

Retention of Metabolites of 2',3'-Didehydro-3'-Deoxy-4'-Ethylnylthymidine, a Novel Anti-Human Immunodeficiency Virus Type 1 Thymidine Analog, in Cells[∇]

Xin Wang,¹ Hiromichi Tanaka,² Masanori Baba,³ and Yung-chi Cheng^{1*}

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06520¹; School of Pharmaceutical Sciences, Showa University, Tokyo 142-8555, Japan²; and Division of Antiviral Chemotherapy, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima 890-8544, Japan³

Received 5 March 2009/Returned for modification 6 May 2009/Accepted 20 May 2009

2',3'-Didehydro-3'-deoxy-4'-ethylnylthymidine (4'-Ed4T), a novel thymidine analog, has more potent anti-human immunodeficiency virus type 1 (HIV-1) activity than its progenitor, stavudine (d4T). The profile of the intracellular metabolites of 4'-Ed4T was qualitatively similar to that of zidovudine (AZT) but not to that of d4T, while after drug removal it showed more persistent anti-HIV activity than AZT or d4T in cell culture. When CEM cells were exposed to various concentrations of 4'-Ed4T, 4'-Ed4T was efficiently taken up by the cells and was readily phosphorylated to 4'-Ed4T monophosphate (4'-Ed4TMP), 4'-Ed4T diphosphate (4'-Ed4TDP), and 4'-Ed4T triphosphate (4'-Ed4TTP). Most importantly, 4'-Ed4TTP, the active metabolite of 4'-Ed4T, persisted significantly longer than 4'-Ed4TDP and 4'-Ed4TMP after drug removal. We further investigated the efflux profiles of 4'-Ed4T in the comparison with those of AZT in CEM cells. After drug removal, both 4'-Ed4T and AZT were effluxed from the cells in a time- and temperature-dependent manner. However, the efflux of 4'-Ed4T from cells was much less efficient than that of AZT. 4'-Ed4T was effluxed from cells only in its nucleoside form, while AZT was effluxed from cells in both its nucleoside and monophosphate forms. The mechanism-of-action study showed that the efflux of 4'-Ed4T or AZT nucleoside might be due to unknown nucleoside transporters which were not related to the equilibrative nucleoside transporters, while the efflux of AZT monophosphate might be due to multidrug resistance protein 4 (MRP4/ABCC4). The results demonstrated that no detectable 4'-Ed4TMP efflux and the less efficient efflux of 4'-Ed4T nucleoside from cells might be one of the biochemical determinants of its persistent antiviral activity in cell culture.

Significant progress in the treatment of human immunodeficiency virus type 1 (HIV-1) infection has been achieved by the advent of antiretroviral therapy, which targets different steps in the viral replication cycle with multiple inhibitors (25). Antiretroviral therapy with these inhibitors has brought about a significant decrease in plasma viremia to undetectable levels and has considerably improved the survival of infected individuals (18). Nucleoside reverse transcriptase (RT) inhibitors (NRTIs) were the first therapeutic agents to demonstrate a clinical role in the treatment of HIV-1 infection, and they continue to play a central role in the treatment of HIV infections (<http://AIDSinfo.nih.gov>). However, their therapeutic benefits are limited by the rapid emergence of drug-resistant viral mutants and delayed toxicity through inhibition of the host DNA polymerase (8, 9). Therefore, there is an urgent need to develop novel antivirals that can inhibit the replication of drug-resistant HIV-1 isolates while displaying favorable pharmacological and toxicity profiles.

Our continuous efforts to search for novel NRTIs with potent anti-HIV-1 activity, low toxicity, and a lack of cross-resistance to existing anti-HIV-1 agents have recently identified the 4'-substituted nucleoside analog 2',3'-didehydro-3'-deoxy-4'-

ethylnylthymidine (4'-Ed4T) (6). 4'-Ed4T is structurally related to stavudine (d4T), while it is more potent against HIV-1 replication than its progenitor, d4T (4, 13). It also showed much less cytotoxicity than d4T in cell culture studies, and 4'-Ed4T triphosphate (4'-Ed4TTP) had no or only a weak inhibitory effect on major host DNA polymerases (24). Moreover, 4'-Ed4T was found to be active against multidrug-resistant HIV-1 clinical isolates, while a unique pattern of RT resistance mutations (P119S, T165A, and M184V) in the virus was observed under the selection pressure of 4'-Ed4T in vitro (13). 4'-Ed4T is currently being evaluated in a phase Ib clinical trial for the treatment of patients with HIV-1 infection. Like other NRTIs, 4'-Ed4T can be phosphorylated in vivo stepwise into its monophosphate, diphosphate, and triphosphate metabolites by host cellular kinases. So far, no other metabolites, in addition to its three phosphate forms, have been found. The phosphorylation of 4'-Ed4T requires enzymes similar to those used by other thymidine analogs; however, the efficiencies are different for the different analogs. 4'-Ed4T is phosphorylated by human thymidine kinase 1 to the 4'-Ed4T monophosphate (4'-Ed4TMP) with an efficiency fourfold higher than that of d4T. 4'-Ed4TMP is phosphorylated by thymidylate kinase, while several enzymes, including nucleoside diphosphate kinase, pyruvate kinase, and 3-phosphoglycerate kinase, could phosphorylate 4'-Ed4T diphosphate (4'-Ed4TDP) to 4'-Ed4TTP (4, 7).

The profiles of the 4'-Ed4T metabolites are qualitatively similar to the profile of zidovudine (AZT), with the monophosphate metabolite being the major metabolite, in contrast to the

* Corresponding author. Mailing address: Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, SHM B226, New Haven, CT 06520. Phone: (203) 785-7119. Fax: (203) 785-7129. E-mail: ycheng@yale.edu.

[∇] Published ahead of print on 26 May 2009.

profile of d4T, the formation of total metabolites of which is relatively poor (7). The antiviral activity of 4'-Ed4T in cells persisted even 48 h after removal of the drug from culture, in contrast to the persistence of the antiviral activities of AZT and d4T (14). In this study, we found that no efflux of 4'-Ed4TMP and the less efficient efflux of 4'-Ed4T nucleoside from cells after drug removal might be one of the biochemical determinants for the longer retention of the 4'-Ed4T metabolites and the persistent antiviral activity of 4'-Ed4T in cell culture.

MATERIALS AND METHODS

Compounds. 4'-Ed4T was synthesized in the laboratory of Hiromichi Tanaka, School of Pharmaceutical Sciences, Showa University, Tokyo, Japan (6). Thymidine and AZT were purchased from Sigma-Aldrich Corp. (St. Louis, MO). The purity of these compounds was verified by high-pressure liquid chromatography (HPLC) analysis. Dipyridamole was purchased from Sigma-Aldrich Corp. Radiochemicals, including [5'-³H]4'-Ed4T and [methyl-³H]AZT, were purchased from Moravex Biochemicals Inc. (Brea, CA). All other chemicals used were of analytical grade or higher.

Cell lines. The CEM cell line (a CD4⁺ T-cell line) was received from the AIDS Research and Reference Reagent Program of the National Institutes of Health and was contributed by Robert Gallo. The cells were grown and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 µg/ml streptomycin (culture medium). The Tet-On RKO cell line was a gift from Edward Chu (Yale University School of Medicine). The cells were cultured at 37°C in the presence of a humidified 5% CO₂ atmosphere.

Analysis of intracellular metabolites of 4'-Ed4T. To evaluate the intracellular 4'-Ed4T metabolites, CEM cells (1 × 10⁶ cells/ml) were incubated with various concentrations (2, 5, 10 µM) of [³H]4'-Ed4T (250 mCi/mmol) in RPMI 1640 medium supplemented with 10% dialyzed FBS, 100 U/ml penicillin G, and 100 µg/ml streptomycin (experimental medium) at 37°C in 5% CO₂ for 12 h. At different time intervals (2, 4, 8, 12 h), aliquots of the cells were harvested by centrifugation and washed three times in ice-cold phosphate-buffered saline (PBS) containing 20 µM dipyridamole. The cell pellets were extracted with 70% (vol/vol) cold methanol. The methanol-soluble extracts were brought to dryness in a Speedvac centrifuge connected to a lyophilizer (Labconco Corp., Kansas City, MO). The samples were dissolved in water and analyzed by HPLC, as described below.

To evaluate the retention of the radioactive metabolites of 4'-Ed4T, CEM cells (1 × 10⁶ cells/ml) were treated with various concentrations (2, 5, 10 µM) of [³H]4'-Ed4T (250 mCi/mmol) for 15 h, as described previously (14). The cells were then washed with ice-cold PBS and resuspended in fresh experimental medium without drugs. Samples were taken after 0, 2, 4, 8, 12, and 24 h of incubation; and the intracellular metabolites of 4'-Ed4T were determined by HPLC, as described below.

Efflux assay. To evaluate the efflux of intracellular thymidine analog metabolites, CEM cells (1 × 10⁶ cells/ml) were preincubated with 1 µM (500 mCi/mmol) of [³H]AZT or [³H]4'-Ed4T in medium comprised of RPMI 1640 with experimental medium at 37°C in 5% CO₂ for 15 h. The cells were then washed with ice-cold PBS and resuspended in fresh experimental medium without drugs. At different time intervals (0, 30, 60, 120 min), the cells and the media were harvested by centrifugation. The cells were then washed three times with ice-cold PBS containing 20 µM dipyridamole (Sigma) and extracted with 70% (vol/vol) cold methanol. The media were first concentrated with a Speedvac centrifuge connected to a Labconco lyophilizer and then extracted with 70% (vol/vol) cold methanol. The methanol-soluble extracts were brought to dryness in a Speedvac centrifuge connected to a Labconco lyophilizer. The samples were dissolved in water, and the metabolites in the extracts were determined by a DE-81 disc (Whatman, Clifton, NJ) assay.

To investigate the effect of the amount of the intracellular monophosphate metabolites on their efflux, CEM cells were cultured with various concentrations of [³H]AZT or [³H]4'-Ed4T with double the radiospecificity (1,000 mCi/mmol) used in the assay described above. After 15 h, the cells were then washed with ice-cold PBS and resuspended in fresh experimental medium without drugs. The cells and media were harvested after 2 h of incubation and extracted as described above. The metabolites in the extract were determined by the DE-81 disc assay and HPLC analysis, as described below.

To investigate the effect of dipyridamole on the efflux of 4'-Ed4T or AZT and

their metabolites, CEM cells were cultured with 2 µM of [³H]AZT or [³H]4'-Ed4T (500 mCi/mmol). After 15 h, the cells were then washed with ice-cold PBS and resuspended in fresh experimental medium with different concentrations of dipyridamole. The cells and media were harvested after 2 h of incubation and were extracted as described above. The metabolites in the extracts were determined by the DE-81 disc assay.

DE-81 disc assay for nucleotide metabolites. The DE-81 disc assay was performed according to previous reports (1, 10). A 30- to 50-µl aliquot from each extract was spotted onto DE-81 discs. The unwashed discs were used for the measurement of total metabolites. The discs were washed by two different methods: they were washed three times with 95% ethanol for 5 min to remove the nucleoside and subsequently dried, or they were washed three times with washing solutions (1 mM ammonium formate plus 4 M formic acid) for 5 min and once with 95% ethanol for 3 min to remove the nucleoside and monophosphate and subsequently dried. For both methods, the improvement in the efficiency of detection of ³H-labeled radioactive nucleotides was achieved by elution of the compound from the discs by incubation with 1 ml of 0.1 N HCl containing 2 M NaCl for 20 min and addition of 10 ml of Safescint scintillation cocktail (American Bioanalytical, Natick, MA) before measurement in a beta scintillation counter (Beckman Coulter Inc., Fullerton, CA). The accuracy and the quality of the DE-81 disc assay were confirmed by measuring the same extract by the HPLC method in parallel (data not shown). All experiments were carried out in duplicate, and each time the extract from culture medium with the same radiospecificity of [³H]AZT or [³H]4'-Ed4T was used as a standard. All data shown are representative of those from three independent experiments. The amounts of intracellular and extracellular metabolites are expressed as pmol/10⁶ cells/ml.

HPLC. HPLC analysis was performed according to previous reports (3, 14). A 50-µl aliquot of each extract was autoinjected into the HPLC column. The metabolites in the soluble fraction were analyzed by HPLC (Shimadzu, Braintree, MA), and the HPLC column was connected to a radiometric detector (flow scintillation analyzer 150TR; Packard) by use of a Partisil SAX column (Whatman). The nucleotides were eluted with a gradient of H₂O to 300 mM potassium phosphate buffer at a flow rate of 1.5 ml/min. The nucleoside and nucleotide peaks were determined by using a diode array detector with a channel set at 265 nm, the maximum λ for 4'-Ed4T. The effluent from the UV detector directly entered an in-line radio spectrophotometer, where it was mixed with 3 ml/min of Monoflow 5 scintillation cocktail (National Diagnostics, Atlanta, GA). This system allowed the detection of nucleoside metabolites that were below the level of UV detection, and the performance parameters have been published previously (3).

Establishment of MRP4 knockdown cell line. The cells of the RKO human colorectal carcinoma cell line were grown in RPMI 1640 medium with 10% (tetra-cycline-free) FBS (Clontech, Mountain View, CA) and were transfected with the pcDNA6/TR vector. Permanent cell lines containing pcDNA6/TR were selected by 5 µg/ml blasticidin. In the downregulated MRP4 cell lines, a short hairpin RNA (shRNA) sequence for the downregulation of MRP4 was designed by using the software provided by Invitrogen. The cDNA oligonucleotide MRP4 (5'-GCACAG AAGCCTTCTTTAAACAGagaTGTTAAAGAAGGCTTCTGTGC-3') (SENSE sequence-loop sequence-ANTISENSE sequence) was cloned into pENTR/H1/TO to express the shRNA. Clones were selected by using 400 µg/ml of phleomycin (Zeocin; Invitrogen). Doxycycline was used to induce shRNA expression. The level of protein expression was determined by Western blotting.

Efflux of monophosphate metabolite in MRP4-knockdown cell line. The impact of MRP4 expression on the efflux of 4'-Ed4TMP and AZT monophosphate (AZTMP) was examined by downregulating the level of MRP4 expression in RKO cells. The RKO/shMRP4 cells were seeded at 5 × 10⁵ cells per culture dish in the presence of 10 ng/ml of doxycycline for shRNA induction. After 72 h of induction, 5 µM (500 mCi/mmol) of [³H]4'-Ed4T or 2 µM (500 mCi/mmol) of [³H]AZT was added to the cells and left for 16 h. The cells were then washed with ice-cold PBS and resuspended in fresh experimental medium without drugs. The cells and media were harvested after 2 h of incubation and extracted as described above. The levels of metabolites in the extracts were determined by the DE-81 disc assay.

Impact of dipyridamole on drug uptake. Dipyridamole, which is an inhibitor of equilibrative nucleoside transporters (ENTs), was used in the drug uptake studies (2, 11). A total of 2 × 10⁶ CEM cells were incubated with various concentrations of dipyridamole at 37°C for 15 min prior to the uptake assays. [³H]thymidine, [³H]AZT, and [³H]4'-Ed4T (50 mCi/mmol) were added to the cells for times ranging from 1 to 30 min. Uptake was terminated after the addition of ice-cold PBS containing 20 µM dipyridamole and the placement of the cells on ice. The cells were then washed five times with cold PBS containing 20 µM dipyridamole and solubilized with 1% Sarkosyl (Fluka Chemie AG, Buchs, Switzerland). The amount of radioactivity was determined in a liquid scintillation counter.

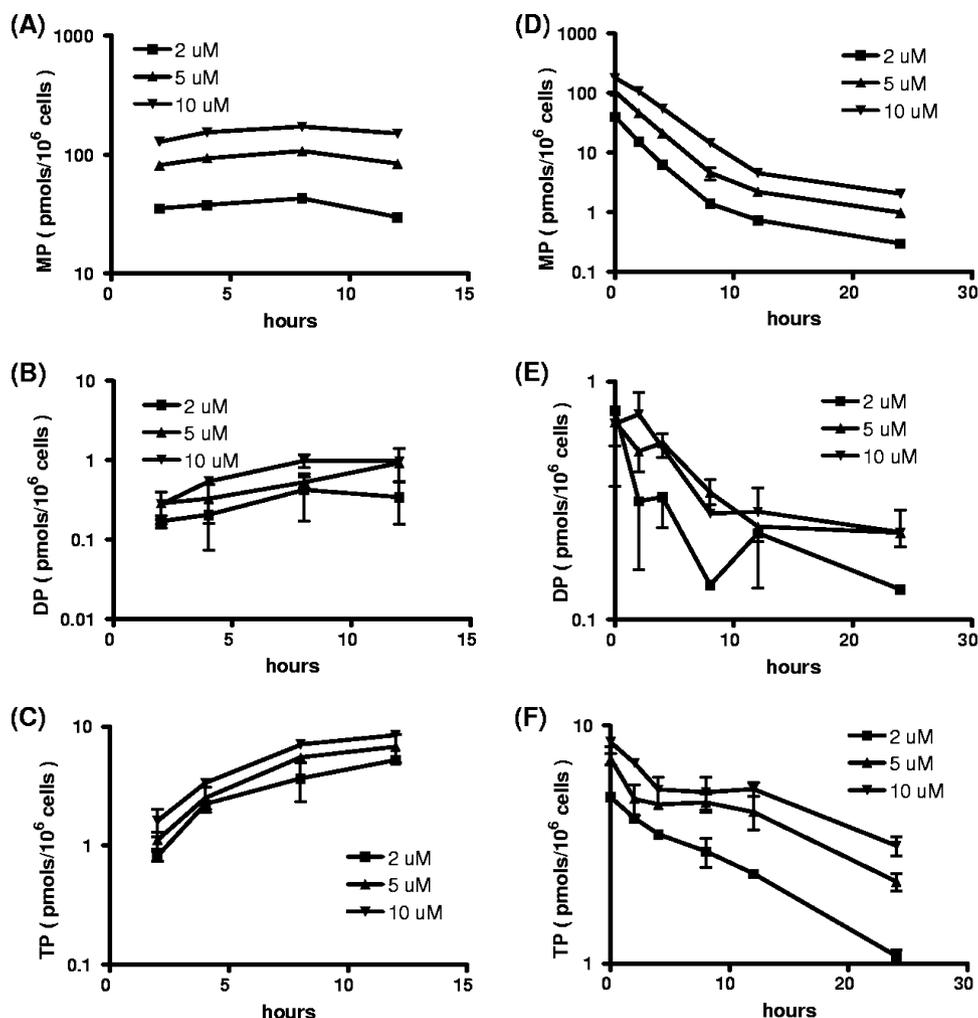


FIG. 1. (A to C) Time- and concentration-dependent intracellular accumulation of 4'-Ed4T radioactive metabolites in CEM cells. CEM cells were incubated in the presence of various concentrations of [³H]4'-Ed4T. At different time intervals, the cells were harvested and extracted with 70% cold methanol. The extracts were subjected to HPLC analyses. The radioactivity detected was calculated as the number of picomoles/10⁶ cells for 4'-Ed4TMP (A), 4'-Ed4TDP (B), and 4'-Ed4TTP (C). The data points represent the means \pm standard deviations of triplicate determinations. (D to F) Intracellular persistence of 4'-Ed4T radioactive metabolites in CEM cells. CEM cells were incubated with various concentrations of [³H]4'-Ed4T for 15 h and thoroughly washed to remove the extracellular drug. After further incubation of the cells for 0, 2, 4, 8, 12, and 24 h, cell extracts were prepared and subjected to HPLC analyses. The radioactivity detected was calculated as the numbers of picomoles/10⁶ cells for 4'-Ed4TMP (D), 4'-Ed4TDP (E), and 4'-Ed4TTP (F). The data points represent the means \pm standard deviations of triplicate determinations.

RESULTS

Intracellular pharmacokinetics of metabolites of 4'-Ed4T in CEM cells. In order to get insight into the persistent antiviral activity of 4'-Ed4T in cells after drug removal, we first determined the amounts of the intracellular metabolites of 4'-Ed4T by the use of different concentrations of [³H]4'-Ed4T in CEM cells at different time intervals and HPLC. The identities of the peaks of the radiolabeled nucleotides of the 4'-Ed4T metabolites were determined with authentic elution time standards of unlabeled nucleotides of 4'-Ed4T (data not shown). When CEM cells were cultured in the presence of 2 μ M [³H]4'-Ed4T, the monophosphate, diphosphate, and triphosphate metabolites of 4'-Ed4T accumulated in the cells in a time-dependent manner; and their levels reached a plateau at about 8 to 12 h (Fig. 1A to C). When the cells were exposed to higher concentrations (5 and 10 μ M) of [³H]4'-Ed4T, the amounts of the

total metabolites and 4'-Ed4TMP increased at all time points (Fig. 1A and data not shown), while the amounts of 4'-Ed4TDP and 4'-Ed4TTP increased disproportionately in comparison with the amount of 4'-Ed4TMP.

In order to examine whether the pharmacokinetics of 4'-Ed4T potentially supported a once- or twice-a-day regimen, we further determined the retention of intracellular 4'-Ed4T metabolites at various concentrations of drug pretreatment. The amounts of each of the intracellular metabolites of 4'-Ed4T were determined by HPLC at 0, 2, 4, 8, 12, and 24 h after drug removal, as shown in Fig. 1D to F. The intracellular level of 4'-Ed4TTP persisted much longer than the intracellular levels of 4'-Ed4TMP and 4'-Ed4TDP for all concentrations of 4'-Ed4T used for pretreatment. When CEM cells were pretreated with 2 μ M [³H]4'-Ed4T, the intracellular levels of 4'-Ed4TMP, -DP, and -TP at 8 h after 4'-Ed4T removal remained at 3%,

18%, and 59% of the values at 0 h, respectively, while at 24 h after drug removal, the intracellular levels of 4'-Ed4TMP, -DP, and -TP were 1%, 18%, and 21% of the values at 0 h, respectively. The data showed that the half-lives ($t_{1/2}$ s) of 4'-Ed4TMP, -DP, and -TP were 1.4, 2.4, and 9.7 h, respectively. These results indicate that the longer retention of intracellular 4'-Ed4TTP might contribute to the persistent antiviral activity after drug removal (Fig. 1D to F). When the cells were pre-treated with higher concentrations (5 and 10 μ M) of [3 H]4'-Ed4T, the retention times of all three intracellular metabolites slightly increased. However, these increases were not statistically significant.

Efflux profile of 4'-Ed4T in CEM cells. The efflux profiles of the 4'-Ed4T metabolites were qualitatively similar to the efflux profile of AZT, with the major metabolites being of the mono-phosphate form (7). Although the total levels of the intracellular metabolites of 4'-Ed4T were only about one-third of those of the intracellular metabolites of AZT, the antiviral activity of 4'-Ed4T in cells persisted much longer after its removal from cell culture than the activity of AZT (14). To evaluate the biochemical determinants of the persistent antiviral activity of 4'-Ed4T, the efflux profile of 4'-Ed4T was compared with that of AZT in CEM cells. After the removal of drug from cell culture, both 4'-Ed4T and AZT effluxed from the cells in a temperature- and time-dependent manner (Fig. 2A and B). When the components of the effluents were examined, the nucleoside was found to be the major effluent in the medium for both 4'-Ed4T and AZT. In addition, the AZTMP metabolite also effluxed into the medium as a function of time, but no 4'-Ed4TMP was detected in the medium (Fig. 2C). The efflux of AZT was much faster and more efficient than that of 4'-Ed4T with time (Fig. 2C). After 30 min of cultivation, about 35.3% of the AZT effluxed from the cells, while only 9.3% of the 4'-Ed4T was found in the medium. Moreover, even after 120 min, only 39.4% of the 4'-Ed4T effluxed from the cells (Fig. 2C).

To further confirm qualitatively the efflux profiles of 4'-Ed4T and AZT, the medium extract was further concentrated and analyzed by HPLC. As shown in Fig. 3A and C, according to the retention times, the nucleosides for both 4'-Ed4T and AZT were effluxed into the medium, while only AZTMP and not 4'-Ed4TMP was detected in the medium. The peak for 4'-Ed4TMP in Fig. 3C may have come from contamination or the lysis of dead cells during the harvesting of the medium, since there was no difference between the peak for the background at 0 h and efflux at 2 h (Fig. 3C, inset). Since there was a threefold greater level of AZTMP accumulation than 4'-Ed4TMP accumulation in the CEM cells when they were exposed to the same concentration of drugs, we further examined whether the efflux of AZTMP but not that of 4'-Ed4TMP was due to the larger amount of AZTMP in the cells. When CEM cells were exposed to 0.1 μ M of AZT, they accumulated less AZTMP (5.7 pmol/ 10^6 cells) than the amount of 4'-Ed4TMP (9.0 pmol/ 10^6 cells) that accumulated in the cells exposed to 1 μ M of 4'-Ed4T. Under those conditions, the efflux of AZTMP could still be clearly detected in the medium (Fig. 3B), which indicates that the lack of efflux of 4'-Ed4TMP from cells was not due to the lower intracellular concentration of 4'-Ed4TMP.

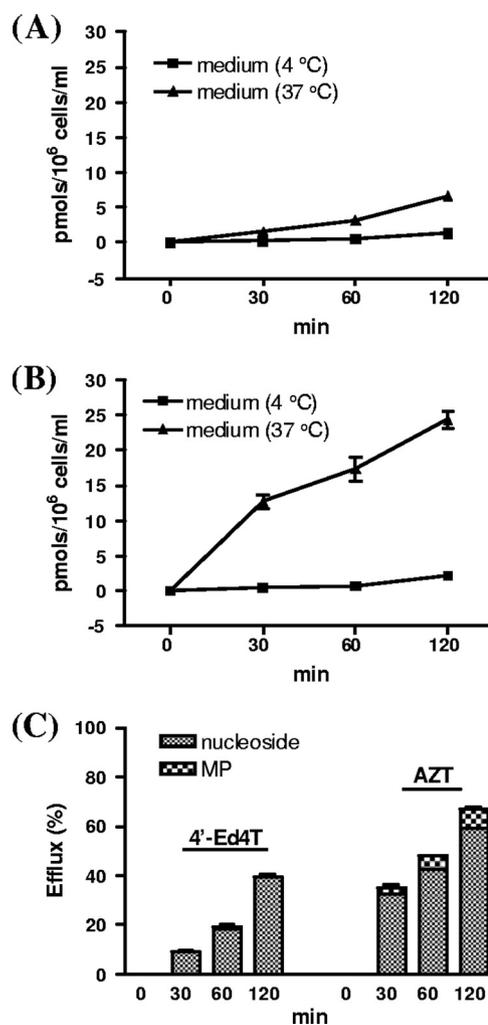


FIG. 2. Time- and temperature-dependent efflux of 4'-Ed4T (A), AZT (B), and/or their metabolites from CEM cells. CEM cells were preincubated with 1 μ M of [3 H]4'-Ed4T and [3 H]AZT for 15 h. The cells were then washed with PBS and resuspended in fresh medium without drugs. At different time intervals, the cells and media were harvested and extracted with 70% cold methanol. The metabolites in the extracts were determined by the DE-81 disc assay. The amounts of extracellular 4'-Ed4T (A), AZT (B), and/or their metabolites in the medium are shown. (C) Ratio of efflux of 4'-Ed4T, AZT, and/or their metabolites as a percentage of the total metabolites.

Mechanism of 4'-Ed4T and AZT efflux. To further characterize the mechanism of 4'-Ed4T and AZT efflux, the effect of dipyridamole, an inhibitor of ENTs, on the efflux of 4'-Ed4T and AZT from CEM cells was examined. As shown in Fig. 4A, the amount of AZT nucleoside and AZTMP in the medium gradually decreased with increasing concentrations of dipyridamole. However, it did not have any effect on 4'-Ed4T efflux. AZTMP was more sensitive to the inhibitory effect of dipyridamole. At a concentration of 20 μ M, dipyridamole almost completely abolished the efflux of AZTMP.

ENTs are bidirectional nucleoside transporters which are sensitive to the inhibitory effect of dipyridamole (15). In order to confirm whether the efflux of 4'-Ed4T and AZT nucleoside was due to the ENTs, the effect of dipyridamole on the uptake of 4'-Ed4T and AZT was examined. As shown in Fig. 4C, the

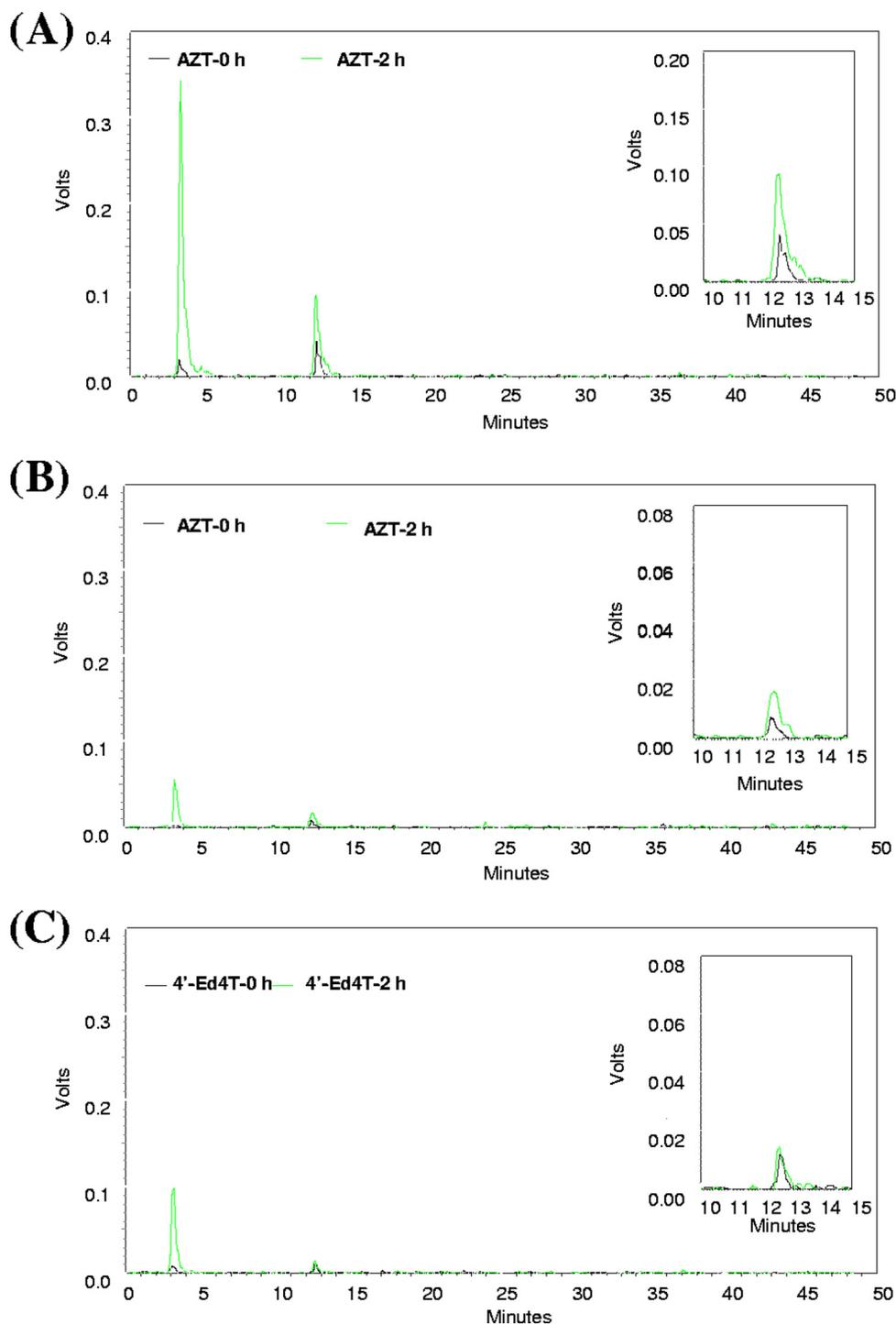


FIG. 3. Efflux of 4'-Ed4T, AZT, and/or their metabolites from CEM cells. CEM cells were preincubated with 1 μ M (A) or 0.1 μ M (B) of [3 H]AZT or 1 μ M of [3 H]4'-Ed4T (C) for 15 h. The cells were then washed with PBS and resuspended in fresh medium for another 2 h. The harvested media were concentrated and then extracted with 70% cold methanol. The extracts were subjected to HPLC. The insets in each panel are enlargements of the areas for the monophosphate peaks.

uptake of thymidine was inhibited by dipyridamole in a dose-dependent manner, while up to 100 μ M of dipyridamole did not show any significant inhibitory effect on the uptake of 4'-Ed4T or AZT. This result indicates that the efflux of 4'-Ed4T and AZT nucleoside might be due to an unknown dipyridamole-sensitive nucleoside transporter(s) but not those defined bidirectional dipyridamole-sensitive ENTs.

It is reported that multidrug resistance protein 4 (MRP4/ABCC4) or breast cancer resistance protein (BCRP/ABCG2) could efflux AZTMP and confer resistance to AZT (20, 22, 23).

Downloaded from <http://aac.asm.org/> on January 16, 2021 by guest

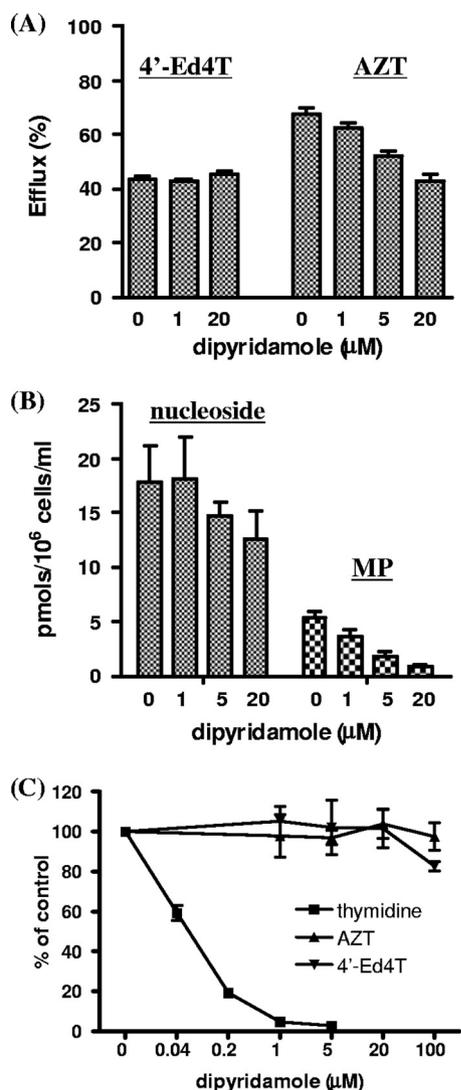


FIG. 4. Effect of the nucleoside inhibitor dipyrindamole on the efflux and influx of 4'-Ed4T and AZT in CEM cells. For the efflux study, CEM cells were cultured with 2 μ M of [3 H]AZT or [3 H]4'-Ed4T. After 15 h, the cells were washed and resuspended in fresh medium with different concentrations of dipyrindamole. The cells and media were harvested after 2 h of incubation and extracted with 70% cold methanol. The metabolites in the extracts were determined by the DE-81 disc assay. (A) Effect of dipyrindamole on the efflux of 4'-Ed4T and AZT; (B) effect of dipyrindamole on the efflux of AZT nucleoside and its monophosphate metabolite; (C) effect of dipyrindamole on the influx of thymidine, 4'-Ed4T, and AZT in CEM cells. For the influx study, CEM cells were incubated with various concentrations of dipyrindamole at 37°C for 15 min prior to the uptake assays. [3 H]thymidine, [3 H]AZT, or [3 H]4'-Ed4T was added to the cells for times ranging from 1 to 30 min. Uptake was terminated by the addition of ice-cold PBS containing 20 μ M dipyrindamole. The cells were washed with the same buffer and solubilized with 1% Sarkosyl. The radioactivity was determined in a liquid scintillation counter.

We checked the expression of those transporters in CEM cells by an RT-PCR method. In accordance with the findings presented in a previous report, the expression of MRP4/ABCC4 but not that of BCRP/ABCG2 could be detected in CEM cells (data not shown) (16). We further studied the relative contributions of MRP4 to the efflux of 4'-Ed4TMP and AZTMP in

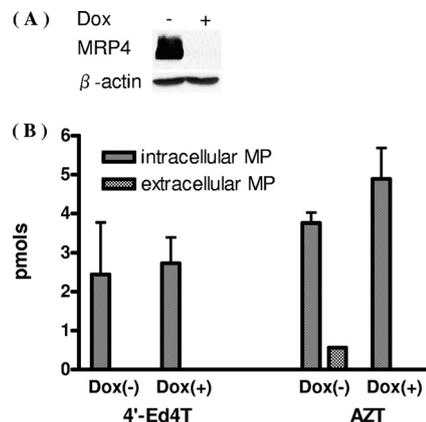


FIG. 5. Effect of downregulation of MRP4 on the efflux of 4'-Ed4TMP and AZTMP. (A) Expression of MRP4 in Tet-On RKO/shMRP4 cells, as detected by Western blotting. Actin was used as the internal control. (B) Doxycycline (Dox)-induced Tet-On RKO/shMRP4 cells were pretreated with 5 μ M of [3 H]4'-Ed4T or 2 μ M of [3 H]AZT for 16 h. The cells were then washed with ice-cold PBS and resuspended in fresh medium. The cell and medium extracts were prepared and subjected to the DE-81 disc assay. The radioactivity detected was calculated as the numbers of picomoles of total intracellular or extracellular monophosphate metabolites for 4'-Ed4T and AZT.

cells. The impact of MRP4 expression on the efflux of 4'-Ed4TMP or AZTMP was examined by downregulating the level of MRP4 expression with doxycycline in stable Tet-On RKO/shMRP4 cells. RKO/shMRP4 cells were cultured with 10 ng/ml of doxycycline for 72 h. Total cell extracts were prepared and analyzed by Western blotting with monoclonal MRP4 antibody by using actin as an internal control. As shown in Fig. 5A, the expression of MRP4 was downregulated to an undetectable level in the presence of 10 ng/ml of doxycycline. Although the downregulation of MRP4 expression completely abolished the efflux of AZTMP into medium from RKO/shMRP4 cells, it did not show any impact on the efflux of 4'-Ed4TMP (Fig. 5B). The intracellular AZTMP level also slightly increased when MRP4 expression was downregulated in those cells. In addition, the downregulation of MRP4 expression did not affect the efflux of 4'-Ed4T or AZT nucleoside (data not shown). This result indicates that the expression of MRP4 in CEM cells might play an important role in the efflux of AZTMP.

DISCUSSION

Novel treatment strategies are needed to overcome HIV-1 drug resistance and delayed toxicity issues. 4'-Ed4T has potent anti-HIV-1 activity (4, 6), maintains considerable activity against HIV-1 mutant strains carrying the K65R or the Q151M complex, and is less inhibitory to mitochondrial DNA synthesis in cell culture than its progenitor, d4T (13, 24). Furthermore, 4'-Ed4T has a superior persistence of antiviral activity even after drug removal in comparison with the persistence of the activities of other RT inhibitors, including d4T and AZT (14). Considering that less frequent dosing could enhance adherence to treatment (5, 21), we examined whether the pharmacokinetics of 4'-Ed4T potentially supported a once- or a twice-

a-day regimen by determining the profiles of anabolic phosphorylation of 4'-Ed4T in CEM cells. Consistent with the findings of our previous study (14), the data from the present study with the CEM cell line showed that 4'-Ed4T efficiently underwent cellular uptake and was readily phosphorylated to 4'-Ed4TMP, 4'-Ed4TDP, and 4'-Ed4TTP in a time- and concentration-dependent manner (Fig. 1A to C). The amounts of 4'-Ed4TMP and 4'-Ed4TDP reached a plateau in 8 to 12 h, while the amount of 4'-Ed4TTP still slightly increased between 8 and 12 h. A longer-term study of the profile of intracellular 4'-Ed4TTP is needed in the future. Most importantly, 4'-Ed4TTP, the active metabolite of 4'-Ed4T, persisted significantly longer ($t_{1/2}$, 8.0 to 9.7 h) than 4'-Ed4TDP ($t_{1/2}$, 2.4 to 5.1 h) and 4'-Ed4TMP ($t_{1/2}$, 1.4 to 2.4 h) after drug removal. It was reported that the intracellular $t_{1/2}$ s of the triphosphate forms of d4T, AZT, dideoxycytosine, abacavir, lamivudine, dideoxyinosine, and tenofovir disoproxil fumarate were 3.5, 2.8, 2.6, 3.3, 10.5 to 15.5, 25 to 40, and 15.4 h, respectively (12, 19). Compared with the $t_{1/2}$ s of these FDA-approved drugs, the intracellular $t_{1/2}$ of 4'-Ed4TTP was in the middle of the range of those $t_{1/2}$ s, and these results suggest its favorable intracellular pharmacokinetics and the possibility for less frequent dosing in clinical studies.

The profile of the phosphorylated metabolites of 4'-Ed4T in T-lymphoid cells is more similar to that of AZT than it is to the progenitor of 4'-Ed4T, d4T, with the monophosphate being the major metabolite of 4'-Ed4T in the cells (7). Interestingly, cells exposed to 4'-Ed4T accumulated total metabolites at a level about threefold less than that of cells exposed to AZT, while 4'-Ed4T showed a much longer persistence of antiviral activity than AZT after drug removal (14). Nakata et al. reported that for CEM cells exposed to AZT, not only the intracellular levels of the accumulated AZTMP but also the intracellular levels of AZT diphosphate (AZTDP) and AZT triphosphate (AZTTP) rapidly declined (12). The $t_{1/2}$ s of AZTMP, AZTDP, and AZTTP were 1.4, 1.6, and 2.8 h in CEM cells, respectively. The intracellular $t_{1/2}$ s of all three 4'-Ed4T phosphates persisted longer than those of the AZT phosphates in CEM cells. In particular, the intracellular $t_{1/2}$ of the active metabolite, 4'-Ed4TTP (8.0 to 9.7 h), was much greater than that of AZTTP (2.8 h) (Fig. 1F) (12). These data suggest that both 4'-Ed4TMP and 4'-Ed4TDP are catabolized slowly and, therefore, that they undergo further phosphorylation to replenish the critical concentration of 4'-Ed4TTP. The data also suggest that 4'-Ed4TTP might be more difficult to catabolize intracellularly than AZTTP. Once it is formed, 4'-Ed4TTP may remain relatively stable and active in cells and able to inhibit HIV-1 replication. The relative longer $t_{1/2}$ and greater accumulation of 4'-Ed4TTP in cells could result in higher cytotoxicity. Nevertheless, 4'-Ed4T showed much less cytotoxicity than d4T and AZT in cell culture studies and might be due to no or only a weak inhibitory effect of 4'-Ed4TTP on major host DNA polymerases (24).

Most of the drugs currently used for the treatment of HIV-1 infection need to cross cell membranes to exhibit pharmacological activity. Thus, host factors, including drug transporters and drug-metabolizing enzymes, might affect intracellular drug concentrations, limiting the ability of drug regimens to inhibit HIV-1 replication. The molecular identification of the proteins involved in nucleoside or nucleotide translocation across the

plasma membrane is still incomplete (15, 17). This is an important bottleneck in the analysis of the putative relationship between drug availability and transport processes. It is indeed probable that the intracellular concentration of phosphorylated metabolites of nucleoside-derived drugs is dependent not only on metabolism but also on the balance between nucleoside influxes and nucleoside or nucleotide efflux, mostly in their monophosphate form, through the proteins MRP/ABCC or BCRP/ABCG2 (20, 22, 23). With the observed superior persistence of intracellular metabolites and antiviral activity, we postulate that less efflux of 4'-Ed4T or its metabolites may also play a role in the longer persistence of 4'-Ed4TTP in the cells after drug removal. The present data from studies with CEM cells showed that both 4'-Ed4T and AZT are effluxed from cells mainly in their nucleoside form, while the efflux of 4'-Ed4T is very slow and less efficient in comparison with that of AZT (Fig. 2C). Our data suggest that nucleoside carrier-mediated transport may contribute to the efflux of 4'-Ed4T and AZT on the basis of various lines of evidence. First, the process is temperature sensitive. It is widely known that protein-mediated cell processes have a marked decrease in activity at low temperatures. By contrast, a simple diffusion process would be less affected by temperature changes, so our data indicated a mediated component in 4'-Ed4T and AZT nucleoside efflux (Fig. 2A and B). Second, the downregulation of MRP4 completely abolished the efflux of AZTMP but did not show any effect on the efflux of 4'-Ed4T or AZT nucleoside, further suggesting that the 4'-Ed4T or AZT nucleoside detected in the medium was not due to the dephosphorylation of their monophosphate forms. Third, although ENTs have bidirectional transport activity, no effect of the nucleoside transporter inhibitor dipyrindamole on the uptake of 4'-Ed4T or AZT by CEM cells suggested that the efflux of 4'-Ed4T or AZT nucleoside might not be due to ENTs but might be due to some other unknown nucleoside transporter(s). Fourth, the different effects of dipyrindamole on the efflux of 4'-Ed4T and AZT nucleoside also suggest that two different nucleoside transporters might be contributing to their efflux (Fig. 4C). The lack of persistence of AZT metabolites may also be due to the efflux of AZTMP from cells by MRP4 (Fig. 4 and 5). Unlike AZTMP, the 4'-Ed4TMP that accumulated cannot be effluxed from CEM cells by MRP4. These results suggest that the lack of 4'-Ed4TMP efflux and the less efficient efflux of 4'-Ed4T nucleoside from cells after drug removal might be one of the biochemical determinants of the longer retention of 4'-Ed4TTP and its persistent antiviral activity.

In conclusion, the cellular uptake of the novel 4'-ethynylthymidine analog 4'-Ed4T and its metabolites was similar to that of AZT, but the efflux of 4'-Ed4T and its metabolites differed from that of AZT. Together with its potent anti-HIV-1 activity, lack of cross-resistance with other anti-HIV-1 thymidine nucleoside analogs, and low level of toxicity, 4'-Ed4T may be a promising candidate for chemotherapy for HIV-1 infection.

ACKNOWLEDGMENTS

We thank Rong Hu, Wing Lam, and Ginger E. Dutschman for helpful suggestions. We thank Elijah Paintsil and Ginger E. Dutschman for critical reading of the manuscript.

This work was supported by Public Health Service grant AI-38204 from the National Institutes of Health to Y.-C.C. Y.-C.C. is a fellow of the National Foundation for Cancer Research.

REFERENCES

- Cheng, Y. C., and W. H. Prusoff. 1974. A new rapid assay for measuring deoxycytidylate- and deoxythymidylate-kinase activities. *Anal. Biochem.* **60**: 545–550.
- Dahlig-Harley, E., Y. Eilam, A. R. Paterson, and C. E. Cass. 1981. Binding of nitrobenzylthioinosine to high-affinity sites on the nucleoside-transport mechanism of HeLa cells. *Biochem. J.* **200**:295–305.
- Dutschman, G. E., E. G. Bridges, S. H. Liu, E. Gullen, X. Guo, M. Kukhanova, and Y. C. Cheng. 1998. Metabolism of 2',3'-dideoxy-2',3'-didehydro- β -L(-)-5-fluorocytidine and its activity in combination with clinically approved anti-human immunodeficiency virus β -D(+) nucleoside analogs in vitro. *Antimicrob. Agents Chemother.* **42**:1799–1804.
- Dutschman, G. E., S. P. Grill, E. A. Gullen, K. Haraguchi, S. Takeda, H. Tanaka, M. Baba, and Y. C. Cheng. 2004. Novel 4'-substituted stavudine analog with improved anti-human immunodeficiency virus activity and decreased cytotoxicity. *Antimicrob. Agents Chemother.* **48**:1640–1646.
- Gathe, J. C., Jr., P. Ive, R. Wood, D. Schurmann, N. C. Bellos, E. DeJesus, A. Gladysz, C. Garris, and J. Yeo. 2004. SOLO: 48-week efficacy and safety comparison of once-daily fosamprenavir /ritonavir versus twice-daily nelfinavir in naive HIV-1-infected patients. *AIDS* **18**:1529–1537.
- Haraguchi, K., S. Takeda, H. Tanaka, T. Nitanda, M. Baba, G. E. Dutschman, and Y. C. Cheng. 2003. Synthesis of a highly active new anti-HIV agent 2',3'-dideoxy-3'-deoxy-4'-ethynylthymidine. *Bioorg. Med. Chem. Lett.* **13**:3775–3777.
- Hsu, C. H., R. Hu, G. E. Dutschman, G. Yang, P. Krishnan, H. Tanaka, M. Baba, and Y. C. Cheng. 2007. Comparison of the phosphorylation of 4'-ethynyl 2',3'-dihydro-3'-deoxythymidine with that of other anti-human immunodeficiency virus thymidine analogs. *Antimicrob. Agents Chemother.* **51**:1687–1693.
- Johnson, A. A., A. S. Ray, J. Hanes, Z. Suo, J. M. Colacino, K. S. Anderson, and K. A. Johnson. 2001. Toxicity of antiviral nucleoside analogs and the human mitochondrial DNA polymerase. *J. Biol. Chem.* **276**:40847–40857.
- Kakuda, T. N. 2000. Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. *Clin. Ther.* **22**:685–708.
- Liou, J. Y., G. E. Dutschman, W. Lam, Z. Jiang, and Y. C. Cheng. 2002. Characterization of human UMP/CMP kinase and its phosphorylation of D- and L-form deoxycytidine analogue monophosphates. *Cancer Res.* **62**:1624–1631.
- Liu, S. H., K. L. Grove, and Y. C. Cheng. 1998. Unique metabolism of a novel antiviral L-nucleoside analog, 2'-fluoro-5-methyl- β -L-arabinofuranosyluracil: a substrate for both thymidine kinase and deoxycytidine kinase. *Antimicrob. Agents Chemother.* **42**:833–839.
- Nakata, H., M. Amano, Y. Koh, E. Kodama, G. Yang, C. M. Bailey, S. Kohgo, H. Hayakawa, M. Matsuoka, K. S. Anderson, Y. C. Cheng, and H. Mitsuya. 2007. Activity against human immunodeficiency virus type 1, intracellular metabolism, and effects on human DNA polymerases of 4'-ethynyl-2-fluoro-2'-deoxyadenosine. *Antimicrob. Agents Chemother.* **51**:2701–2708.
- Nitanda, T., X. Wang, H. Kumamoto, K. Haraguchi, H. Tanaka, Y. C. Cheng, and M. Baba. 2005. Anti-human immunodeficiency virus type 1 activity and resistance profile of 2',3'-dideoxy-3'-deoxy-4'-ethynylthymidine in vitro. *Antimicrob. Agents Chemother.* **49**:3355–3360.
- Paintsil, E., G. E. Dutschman, R. Hu, S. P. Grill, W. Lam, M. Baba, H. Tanaka, and Y. C. Cheng. 2007. Intracellular metabolism and persistence of the anti-human immunodeficiency virus activity of 2',3'-dideoxy-3'-deoxy-4'-ethynylthymidine, a novel thymidine analog. *Antimicrob. Agents Chemother.* **51**:3870–3879.
- Pastor-Anglada, M., P. Cano-Soldado, M. Molina-Arcas, M. P. Lostao, I. Larrayoz, J. Martinez-Picado, and F. J. Casado. 2005. Cell entry and export of nucleoside analogues. *Virus Res.* **107**:151–164.
- Peng, X. X., Z. Shi, V. L. Damaraju, X. C. Huang, G. D. Kruh, H. C. Wu, Y. Zhou, A. Tiwari, L. Fu, C. E. Cass, and Z. S. Chen. 2008. Up-regulation of MRP4 and down-regulation of influx transporters in human leukemic cells with acquired resistance to 6-mercaptopurine. *Leuk. Res.* **32**:799–809.
- Podgorska, M., K. Kocbuch, and T. Pawelczyk. 2005. Recent advances in studies on biochemical and structural properties of equilibrative and concentrative nucleoside transporters. *Acta Biochim. Pol.* **52**:749–758.
- Pomerantz, R. J., and D. L. Horn. 2003. Twenty years of therapy for HIV-1 infection. *Nat. Med.* **9**:867–873.
- Robbins, B. L., R. V. Srinivas, C. Kim, N. Bischofberger, and A. Fridland. 1998. Anti-human immunodeficiency virus activity and cellular metabolism of a potential prodrug of the acyclic nucleoside phosphonate 9-R-(2-phosphonomethoxypropyl)adenine (PMPA), bis(isopropylloxymethylcarbonyl)-PMPA. *Antimicrob. Agents Chemother.* **42**:612–617.
- Schuetz, J. D., M. C. Connelly, D. Sun, S. G. Paibir, P. M. Flynn, R. V. Srinivas, A. Kumar, and A. Fridland. 1999. MRP4: a previously unidentified factor in resistance to nucleoside-based antiviral drugs. *Nat. Med.* **5**:1048–1051.
- Sosa, N., C. Hill-Zabala, E. DeJesus, G. Herrera, A. Florance, M. Watson, C. Vavro, and M. Shaefer. 2005. Abacavir and lamivudine fixed-dose combination tablet once daily compared with abacavir and lamivudine twice daily in HIV-infected patients over 48 weeks (ESS30008, SEAL). *J. Acquir. Immune Defic. Syndr.* **40**:422–427.
- Wang, X., T. Furukawa, T. Nitanda, M. Okamoto, Y. Sugimoto, S. Akiyama, and M. Baba. 2003. Breast cancer resistance protein (BCRP/ABCG2) induces cellular resistance to HIV-1 nucleoside reverse transcriptase inhibitors. *Mol. Pharmacol.* **63**:65–72.
- Wang, X., T. Nitanda, M. Shi, M. Okamoto, T. Furukawa, Y. Sugimoto, S. Akiyama, and M. Baba. 2004. Induction of cellular resistance to nucleoside reverse transcriptase inhibitors by the wild-type breast cancer resistance protein. *Biochem. Pharmacol.* **68**:1363–1370.
- Yang, G., G. E. Dutschman, C. J. Wang, H. Tanaka, M. Baba, K. S. Anderson, and Y. C. Cheng. 2007. Highly selective action of triphosphate metabolite of 4'-ethynyl D4T: a novel anti-HIV compound against HIV-1 RT. *Antivir. Res.* **73**:185–191.
- Yeni, P. G., S. M. Hammer, M. S. Hirsch, M. S. Saag, M. Schechter, C. C. Carpenter, M. A. Fischl, J. M. Gatell, B. G. Gazzard, D. M. Jacobsen, D. A. Katzenstein, J. S. Montaner, D. D. Richman, R. T. Schooley, M. A. Thompson, S. Vella, and P. A. Volberding. 2004. Treatment for adult HIV infection: 2004 recommendations of the International AIDS Society—USA Panel. *JAMA* **292**:251–265.