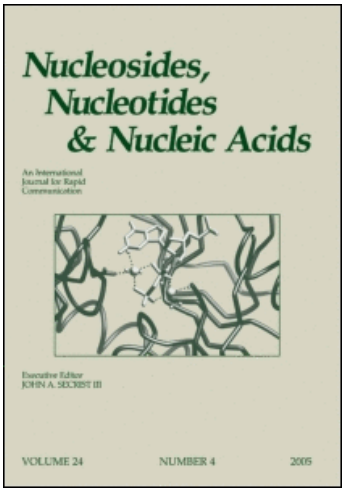


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Monitoring the Intracellular Metabolism of Nucleoside Phosphoramidate Pronucleotides by ^{31}P NMR

Jisook Kim[a]; Daniel P. Drontle[a]; Carston R. Wagner[a]
[a] Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota, USA

Online publication date: 02 October 2004

To cite this Article Kim, Jisook , Drontle, Daniel P. and Wagner, Carston R.(2004) 'Monitoring the Intracellular Metabolism of Nucleoside Phosphoramidate Pronucleotides by ^{31}P NMR ', Nucleosides, Nucleotides and Nucleic Acids, 23: 1, 483 — 493
To link to this Article: DOI: 10.1081/NCN-120028341
URL: http://dx.doi.org/10.1081/NCN-120028341

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 23, Nos. 1 & 2, pp. 483–493, 2004

Monitoring the Intracellular Metabolism of Nucleoside Phosphoramidate Pronucleotides by ^{31}P NMR†

Jisook Kim, Daniel P. Drontle, and Carston R. Wagner*

Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota, USA

ABSTRACT

The intracellular metabolism of 3′-azido-3′-deoxythymidine (AZT)-(L)-tryptophan methyl ester phosphoramidate (L-ATO) and AZT-(L)-phenylalanine methyl ester phosphoramidate (L-APO) by the human T-lymphoblastoid cell line CCRF-CEM (CEM-1.3) and peripheral blood mononuclear cell line (PBMC) was investigated with high field ^{31}P NMR spectroscopy. The AZT amino acid phosphoramidates were shown to accumulate intracellularly and to be readily converted into AZT-MP by both tissues types. Thus, the efficient delivery of nucleoside monophosphates to cells can be facilitated by nucleoside phosphoramidate pronucleotides.

Key Words: Prodrugs; Antiviral agent; AZT; Phosphoramidate; ^{31}P NMR.

†In honor and celebration of the 70th birthday of Professor Leroy B. Townsend.

*Correspondence: Dr. Carston R. Wagner, Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55454, USA; Fax: (612) 624-0139; E-mail: wagne003@tc.umn.edu.

DOI: 10.1081/NCN-120028341 1525-7770 (Print); 1532-2335 (Online)

INTRODUCTION

Various nucleoside analogs are known for their role as antiviral or anticancer agents for the treatment of cancer and viral diseases.[1–4] The mechanism of action of these analogs has been studied extensively and the most probable mechanism is believed to be inhibition of viral or transformed DNA chain elongation and/or ultimate termination. This mechanism requires that, after cellular uptake, nucleosides must be converted into their 5′-mono, di, and triphosphates by cellular nucleoside kinases.[5–9] Consequently, the efficacy of nucleoside analogs as antiviral and anticancer agents is limited by their ability to serve as substrates for nucleoside kinases. Decreased activity of cellular kinases has been associated with a resistance of target tissues toward these agents.[10–16] In principle, treatment with nucleoside 5′-monophosphate could overcome the limitations of nucleoside analogs. However, nucleoside monophosphates have poor cell permeability and are excellent substrates for plasma and extracellular phosphatases.[17–19]

Numerous pronucleotide approaches have been devised in an effort to improve nucleoside/nucleotide analogs.[20–25] For example, nucleoside amino acid phosphoramidates have shown promise as potent antiviral and/or anticancer agents with enhanced activity and reduced cytotoxicity.[26–30] Specifically, we have demonstrated that 3′-azido-3′-deoxythymidine (AZT) amino acid phosphoramidate derivatives exhibited potent antiviral and anticancer activity with less cytotoxicity and an improved plasma half-life compared to AZT (Figure 1).[31,32]

Previously, we have shown that AZT amino acid phosphoramidates are able to generate phosphorylated AZT.[31] Two possible decomposition pathways can lead to the generation of 3′-azido-3′-deoxythymidine monophosphate (AZT-MP) (Scheme 1).[33] In the first pathway, the P–O bond can be hydrolyzed, which would liberate the nucleoside AZT. This free nucleoside can then be rephosphorylated by thymidine kinase to form AZT-MP. In the second pathway, the P–N bond can be cleaved to yield AZT-MP directly. For both cases, the further phosphorylation of AZT-MP will generate

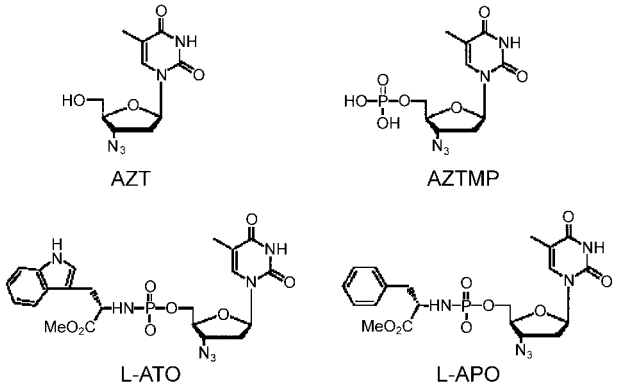
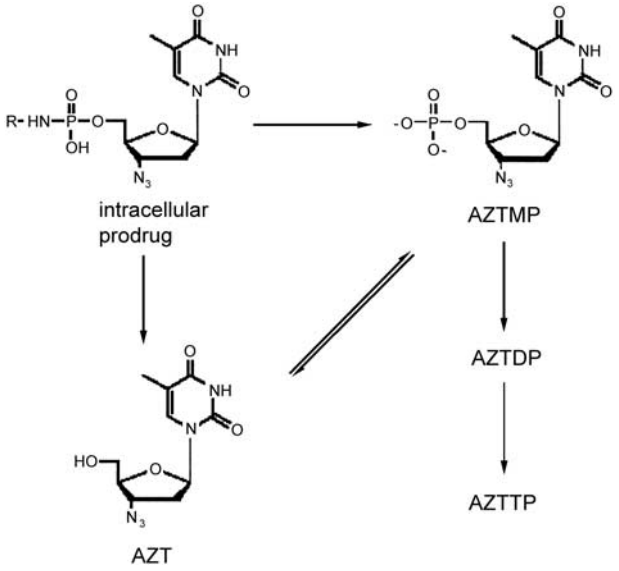


Figure 1. Structures of AZT, AZT-MP, L-ATO and L-APO.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016



Scheme 1. Proposed intracellular metabolism of AZT amino acid phosphoramidate.

AZT-TP which will compete with thymidine triphosphate for the active sites of DNA polymerases. Thus, it is critical to identify the major metabolite of AZT amino acid phosphoramidates and to investigate cell line differences in metabolism.

In this context, Imbach and coworkers have developed a method for determining the decomposition of phosphoramide pronucleotides by cell extracts with LC-MS.[34] Although applicable to a broad array of nucleoside pronucleotides, the sensitivity of this method is insufficient for quantitating intracellular prodrug metabolism. To address this issue, our laboratory developed a coupled radioimmunoassay (RIA)-HPLC to quantitate the intracellular metabolism of AZT phosphoramidates.[31] This approach was sensitive enough to examine intracellular AZT phosphoramidate decomposition. Nevertheless, the requirement for a specific nucleoside RIA and the tediousness of the protocol has limited the applicability of this methodology.

Recently, Di Vito and coworkers have demonstrated that ^{1}H and ^{31}P nuclear magnetic resonance (NMR) can be used as a tool to monitor the intracellular metabolism of AZT.[33] While NMR is not typically used for studying drug metabolism, this technique offers several advantages for phosphorylated species over the more conventional methodologies such as HPLC, LC/MS or GC. First, the preparation of samples for analysis should be simple, efficient and not dependent on chromatographic separation steps. Second, the protocol should allow the simultaneous monitoring of multiple phosphorylated metabolites and the parent pronucleotide. Considering these advantages, we explored the utility of ^{31}P NMR spectroscopy as a tool for monitoring the intracellular behavior of pronucleotides by examining the cellular uptake and decomposition of AZT amino acid phosphoramidates by lymphocytes.

MATERIALS AND METHODS

Chemicals

AZT was kindly donated from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Trimethylphosphate (TMP) and AZT-MP were purchased from Sigma. Ethylenediaminetetracetic acid (EDTA, free acid) and 4-morpholineethanesulfonic acid monohydrate (MES) were purchased from Fisher. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was purchased from Cambridge Isotope Laboratory Inc. L-ATO and L-APO were synthesized as previously described.[31,32] RPMI-1640, fetal bovine serum (FBS), and trypan blue stain (0.4%) in saline (0.85%) were purchased from GIBCO (Grand Island, NY, USA). Sterile stock solutions of penicillin G, streptomycin, human interleukin-2, and phytohemagglutinin have been prepared with sterilizing filters.

Culture of Cells

CCRF-CEM cells (human T-lymphoblastoid leukemia cell lines, American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum, penicillin G (Fisher) (100 U/mL), streptomycin (Sigma) (10 μg/mL), and human interleukin-2 (IL-2) (Boehringer Mannheim, IN) (10 U/mL).

PBMC cells were kindly donated by the AIDS CLINICAL TRIALS UNIT (ACTU) in the department of Laboratory-medicine and Pathology at the University of Minnesota and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 U/mL), and streptomycin (100 μg/mL). Cultures were supplemented with phytohemagglutinin (PHA, Sigma, MI) (10 μg/mL) and IL-2 (10 U/mL).

Incubation of Cells with AZT and Prodrug Analogs L-ATO and L-APO

Cells were suspended in a tissue culture flask with a density of 10^6 cells/mL in fresh growth media. The final concentration of test compounds was either 0.5 mM or 2.5 mM. For each experiment, 100 million cells were used for the incubation. The cultures were incubated in a 10% CO_2/air incubator at 37°C for 20 hr. After cells were counted using the trypan blue dye exclusion method, cells were centrifuged to form a cell pellet (1,500 rpm, 10 min, room temperature) and the supernatant removed. The cell pellet was resuspended in 5 mL 60% methanol and stored overnight at −20°C. On the next day, cell debris and the supernatant were separated by centrifugation at 12,000 rpm for 10 min at 4°C and microcentrifuged for 10 sec at 0°C. The supernatant was transferred to a 1.5 mL eppendorf and lyophilized.

^{31}P NMR Spectroscopic Analysis of Cell Samples

Dried cell extract above was reconstituted in 600 μL of D_2O buffer adjusted to pH 6.25 ± 0.5 containing 20 mM MES, 50 mM EDTA, 1 mM TMP and 1 mM DSS and

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

then transferred into a Wilmad 5 mm O.D. NMR tube. The NMR data was collected at 25°C on a Varian Inova 600 MHz spectrometer at the frequency of 243 MHz at gated proton decoupling mode. For each spectrum, 3,000–5,000 scans were collected using 1.5–3 sec delay time (d_1). The identification of peaks due to AZT-MP and AZT-(L)-amino acid phosphoramidates methyl ester was carried out by adding known quantities of authentic AZT-MP and AZT-(L)-amino acid phosphoramidates methyl ester to the cell extract. Each ^{31}P NMR spectrum was referenced to TMP. The integration of peaks due to AZT-MP and prodrug was measured three times and the average value was considered in calculating the concentration of AZT-MP and prodrug. All values for the intracellular concentration of prodrugs and their metabolite represent duplicate measurements.

RESULTS

CEM cells and PBMCs were incubated with AZT, AZT-(L)-tryptophan methyl ester phosphoramidate (L-ATO) or AZT-(L)-phenylalanine methyl ester phosphoramidate (L-APO) for 20 hr to determine possible differences in intracellular uptake and metabolism for these cell lines. The methanolic cell extracts were monitored by ^{31}P NMR spectroscopy, and the intracellular concentrations of the phosphoramidate and AZT-MP were detected simultaneously. For each experiment an equivalent number of cells were incubated in the absence of AZT or the phosphoramidates as a control. As previously observed by Vito and coworkers, AMP, ADP, ATP, and Pi can be easily observed in CEM cells by ^{31}P NMR from −20–5 ppm (Figure 2A).[33] Similar amounts of the adenosine phosphorylated species were also observed for PBMCs (Figure 2B). Significant amounts of intracellular mono-, di- and triphosphate cytosine, guanosine, thymidine and uridine were not detected. This observation is consistent with the fact that the major intracellular metabolite is phosphorylated adenosine.[33,35] Optimization of the chemical shifts by pH titration succeeded in resolving the phosphoramidates (≈8 ppm) and AZT-MP (3.5 ppm) from the other intracellular phosphorylated species. Verification and quantitation of the intracellular amounts of AZT-MP, L-ATO, and L-APO was accomplished by the addition of known concentrations of authentic AZT-MP, L-ATO, and L-APO followed by conversion of the original peak area to the sample concentration.

Incubation of CEM cells and PBMCs with 2.5 mM AZT (Figure 2D) for 20 hr revealed significant accumulation of AZT-MP. Although, little difference was observed between the intracellular levels of L-ATO and L-APO for PBMCs (Figure 2G/2H), nearly twice as much L-ATO was observed in CEM cells than L-APO. (Figure 2E/2F) In contrast, the amounts of intracellular AZT-MP for CEM cells and PBMCs was greater for cells incubated with AZT and L-ATO, than L-APO (Figure 3). Approximately 10–16% of the intracellular phosphorylated AZT (phosphoramidate + AZT-MP) was found to be AZT-MP, when either CEM cells or PBMCs were incubated with either phosphoramidate. High field ^{1}H NMR revealed lower amounts of AZT than AZT-MP accumulated in cells incubated with the phosphoramidates (data not shown).

To determine the effect of concentration on phosphoramidate uptake and metabolism, CEM cells were incubated with 0.5 and 2.5 mM AZT, L-ATO and L-APO. As has been observed previously, the amount of AZT-MP generated from either

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

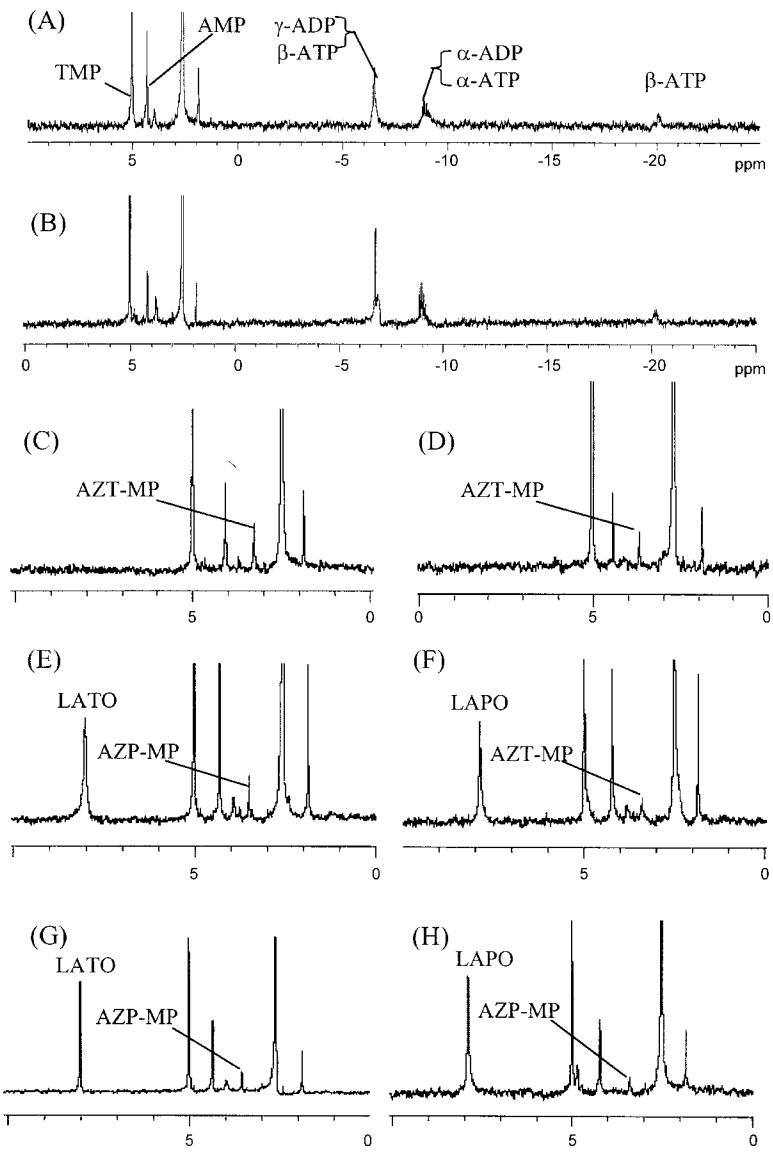


Figure 2. ^{31}P NMR spectra of methanolic extracts of (A) CEM for control experiment, (B) PBMC for control experiment, (C) CEM incubated at 2.5 mM AZT for 20 hr, (D) PBMC incubated at 2.5 mM AZT for 20 hr (E) CEM incubated at 2.5 mM L-ATO for 20 hr, (F) CEM incubated at 2.5 mM L-APO for 20 hr, (G) PBMC incubated at 2.5 mM L-ATO for 20 hr, (H) PBMC incubated at 2.5 mM L-APO for 20 hr.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

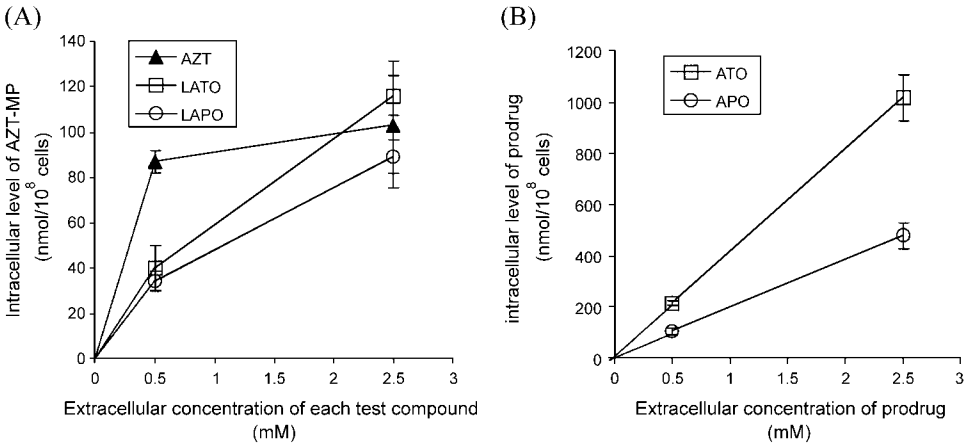


Figure 3. (A) Extracellular concentration of prodrug vs. intracellular concentration of AZT-MP for CEM cell line. (▲); incubated with AZT, (□); incubated with L-ATO, (○); incubated with L-APO. (B) Extracellular concentration of prodrug vs. intracellular concentration of prodrug for CEM cell line. (□); incubated with L-ATO, (○); incubated with L-APO. Each data point represents the average of two independent experiments.

concentration of AZT was not significantly different, while cells incubated with the phosphoramidates accumulated only 50% of the amount of AZT-MP observed for AZT at 0.5 mM. Similar levels of AZT-MP were observed, however, for cells incubated with 2.5 mM AZT, LATO and L-APO. Similar to AZT-MP the intracellular concentration of the phosphoramidates increased linearly, without a noticeable plateau (Figure 4).

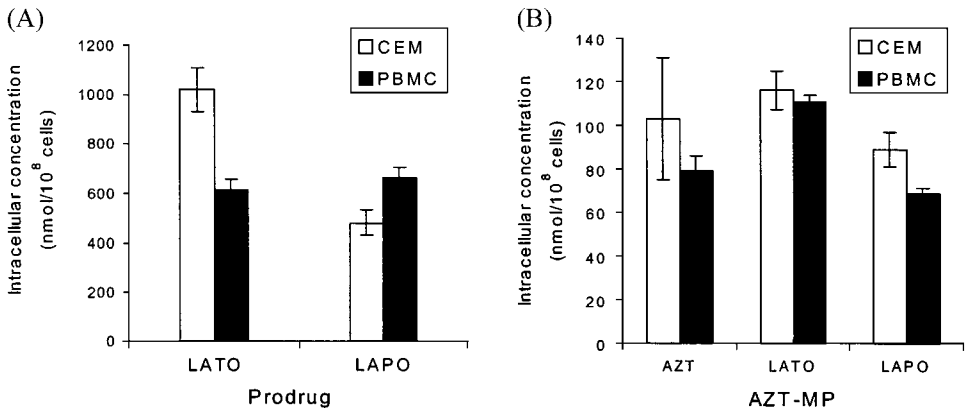


Figure 4. (A) Intracellular concentration of prodrug (nmol/100 million cells) detected by NMR spectroscopy in methanolic extracts of CEM cells and PBMCs incubated at 2.5 mM of L-ATO or L-APO for 20 hr. (B) Intracellular concentration of AZT-MP (nmol/100 million cells) of CEM cells and PBMCs incubated at 2.5 mM of AZT, L-ATO or L-APO for 20 hr.

DISCUSSION

Previously, we have reported that AZT phosphoramidate derivatives appeared to be converted to AZT-MP or AZT, and further metabolized to the triphosphate.[31] This was confirmed by intracellular metabolism studies that relied on a coupled radioimmunoassay-reverse phase HPLC assay. The current study reports on the use of ^{31}P as an alternative method to monitor the cellular uptake and metabolism of AZT amino acid phosphoramidates by CEM cells and PBMCs.

As confirmed by ^{31}P NMR experiments, AZT-MP is a major metabolite of L-ATO and L-APO in both CEM cells and PBMCs. Neither AZT-DP nor AZT-TP was detected (data not shown), consistent with previous reports of the low accumulation of these metabolites in lymphocytic tissues.[36] Significant accumulation of the phosphoramidates was observed even at the lowest concentration. Whether the mechanism of cellular uptake relies on a non-facilitated or facilitated process remains to be determined. Nevertheless, the linear increase in the intracellular accumulation of the phosphoramidates observed at mM concentrations implies that either the test concentrations are below the K_m of the cellular transporter or cellular uptake is non-saturable. The observed differences in the accumulated levels of phosphoramidate in CEM cells, which were not observed for PBMCs, may indicate transport differences between normal and tumor tissue. The generality of this finding remains to be determined.

When compared to the parent nucleoside, CEM cells and PBMCs generated comparable levels of AZT-MP from L-ATO and L-APO only at the highest extracellular concentration, suggesting that AZT was more efficiently processed to the monophosphate than either phosphoramidate. Although, we are unable to delineate the extent to which cellular metabolism relies on P–N bond hydrolysis, the ability of high field ^{31}P NMR or LC/MS to distinguish between ^{18}O labeled AZT-MP phosphate and ^{16}O AZT-MP (0.02 ppm difference by ^{31}P NMR or 2 mass unit difference by MS) will enable future experiments to address this question.3 Regardless, the ability of substantial amounts of the phosphoramidate to accumulate in cells implies that they are capable of serving as a long-lived intracellular depot form of AZT-MP.

In summary, we have demonstrated that high field ^{31}P NMR can be used to quantitate the intracellular behavior of AZT phosphoramidates. With only minor modifications, this approach should be generally applicable to other pronucleotides. The ability of ^{31}P NMR to simultaneously monitor all phosphorylated species derived from a cellular sample, without the need for chromatographic separation and extensive sample preparation is experimentally advantageous providing similar detection limit (nmol range) to conventional LC/MS. Indeed this approach should complement more highly sensitive, but labor intensive approaches that relie on LC-MS and coupled RIA-HPLC.

ACKNOWLEDGMENTS

The authors are thankful to Dr. Balfour's group in ACTU unit in the Department of Lab-medicine and Pathology at the University of Minnesota for providing PBMCs. We are also grateful to Dr. David Live and Dr. Beverly Ostrowski for their assistance

in NMR technique. NMR instrumentation was provided with funds from the NSF (BIR-961477), the University of Minnesota Medical Scholl, and the Minnesota Medical Foundation. This research is supported by NIH-NCI (CA 89615).

REFERENCES

1. Herdewijn, P. Structural requirements for antiviral activity in nucleosides. Drug Discov. Today **1997**, *2*, 235.
2. De Clercq, E. Antiviral activity spectrum and target of action of different classes of nucleoside. Nucleosides Nucleotides **1994**, *13*, 1271.
3. Cheson, B.D.; Keating, M.J.; Plunkett, W. *Nucleoside Analogs in Cancer Therapy*; Marcel Dekker, Inc.: New York, 1997; Vol. 12.
4. Chabner, B.A.; Longo, D.L. *Cancer Chemotherapy and Biotherapy*; Lippincott-Raven: New York, 1996.
5. Vrang, L.; Bazin, H.; Remaud, G.; Chattopadhyaya, J.; Oberg, B. Inhibition of the reverse transcriptase from HIV by 3′-azido-3′deoxythymidine triphosphate and its threo analogue. Antivir. Res. **1987**, *7*, 139–149.
6. Mitsuya, H.; Jarett, R.F.; Matsukura, M.; Veronese, F.D.M.; De Vico, A.L.; Sarngadharan, M.G.; Johns, D.G.; Reitz, M.S.; Broder, S. Long-term inhibition of human T-lymphotropic virus type III lymphadenoparthy-associated virus (human immunodeficiency virus) DNA synthesis and RNA expression in T cells protected by 2′,3′-dideoxynucleosides in vitro. Proc. Natl. Acad. Sci. U. S. A. **1987**, *84*, 2033–2037.
7. Furman, P.A.; St. Clair, M.H.; Weinhold, K.; Rideout, J.L.; Freeman, G.A.; Nusinoff, L.S.; Bolognesi, D.P.; Broder, S.; Mitsuya, H.; Barry, D.W. Phosphorylation of 3′-azido-3′deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. U. S. A. **1986**, *83*, 8333–8337.
8. De Clercq, E. New perspectives for the treatment of HIV infections. Collect. Czechoslov. Chem. Commun. **1998**, *63*, 449–479.
9. Cheng, Y.C.; Dutschman, G.E.; Bastow, K.F.; Sarngadharan, M.G.; Ting, R.Y. Human immunodeficiency virus reverse transcriptase: general properties and its interactions with nucleoside triphosphate analogs. J. Biol. Chem. **1987**, *262*, 2187–2189.
10. Avramis, V.I.; Kwock, R.; Solorzano, M.M.; Gomperts, E. Evidence of in vitro development of drug resistance to azidothymidine in T-lymphocytic leukemia cell lines (Jurkat E6-1/AZT-100) and in pediatric patients with HIV-1 infection. J. Acquir. Immune Defic. Syndr. **1993**, *6*, 1287–1296.
11. Darby, G.; Field, H.J. Altered substrate specificity of herpes simplex virus thymidine dinase confers acyclovir resistance. Nature **1981**, *289*, 81–83.
12. Hall, A.; Cattan, A.R.; Proctor, S.J. Mechanisms of drug resistance in acute leukemia. Leuk. Res. **1989**, *13*, 351–356.
13. Antonelli, G.; Turriziani, O.; Verri, A.; Narciso, P.; Ferri, F.; D'Offizi, G.; Dianzini, F. Long-term exposure to zidovudine affects in vitro and in vivo the efficiency of thymidine kinase. AIDS Res. Hum. Retrovir. **1996**, *12*, 223–228.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

14. Flasshove, M.; Strumberg, D.; Ayscue, L. Structural analysis of the deoxycytidine kinase gene in patients with acute myeloid leukemia and resistance to cytosine arabinoside. Leukemia **1994**, *8*, 780–785.
15. Reichard, P.; Skold, O.; Klein, G. Studies on resistance against 5-fluorouracil. I. Enzymes of the uracil pathway during the development of resistance. Cancer Res. **1962**, *22*, 235–243.
16. Jacobsson, B.; Britton, S.; He, Q. Decreased thymidine kinase levels in peripheral blood cells from HIV-seropositive individuals: implications for zidovudine metabolism. AIDS Res. Hum. Retrovir. **1995**, *11*, 805–811.
17. Schrecker, A.W.; Goldin, A. Antitumor effect and mode of action of 1-β-d-arabinofuranosylcytosine 5′-phosphate in leukemia L 1210. Cancer Res. **1968**, *28*, 802–803.
18. LePage, G.A.; Naik, S.R.; Katakkar, S.B.; Khaliq, A. 9-Beta-D-arabinofuranosyladenine 5′-phosphate metabolism and excretion in humnas. Cancer Res. **1975**, *35* (111), 3036–3040.
19. Leibman, K.C.; Heidelberg, C. The metabolism of ^{32}P-labeled ribonucleotides in tissue slices and cell suspensions. J. Biol. Chem. **1955**, *216*, 823–830.
20. Meier, C. Pronucleotides recent advances in the design of efficient tools for the delivery of biologically active nucleoside monophosphates. Synth. Lett. **1998**, 233–242.
21. Lorcy, M.; Meier, C.; De Clercq, E.; Balzarini, J. Cyclo-saligenyl-5-fluoro-2′-deoxyuridinemonophosphate (cycloSal-FdUMP)—a new prodrug approach for FdUMP. Nucleosides Nucleotides **1997**, *16*, 1303–1306.
22. McGuigan, C.; Devine, K.G.; O'Connor, T.J.; Galpin, S.A.; Jeffries, D.J.; Kinchington, D. Synthesis and evaluation of some novel phosphoramidate derivatives of AZT as anti-HIV compounds. Antivir. Chem. Chemother. **1990**, *1*, 107–113.
23. McGuigan, C.; Sheeka, H.M.; Mahmood, N.; Hay, A.J. Phosphate derivatives of d4T as inhibitors of HIV. Bioorg. Med. Chem. Lett. **1993**, *3*, 1203–1206.
24. Turcotte, J.G.; Srivastava, S.P.; Steim, J.M.; Calabresi, P.; Tibbetts, L.M.; Chu, M.Y. Cytotoxic liponucleotide analogs. II. Antitumor activity of CDP-diacylglycerol analogs containing the cytosine arabinoside moiety. Biochem. Biophys. **1980**, *619*, 619–631.
25. Hong, C.I.; Kirisits, A.J.; Nechaev, A.; Buchheit, D.J.; West, C.R. Synthesis and comparison of antitumor activity of 1-beta-D-arabinofuranosylcytosine conjugates of corticosteroids and selected lipophilic alcohols. J. Med. Chem. **1985**, *28*, 171–177.
26. McIntee, E.J.; Remmel, R.P.; Schinazi, R.F.; Abraham, T.W.; Wagner, C.R. Probing the mechanism of action and decomposition of amino acid phosphomonoester amidates of antiviral nucleoside prodrugs. J. Med. Chem. **1997**, *40*, 3323–3331.
27. Wagner, C.R.; Chang, S.-I.; Griesgraber, G.W.; Song, H.; McIntee, E.J.; Zimmerman, C.L. Antiviral nucleoside drug delivery via amino acid phosphoramidates. Nucleosides Nucleotides **1999**, *18*, 913–919.
28. Molema, G.; Jansen, R.W.; Pauwels, R.D. Targeting of antiviral drugs to T4-lymphocytes. Anti-HIV activity of neoglycoprotein-AZTMP conjugates in vitro. Biochem. Pharmacol. **1990**, *40*, 2603–2610.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

29. Jones, A.S.; McGuigan, C.; Walker, R.T.; Balzarini, J.; De Clercq, E. Synthesis, properties, and biological activity of some nucleoside cyclic phosphoramidates. J. Chem. Soc., Perkin Trans. **1984**, *40*, 2603–2610.
30. Abraham, T.W.; Wagner, C.R. A phosphoramidate-based synthesis of phosphoramidate amino acid diesters of antiviral nucleosides. Nucleosides Nucleotides **1994**, *1994*, 1891–1903.
31. Chang, S.-I.; Griesgraber, G.W.; Southern, P.J.; Wagner, C.R. Amino Acid phosphoramidate monoesters of 3′-azido-3′-deoxythymidine: relationship between antiviral potency and intracellular metabolism. J. Med. Chem. **2001**, *44*, 223–231.
32. Iyer, I.V.; Griesgraber, G.W.; Radmer, M.R.; McIntee, E.J.; Wagner, C.R. Synthesis, in Vitro anti-breast cancer activity, and intracellular decomposition of amino acid methyl ester and alkyl amide phosphoramidate monoesters of 3′-azido-3′-deoxythymidine (AZT). J. Med. Chem. **2000**, *43*, 2266–2274.
33. Vito, M.D.; Bozzi, A.; Ferretti, A.; Cianfriglia, M.; Stefano, B.; Signoretti, C.; Lenti, L.; d'Agostino, F.; Strom, R.; Podo, F. Intracellular metabolism of 3′-azido-3′-deoxythymidine (AZT): a nuclear magnetic resonance study on T-lymphoblastoid cell lines with different resistance to AZT. Biochem. Pharmacol. **1997**, *54*, 979–990.
34. Lefebvre, I.; Perigaud, C.; Pompon, A.; Aubertin, A.M.; Girardet, J.L.; Kirn, A.; Gosselin, G.; Imbach, J.L. Mononucleoside phosphotriester derivatives with S-acyl-2-thioethyl bioreversible phosphate-protecting groups: intracellular delivery of 3′-azido-2′,3′-dideoxythymidine 5′-monophosphate. J. Med. Chem. **1995**, *38* (20), 3941–3950.
35. Huang, D.; Zhang, Y.; Chen, X. Analysis of intracellular nucleoside triphosphate levels in normal and tumor cell lines by high-performance liquie chromatography. J. Chromatogr., B **2003**, *784*, 101–109.
36. Balzarini, J.; Herdewijn, P.; De Clercq, E. 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. **1989**, *264* (15), 6127–6133.

Received August 12, 2003
Accepted November 3, 2003

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Request Permission or Order Reprints Instantly!