ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1992, p. 2473–2477
0066-4804/92/112473-05$02.00/0

Vol. 36, No. 11

Intracellular Zidovudine (ZDV) and ZDV Phosphates as Measured by a Validated Combined High-Pressure Liquid Chromatography–Radioimmunoassay Procedure

J. T. SLUSHER,[1,2] S. K. KUWAHARA,[1,2]* F. M. HAMZEH,[1,2] L. D. LEWIS,[1,2]†
D. M. KORNHAUSER,[1,2,3]‡ AND P. S. LIETMAN[1,2,3]

Division of Clinical Pharmacology[1] and Departments of Medicine[2] and of Pharmacology and Molecular Sciences,[3] The Johns Hopkins University School of Medicine, Baltimore, Maryland 21287-5554

Received 27 April 1992/Accepted 7 September 1992

In vitro studies of zidovudine (ZDV) phosphorylation may not accurately reflect the in vivo dose-response relationship, which is crucial to determining the relationship between ZDV exposure, efficacy, and toxicity. However, measurement of ZDV phosphorylated anabolites in peripheral blood mononuclear cells (PBMCs) from ZDV-treated human immunodeficiency virus (HIV)-infected patients would be extremely useful in the more appropriate utilization of ZDV in the treatment of HIV infection. We developed a specific and sensitive combined high-pressure liquid chromatography (HPLC)–radioimmunoassay (RIA) procedure for the determination of ZDV, ZDV-monophosphate, ZDV-diphosphate, and ZDV-triphosphate in PBMCs taken from ZDV-treated HIV-infected patients. ZDV and its anabolites were extracted from washed, Ficoll-Paque-isolated PBMCs and then separated by HPLC using a strong anion-exchange column. The anabolites were then hydrolyzed to ZDV with acid phosphatase. ZDV was then measured by using a modified commercially available RIA protocol. Our method was validated by measuring [^{3}H]ZDV anabolites generated in Molt-4 cells radioisotopically and simultaneously by the combined HPLC-RIA procedure. The ZDV determinations correlated well (r^2 = 0.97) over the range of 0.037 to 5.2 pmol (10 to 1,400 pg) per assay tube. Furthermore, we defined the stability of ZDV anabolites during ficoll isolation and the recovery after extraction and cleanup. We then measured intracellular parent ZDV and its phosphorylated anabolites in PBMCs from six ZDV-treated HIV-infected patients (PBMCs were taken 2 h after a 300-mg oral dose). The mean concentrations (± standard deviations) of parent and of mono-, di-, and triphosphates were 0.15 ± 0.08, 1.4 ± 1, 0.082 ± 0.02, and 0.081 ± 0.03 pmol/10^6 PBMC, respectively (one pmol/10^6 PBMC represents a concentration of approximately 1 μM). Concurrent serum ZDV concentrations were between 1.3 and 7.1 μM. This method should provide a useful tool for evaluating in vivo pharmacokinetics of ZDV anabolites in PBMCs and possibly other cell types, even at the low doses of ZDV currently administered therapeutically.

3′-Azido-3′-deoxythymidine (zidovudine) (ZDV) is a pyrimidine analog first synthesized in the mid 1960s (7) and shown to be active against human immunodeficiency virus (HIV) in 1985 (10). ZDV prolongs survival and reduces the rate of disease progression in HIV-infected patients (5). However, like several other anti-HIV nucleoside analogs, it is a prodrug and requires intracellular anabolic phosphorylation to be converted into its active form. ZDV enters cells by diffusion (8), after which it is first converted by a cellular thymidine kinase to ZDV-monophosphate (ZDV-MP). The ZDV-MP is then further phosphorylated by a cellular thymidylate kinase to ZDV-diphosphate (ZDV-DP). Although thymidylate kinase has a high affinity for ZDV-MP, it also has a low maximum rate of metabolism for its conversion to ZDV-DP, thus making conversion of ZDV-MP to ZDV-DP the rate-limiting reaction in the anabolic pathway (6). ZDV-DP is then further anabolized by other cellular nucleoside diphosphate kinases to ZDV-triphosphate (ZDV-TP). ZDV-TP acts as a competitive inhibitor of the HIV-encoded reverse transcriptase and in addition causes chain termination when incorporated into nascent proviral DNA (6).

Quantification of intracellular levels of ZDV-TP, which is the active metabolite, and defining the time course of ZDV-TP formation and degradation are of paramount importance for understanding the relationships between intracellular levels of ZDV-TP and antiviral activity. Intracellular pharmacokinetics and pharmacodynamic parameters of ZDV-TP would presumably be much more accurate indicators of efficacy and toxicity than extracellular pharmacokinetic parameters.

Attempts at measuring ZDV and its phosphorylated anabolites have been reported by Toyoshima et al. (16), who utilized a high-pressure liquid chromatography (HPLC) system with column switching and UV detection. Kuster et al. (9), using a coupled HPLC-radioimmunoassay (RIA) method, also measured ZDV and ZDV phosphates in HIV-infected patients. These methods, however, have not been thoroughly validated, and they lack the sensitivity (limit of detection, 0.1 pmol/10^6 peripheral blood mononuclear cells [PBMC]) needed for the study of the time course of ZDV anabolism.

Our study describes the development and validation of a specific and sensitive assay for measurement of ZDV and its phosphorylated anabolites from PBMCs of ZDV-treated HIV-infected patients. Our assay utilizes a combined HPLC-

* Corresponding author.
† Present address: 39 Woodfield Rd., Rudgewick, West Sussex RH12 3EP, England.
‡ Present address: Dupont Merck Pharmaceutical Co., Newark, DE 19714.

RIA method with modification of the commercially available RIA protocol.

MATERIALS AND METHODS

Ficoll-Paque was purchased from Pharmacia LKB Biotechnology Inc., Piscataway, N.J. ZDV and acid phosphatases (types XA and IV-S) were purchased from Sigma Chemical Co., St. Louis, Mo. [*methyl*-^{3}H]ZDV (20 Ci/mmol) was purchased from Moravek Biochemicals Inc., Brea, Calif. Hi-ionic Fluor was purchased from Packard, Meriden, Conn. The ZDV RIA kit was obtained from Incstar, Stillwater, Minn. All other chemicals were of the highest grade possible. RPMI 1640 medium and fetal bovine serum were obtained from GIBCO, Grand Island, N.Y. Molt-4 cells were obtained from American Tissue Culture Collection, Rockville, Md.

Human PBMCs from ZDV-treated HIV-infected patients. PBMCs were isolated from healthy volunteers or HIV-infected patients who were receiving ZDV. The duration of previous ZDV therapy at the time of the study ranged from 1 to 8 months. The subjects were fasted overnight and then received a single 300-mg oral dose of ZDV. Fifty milliliters of venous blood was sampled 2 h after the dose. PBMCs were isolated by layering two 25-ml aliquots of blood onto 25 ml of cold (4°C) Ficoll-Paque and centrifuging them at 600 × *g*. All patient volunteers gave written informed consent, and the protocol was approved by the Institutional Review Board of the Johns Hopkins Medical Institutions.

Culture of human PBMCs and Molt-4 cells and extraction of nucleotides. Molt-4 cells or PBMCs were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. The initial cell densities were 4×10^5/ml for Molt-4 cells and 1×10^6/ml for PBMCs. The cells were incubated with 2 μM [^{3}H]ZDV (specific activity, 2,032 to 2,146 dpm/pmol). Incubations were performed at 37°C in 5% CO_2–95% air. The cells were incubated for 6 h, harvested by centrifugation at 600 × *g* for 5 min, washed, and counted. Nucleotides were extracted from PBMCs or Molt-4 cells with 3 ml of 60% methanol as previously described (14). Dried cell extracts were stored at −80°C until analyzed.

Separation, collection, and hydrolysis of ZDV anabolites. ZDV anabolites were separated by using HPLC with a strong-anion-exchange (SAX) column (Patisil 10-SAX; Whatman) and a method similar to that described by Elion et al. (4). The HPLC system consisted of a Hitachi pump, controller, and integrator; an Anspec UV detector; and a Rheodyne manual injector. Cell extract residues were reconstituted in either 400 to 600 μl (Molt-4) or 150 to 200 μl (PBMC) of 0.01 M KH_2PO_4 and centrifuged at 16,000 × *g* for 10 min, and 100 μl of the supernatant fluid was injected.

Nucleotides were eluted by using a gradient of KH_2PO_4 buffer (pH 4.5). The gradient consisted of 0.01 M KH_2PO_4 for 20 min followed by a linear shift to 1.0 M KH_2PO_4 over 40 min, which was maintained for an additional 15 min. The flow rate was 0.5 ml/min, and the detector was set at a wavelength of 260 nm. The retention times of ZDV and its phosphorylated anabolites were determined by isotope counting of 0.5-ml fractions collected over 90 min. Typical retention times for ZDV and its mono-, di-, and triphosphates were 11, 44, 59, and 78 min, respectively. Pooled fractions containing ZDV and its phosphorylated anabolites were enzymatically digested to ZDV with acid phosphatase at a concentration of 40 U/ml.

Sample cleanup. The products from acid phosphatase digestion were first cleaned up by solid-phase extraction cartridges (Supelco, C-18). The products of enzymatic digestion were washed with 4 ml of 10% methanol and then eluted off the cartridge with 3 ml of 50% methanol. The eluates were dried and then reconstituted in 100 μl of 36% methanol. Further cleaning was done with a reverse-phase HPLC system (Beckman C-18 column [250 by 4.6 mm]). The mobile phase consisted of 36% methanol at a flow rate of 1 ml/min, and the retention time of ZDV on this system was approximately 7 min. The pooled fractions representing the peak (ZDV) were then dried and stored at −20°C until assayed.

RIA. The samples were first reconstituted in either 450 μl (PBMC) or 600 to 1,000 μl (Molt-4) of assay diluent buffer (based on expected levels of ZDV). A commercially available ZDV RIA supplied by Incstar was used with the following modifications to quantify the ZDV: (i) the antibody was diluted 1:3 in assay diluent supplemented with 1% fetal bovine serum, (ii) the samples were preincubated for 2 h with diluted antibody before a 1:6 dilution of the ^{125}I-labeled ZDV solution was added, and (iii) the volume of the second antibody (anti-immunoglobulin G) was reduced to 300 μl. We also modified the ZDV standards to be able to measure ZDV concentrations covering a range of 0.0325 to 10 ng/ml (6.5 to 2,000 pg per assay tube). The standard curve was linear over the range 12.5 to 2,000 pg per RIA assay tube ($r^2 > 0.97$). In order to maximize RIA sensitivity (3), RIA kits were used within 2 weeks of the manufacturer's tracer iodination date. Radioactivity in aliquots from [^{3}H]ZDV-labeled Molt-4 cells, which were used as our validation and recovery controls, were determined just before ZDV was measured by RIA.

RESULTS

Separation of ZDV phosphates by SAX chromatography. Figure 1 compares a SAX chromatogram of thymidine-thymidine phosphate standards detected by UV A_{260} with a typical [^{3}H]ZDV anabolite profile chromatographed under the same conditions. The [^{3}H]ZDV anabolite peak retention times and relative peak areas were similar to those of published chromatograms (1, 2, 15).

The peaks were accordingly identified as ZDV, ZDV-MP, ZDV-DP, and ZDV-TP. These chromatographic conditions allowed for the collection of ZDV anabolites without the cocollection of any thymidine phosphates.

Anabolite stability during Ficoll isolation of PBMCs. To determine whether ZDV anabolites were degraded or lost during Ficoll isolation, PBMCs were incubated with 2 μM [^{3}H]ZDV (2 μCi/ml) for 6 h and split into two fractions. One cell portion was immediately extracted as described above, while the second was relayered onto Ficoll-Paque and isolated as whole blood is isolated. The phosphorylated ZDV anabolites in both samples were similar, suggesting that these anabolites were stable during PBMC separation (data not shown).

Recovery of radioactivity during extraction, separation, and cleanup. Recovery of [^{3}H]ZDV and its metabolites after methanol extraction, as calculated from radioactivity associated with cell debris compared with total radioactivity of the methanol extract, was nearly complete (>98%).

Efficiency of collection from the SAX column was determined by first collecting individual fractions to define the peak and then by collecting pooled fractions representing that peak. The peaks of [^{3}H]ZDV and [^{3}H]ZDV phosphates were identified by measuring radioactivity in 0.5-ml fractions collected over 90 min from the SAX column. Another aliquot of [^{3}H]ZDV and its metabolites was injected, and then

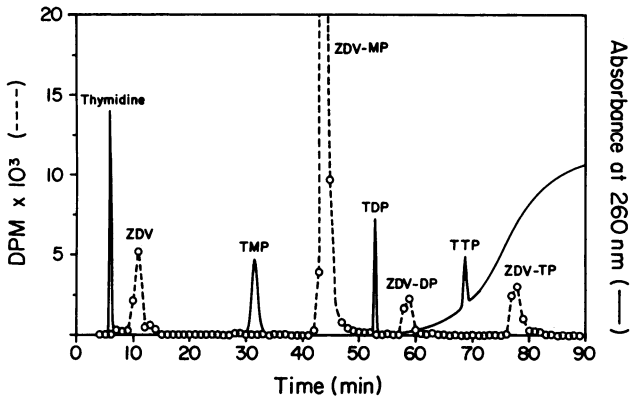

FIG. 1. SAX HPLC chromatogram of [³H]ZDV anabolites from Molt-4 culture overlaid with a chromatogram of thymidine-thymidine nucleotide standards detected by UV at 260 nm.

fractions representing the peaks identified as described above were pooled. Collection efficiency was determined by comparing total radioactivity in the pooled fractions with the sum of radioactivity in the 0.5-ml fractions representing that particular peak as described above (Table 1).

Extraction efficiency represents recovery from the pooled fractions through final sample cleanup. Therefore, extraction efficiency includes the efficiency of hydrolysis, recovery of [³H]ZDV from the solid-phase extraction column, and recovery by HPLC. Extraction efficiency was determined by measuring radioactivity in the pooled fraction and then determining radioactivity in the final reconstituted residue. These findings indicated an extraction efficiency of about 88% (Table 1). The overall recoveries of [³H]ZDV anabolites from Molt-4 cells (Table 1) were calculated by multiplying collection efficiency times extraction efficiency. To determine whether we had similar recovery of ZDV anabolites from PBMCs, [³H]ZDV anabolites from Molt-4 extract were added to the extract from 50×10^6 PBMCs, and then the same series of recovery experiments described above was performed. Recovery results for these experiments, shown in Table 1, suggest that recovery of ZDV anabolites was not affected by endogenous substances (i.e., nucleotides) in PBMCs.

Correlation of RIA with radioisotopic assay. Figure 2 shows a scatter plot of radioisotopic measurements versus concurrent RIA measurements. Correlation between RIA and radioisotopic assay was assessed by weighted (y^{-2}) least-squares analysis of 43 [³H]ZDV samples derived from Molt-4 cell extracts. Amounts measured by RIA and by radioisotopic assay correlated well ($r^2 = 0.97$).

ZDV anabolites in PBMCs from ZDV-treated HIV-infected patients. ZDV anabolites from PBMCs of ZDV-treated HIV-infected patients (2 h after a 300-mg oral dose) were compared with PBMCs from fasting, ZDV-free healthy volunteers. The ZDV-free PBMCs served as the cell background, which was found to be only slightly higher than levels in the buffer blank, and both were well below RIA measurements of patient samples (Table 2).

ZDV phosphates in cultured Molt-4 and PBMCs compared with in vivo PBMC levels. In vitro incubation of PBMCs and Molt-4 cells with [³H]ZDV (Table 3) shows that the ZDV anabolite levels in replicating Molt-4 cell were generally 100-fold higher than the corresponding anabolite levels in PBMCs. Table 4 shows the levels of serum ZDV and intracellular ZDV anabolites in PBMC isolated from seven patients 2 h after they had received 300 mg orally.

TABLE 1. Percent recovery of [³H]ZDV anabolites through collection and extraction

Cells and anabolite	n	Efficiency (mean ± SD)		Overall recovery (mean ± SD)
		Collection	Extraction	
Molt-4				
ZDV	6	88 ± 9	83 ± 9	73 ± 10
ZDV-MP	5	82 ± 6	88 ± 3	72 ± 8
ZDV-DP	6	95 ± 8	88 ± 5	84 ± 11
ZDV-TP	6	89 ± 7	88 ± 8	79 ± 11
PBMCs[a]				
ZDV	3	96 ± 11	76 ± 4	72 ± 4
ZDV-MP	3	86 ± 11	86 ± 5	74 ± 7
ZDV-DP	3	88 ± 5	82 ± 5	73 ± 1
ZDV-TP	2	89 ± 7	85 ± 2	76 ± 5

[a] Dried extract from 25×10^6 to 30×10^6 PBMCs isolated from healthy, ZDV-free subjects was reconstituted with [³H]ZDV anabolites.

DISCUSSION

Attempts at measuring intracellular ZDV-TP from ZDV-treated HIV-patients have not been very successful because intracellular ZDV-TP concentrations are extremely low at therapeutically achieved levels (8, 12–15). ZDV-TP in resting PBMCs exposed to 1.6 μM [³H]ZDV ex vivo for 4 h was 0.002 pmol/10^6 PBMC compared with 0.3 pmol/10^6 in phytohemagglutinin-stimulated PBMCs (15). This suggests that the sensitivity of the RIA protocol, which measures only ZDV, must be improved in order to be able to measure ZDV levels after dephosphorylation of ZDV phosphates.

We first optimized and assessed the sensitivity, specificity, and reproducibility of the commercially available RIA by using extracts from >20 to 10^6 ZDV-free PBMCs as our cell background. The PBMC blank was 15 pg when extract from 20×10^6 ZDV-free PBMCs was used. The detection limit of our method as defined by three times the background level was 0.2 pmol (0.01 pmol/10^6 PBMCs, 20×10^6 cells).

In vitro cell culture using Molt-4 cells incubated with [³H]ZDV (11) provided a convenient tool for validating our measurements in the modified RIA. In addition, the [³H]ZDV-labeled Molt-4 cell extracts allowed us to eliminate cell background when levels measured radioisotopically

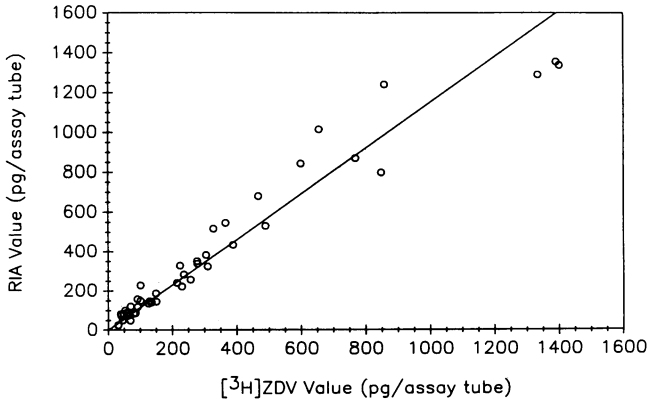

FIG. 2. Correlation between ZDV levels found radioisotopically and simultaneous RIA levels. The equation of the correlation line was $y = 1.15x - 0.92$ ($r^2 = 0.97$).

TABLE 2. ZDV levels measured in buffer blanks and PBMCs from ZDV-free and ZDV-treated volunteers

Sample	n	Mean amt (pg) ± SD/RIA assay tube			
		ZDV	ZDV-MP	ZDV-DP	ZDV-TP
Buffer[a]	3	4 ± 6	5 ± 2	11 ± 12	13 ± 1
PBMC[b]					
Healthy volunteers	5	15 ± 9	17 ± 8[c]	16 ± 8	18 ± 2
ZDV-treated volunteers	9	370 ± 250	2,400 ± 1,700	190 ± 44[d]	190 ± 61

[a] Mobile phase was collected at anabolite retention times.
[b] 18×10^6 to 28×10^6 PBMCs.
[c] $n = 4$ samples.
[d] $n = 6$ samples.

TABLE 4. ZDV and ZDV anabolite levels in PBMCs from ZDV-treated volunteers

Patient no.	Time on ZDV (mo)	Serum ZDV level (μM)	pmol/10^6 cells[a]			
			ZDV	ZDV-MP	ZDV-DP	ZDV-TP
1	5	1.3	0.08	0.70	0.07	0.07
2	2	2.3	0.11	0.85	0.05	0.05
3	6	3.2	0.16	0.84	0.09	NS
4	3	3.0	0.24	1.26	0.10	0.14
5	1	3.4	0.27	0.86	0.09	0.08
6	8	7.1	0.06	3.99	0.09	0.09
7	4	2.2	0.14	1.17	0.09	0.07
Mean	4	3.2	0.15	1.38	0.082	0.082

[a] Assuming a mean cellular volume of 1 pl for PBMCs, then 1 pmol/10^6 cells is equivalent to 1 μM. NS, no sample.

([^{3}H]ZDV, ZDV-MP, ZDV-DP, and ZDV-TP) were much lower than those measured by RIA, suggesting the presence of an interfering substances in PBMC extracts. We found that this interference, which resulted in the high background of ZDV-free PBMCs, was due to the coelution of thymidine phosphates with ZDV phosphates from the SAX column. The ZDV-free PBMC background was reduced markedly when the pH of the mobile phase was lowered, allowing complete separation of thymidine phosphates from ZDV phosphates. Furthermore, [^{3}H]ZDV-labeled Molt-4 cell extracts allowed quantification of recovery at each step. These issues were not thoroughly addressed previously (9, 16).

One problem we did not anticipate was the percent purity of the commercially available [^{3}H]ZDV stock. At the time of receipt, [^{3}H]ZDV is 95% radiochemically pure. It decomposes at a rate of 1.0% per month when stored at −20°C (10a). The effect of freeze-thawing on stability is not known. We found by reverse-phase HPLC that our [^{3}H]ZDV was 87% pure after the study was performed. It was necessary to take into account the percent purity of [^{3}H]ZDV when ZDV concentration was calculated by using the specific activity determined by radioisotopic counting. We believe that the small impurity of [^{3}H]ZDV has minor effects on recovery experiments when absolute radioisotopic counts recovered after separation of the ZDV phosphates are considered. Furthermore, we believe that in the worst scenario, our results may be off by about 13%. This low percentage of error does not substantially affect any of our conclusions from this study, since none of the material presented here was based on such a small increment. However, the small impurity of the [^{3}H]ZDV may explain why extraction recovery for ZDV was consistently less than that of the hydrolyzed ZDV nucleotides.

Toyoshima et al. (16) reported 14 times greater levels of ZDV phosphates in two ZDV-treated HIV-infected patients. They used an HPLC system with column-switching and UV detection. Kuster et al. (9), using an HPLC-RIA system similar to ours, reported levels in PBMCs of HIV-infected patients (2 h after a 250-mg dose of ZDV) about two to five times higher than those in our patients. It is possible that these different levels are due to the methodology or to biological variability. As described in this work, we carefully addressed the questions of recovery and validation, which were clearly not addressed in previous work (9, 16). The possibility of interindividual differences cannot be excluded, since Toyoshima et al. (16) reported some variability between their two patients. Although the two patients had AIDS and were asymptomatic, there is no plausible explanation for why the stages of their HIV infections would result in this difference. However, in vitro results previously described (8, 15) demonstrate that PBMCs stimulated by phytohemagglutinin produced 100 times more ZDV phosphates than did resting PBMCs (8, 15). These findings suggest that the extent of ZDV phosphorylation may depend highly on the degree of activation of the PBMCs. Therefore, it may not be surprising that we found nearly 10-fold-greater levels of ZDV phosphates from HIV-infected subjects than in PBMCs from healthy subjects exposed to similar ZDV concentration in vitro. However, our results and those of Kuster et al. (9) did not show marked interindividual variability to explain the difference of ZDV-TP levels in PBMCs from ZDV-treated HIV-infected patients.

A combined HPLC-RIA method for measuring levels of intracellular ZDV and ZDV phosphates has been developed and validated. The sensitivity of the RIA for ZDV was improved, and the ZDV-free PBMC background was almost eliminated, allowing measurement of intracellular ZDV and ZDV phosphates in AIDS patients.

TABLE 3. ZDV and ZDV anabolite levels in Molt-4 cells and PBMCs cultured in vitro[a]

Cell type	n	Mean amt (pmol/10^6 cells) ± SD			
		ZDV	ZDV-MP	ZDV-DP	ZDV-TP
Molt-4	9	5.9 ± 7	12 ± 7	0.97 ± 0.5	1.6 ± 0.7
PBMC	3	0.05 ± 0.008	0.11 ± 0.009	0.012 ± 0.003	0.011 ± 0.002

[a] Cells or were cultured in 2 μM [^{3}H]ZDV for 6 h.

ACKNOWLEDGMENTS

This work was supported in part by the AIDS Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (grant AI2766806), and the Department of Health and Human Services Food and Drug Administration (grant 223-90-3401).

We thank Kathleen Bell for assistance with the cell culture assays and Denice Jones for patient recruitment and phlebotomy.

REFERENCES

1. **Avramis, V. I., W. Markson, R. L. Jackson, and E. Gomperts.** 1989. Biochemical pharmacology of zidovudine in human T-lymphoblastoid cells (CEM). AIDS **3**:417–422.
2. **Balzarini, J., P. Herdewijn, and E. DeClercq.** 1989. Differential patterns of intracellular metabolism of 2′,3′-didehydro-2′,3′-dideoxythymidine and 3′-azido-2′,3′-dideoxythymidine, two potent anti-human immunodeficiency virus compounds. J. Biol. Chem. **264**:6127–6133.
3. **Campfield, L. A.** 1983. Mathematical analysis of competitive

protein binding assays, p. 125–148. *In* W. D. Odell and P. Franchimont (ed.), Principles of competitive protein-binding assays. John Wiley & Sons, Inc., New York.

4. **Elion, G. B., P. A. Furman, J. A. Fyfe, P. DeMiranda, L. Beauchamp, and H. J. Shaeffer.** 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. Proc. Natl. Acad. Sci. USA **74:**5716–5720.
5. **Fischl, M. A., D. D. Richman, M. H. Grieco, M. S. Gottlieb, P. A. Volberding, O. L. Laskin, J. M. Leedom, J. E. Groopman, D. Mildvan, R. T. Schooley, G. G. Jackson, D. T. Durack, D. King, et al.** 1987. The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. N. Engl. J. Med. **317:**191–197.
6. **Furman, P. A., J. A. Fyfe, M. H. St. Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry.** 1986. 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.
7. **Horwitz, J. P., J. Chua, M. Noel, and V. Nucleosides.** 1964. The monomesylates of 1-(2′-deoxy-B-D-lyxofuranosyl)thymidine. J. Org. Chem. **29:**2074–2078.
8. **Kong, X., Q. Zhu, P. M. Vidal, D. A. Watanabe, B. Polsky, D. Armstrong, M. Ostrander, S. A. Lang, Jr., E. Muchmore, and T. Chou.** 1992. Comparisons of anti-human immunodeficiency virus activities, cellular transport, and plasma and intracellular pharmacokinetics of 3′-fluoro-3′-deoxythymidine and 3′-azido-3′-deoxythymidine. Antimicrob. Agents Chemother. **36:**808–818.
9. **Kuster, H., M. Vogt, B. Joos, V. Nadai, and R. Luthy.** 1991. A method for the quantification of intracellular zidovudine nucleotides. J. Infect. Dis. **164:**773–776.
10. **Mitsuya, H., K. J. Weinhold, P. A. Furman, M. H. St. Clair, S. N. Lehrman, R. C. Gallo, D. Bolognesi, D. W. Barry, and S. Broder.** 1985. 3′-Azido-3′-deoxythymidine (BWA509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*. Proc. Natl. Acad. Sci. USA **82:**7096–7100.

10a. **Moravek, J. (Moravek Biochemicals).** Personal communication.

11. **Starnes, M. C., and Y. C. Cheng.** 1987. Cellular metabolism of 2′,3′-dideoxycytidine, a compound active against human immunodeficiency virus in vitro. J. Biol. Chem. **262:**988–991.
12. **Stretcher, B. N., A. J. Pesce, B. A. Geisler, and W. H. Vine.** 1991. A coupled HPLC/radioimmunoassay for analysis of zidovudine metabolites in mononuclear cells. J. Liq. Chromatogr. **14:**2261–2272.
13. **Stretcher, B. N., A. J. Pesce, J. A. Murray, P. E. Hurtubise, W. H. Vine, and P. T. Frame.** 1991. Concentrations of phosphorylated zidovudine (ZDV) in patient leukocytes do not correlate with ZDV dose or plasma concentrations. Ther. Drug Monit. **13:**335–341.
14. **Stretcher, B. N., A. J. Pesce, J. R. Wermeling, and P. E. Hurtubise.** 1990. *In vitro* measurement of phosphorylated zidovudine in peripheral blood leukocytes. Ther. Drug Monit. **12:**481–489.
15. **Törnevik, Y., B. Jacobsson, S. Britton, and S. Eriksson.** 1991. Intracellular metabolism of 3′-azidothymidine in isolated human peripheral blood mononuclear cells. AIDS Res. Hum. Retroviruses **7:**751–759.
16. **Toyoshima, T., S. Kimura, S. Muramatsu, K. Takahagi, and K. Shimada.** 1991. A sensitive nonisotopic method for the determination of intracellular azidothymidine 5′-mono-, 5′-di, and 5′-triphosphate. Anal. Biochem. **196:**302–307.