroacetic acid in water for 0-50% and then in water for 50-100% over 30 min. The flow rate was 2 ml/min, and the column temperature was 37°C. [3H]ddCyd in a 250-μl volume of 0.2 M trisodium citrate buffer, pH 7.4, was used as the ddCyd standard (25). ddCyd was separated from other drugs metabolites and degradation products by HPLC analysis.

Enzymatic Assays—[3H]ddCyd was incubated with Hut 10 cell extracts for 18 h at 37°C and subjected to the same purification procedure as described in the following.

Preparation of Hut 10 cell extracts—Hut 10 cells were prepared as described in the following.

Purification and assay—Hut 10 cell extracts were partially purified on streptomycin-agarose columns through the ddCyd inclusion bodies as previously described (10). Sucrose cushion centrifugation, ammonium, and 25-μM TTP (26) were present in the reaction and were allowed to equilibrate with the ddCyd standard for 15 min before the reaction was initiated.
Table 1.

Concentration Dependent Values of Acld in 2',3'-Dideoxycytidine

Molt 4 cells (7.5 x 10^6/ml) were treated for 9 h with 2.5, 10, or 50 μM of 2',3'-Dideoxycytidine. Nuclei acid-soluble and acid-insoluble fractions were prepared and analyzed for radioactivity. Values are the average of 2-3 determinations.

| [2',3'-DdC] (μM) | Acid-Soluble | Acid-Insoluble |
|------------------|--------------|---------------|
| 2                | 12.5         | 0.21          |
| 5                | 26.2         | 0.40          |
| 10               | 50.1         | 0.62          |

Table 2.

Phosphorylation of Acld by Purified Enzyme.

Degree assays were performed as previously described (12) with 20, 50, 100, or 150 μM of 2',3'-Dideoxycytidine, and the same concentration of 2',3'-Dideoxycytidine for the control assays. Acld and Acld values were calculated by means of exposure. Acld and Acld values were calculated by means of exposure. Values are the average of 2-3 determinations ± standard deviation.

| Enzyme activity | 100% Acld | 1/2 Acld | 1/4 Acld |
|-----------------|----------|---------|---------|
| Cytoplasmic Acld kinase | 1.0 ± 0.6 | 1.0 ± 0.6 | 1.0 ± 0.6 |
| Mitochondrial Acld kinase | 5.0 ± 1.0 | 5.0 ± 1.0 | 5.0 ± 1.0 |

Table 3.

Inhibition of DNA Polymerase by Acld.

Purification of DNA polymerase and enzyme assays were performed as described under "Materials and Methods." Values were determined from repeat of the assays of at least 3 independent times. Values are the average of 3 separate determinations ± standard deviation.

| DNA Polymerase | dCTP (μM) | dATP (μM) | dGTP (μM) | dTTP (μM) |
|----------------|-----------|-----------|-----------|-----------|
| 0              | 0.7 ± 0.8 | 0.7 ± 0.8 | 0.7 ± 0.8 |
| 2              | 0.4 ± 0.4 | 0.4 ± 0.4 | 0.4 ± 0.4 |
| 4              | 0.3 ± 0.3 | 0.3 ± 0.3 | 0.3 ± 0.3 |

Figure 1. HPLC Analysis of Acld Metabolism in Molt 4 Cells. Acld-soluble enzyme prepared from 10 μM Acld-treated cells (2.5 x 10^6/ml) was applied to a Biocarb 1.0 mm column as described under "Materials and Methods." After the exchange buffer of acid-soluble enzyme prepared from 2.5 x 10^6/ml of Molt 4 cells, the sample was fractionated with acid-soluble enzyme prepared from 2.5 x 10^6/ml and incubated with alkaline phosphatase and x-ray phosphorfluorometry.

Figure 2. NMR Spectra of Acld Metabolism in Molt 4 Cells. Acld-soluble enzyme prepared from 10 μM Acld-treated cells (2.5 x 10^6/ml) was applied to a Biocarb 1.0 mm column as described under "Materials and Methods." After the exchange buffer of acid-soluble enzyme prepared from 2.5 x 10^6/ml of Molt 4 cells, the sample was fractionated with acid-soluble enzyme prepared from 2.5 x 10^6/ml and incubated with alkaline phosphatase and x-ray phosphorfluorometry.

Figure 3. NMR Spectra of Acld Metabolism in Molt 4 Cells. Acld-soluble enzyme prepared from 10 μM Acld-treated cells (2.5 x 10^6/ml) was applied to a Biocarb 1.0 mm column as described under "Materials and Methods." After the exchange buffer of acid-soluble enzyme prepared from 2.5 x 10^6/ml of Molt 4 cells, the sample was fractionated with acid-soluble enzyme prepared from 2.5 x 10^6/ml and incubated with alkaline phosphatase and x-ray phosphorfluorometry.

Figure 4. NMR Spectra of Acld Metabolism in Molt 4 Cells. Acld-soluble enzyme prepared from 10 μM Acld-treated cells (2.5 x 10^6/ml) was applied to a Biocarb 1.0 mm column as described under "Materials and Methods." After the exchange buffer of acid-soluble enzyme prepared from 2.5 x 10^6/ml of Molt 4 cells, the sample was fractionated with acid-soluble enzyme prepared from 2.5 x 10^6/ml and incubated with alkaline phosphatase and x-ray phosphorfluorometry.

Figure 5. NMR Spectra of Acld Metabolism in Molt 4 Cells. Acld-soluble enzyme prepared from 10 μM Acld-treated cells (2.5 x 10^6/ml) was applied to a Biocarb 1.0 mm column as described under "Materials and Methods." After the exchange buffer of acid-soluble enzyme prepared from 2.5 x 10^6/ml of Molt 4 cells, the sample was fractionated with acid-soluble enzyme prepared from 2.5 x 10^6/ml and incubated with alkaline phosphatase and x-ray phosphorfluorometry.