AAC Accepts, published online ahead of print on 26 May 2009
Antimicrob. Agents Chemother. doi:10.1128/AAC.00302-09

AAC00302-09 Version 3

Study of the retention of metabolites of 4'-Ed4T, a novel anti-HIV-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 06520,[1] School of Pharmaceutical Sciences, Showa University, Tokyo 142-8555, Japan,[2] Division of Antiviral Chemotherapy, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima 890-8544, Japan[3]

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: yccheng@yale.edu

Running Title: Study of the retention of metabolites of 4'-Ed4T

ABSTRACT

2', 3'-didehydro-3'-deoxy-4'-ethynylthymidine (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 intracellular 4'-Ed4T metabolites was qualitatively similar to that of zidovudine (AZT), but not to that of d4T, while it showed more persistent anti-HIV activity after drug removal than AZT or d4T in cell culture. When the CEM cells were exposed to various concentrations of 4'-Ed4T, 4'-Ed4T efficiently underwent cellular uptake into the cells and was readily phosphorylated to 4'-Ed4TMP, 4'-Ed4TDP and 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 AZT in CEM cells. After drug removal, both 4'-Ed4T and AZT were effluxed from the cells in a time- and temperature-dependent manner. But 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 nucleoside and monophosphate form. The mechanism study showed that efflux of 4'-Ed4T or AZT nucleoside might due to unknown nucleoside transporters which were not related to the equilibrative nucleoside transporters, while the efflux of AZTMP might due to the multidrug resistance protein 4 (MRP4/ABCC4). The results demonstrated that no detectable 4'-Ed4TMP efflux and less efficient efflux of 4'-Ed4T nucleoside from cells might be one of the biochemical determinants for its persistent antiviral activity in the cell culture.

Keywords: 4'-Ed4T, AZT, efflux, nucleoside, monophosphate

Significant progress in the treatment of human immunodeficiency virus type 1 (HIV-1) infection have been achieved by the advent of antiretroviral therapy (ART), which targets different steps in the viral replication cycle with multiple inhibitors (25). ART with these inhibitors has brought about a significant decrease of plasma viremia to undetectable levels and has considerably improved the survival of infected individuals (18). Nucleoside reverse transcriptase inhibitors (NRTIs) were the first therapeutic agents to demonstrate clinical role in the treatment of HIV-1 infection, and they continue to play a central role in the treatment of HIV (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 drug-resistant HIV-1 replication 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'-ethynylthymidine (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'-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 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 (MP), diphosphate (DP), and triphosphate (TP) 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 (TK1) to the monophosphate with an efficiency fourfold higher than that of d4T. 4'-Ed4T monophosphate (4'-Ed4TMP) is phosphorylated by thymidylate kinase (TMPK) while several enzymes, including nucleoside diphosphate kinase (NDPK), pyruvate kinase, and 3-phosphoglycerate kinase (PGK), could phosphorylate 4'-Ed4TDP to 4'-Ed4TTP (4, 7).

The profile of 4'-Ed4T metabolites was qualitatively similar to that for zidovudine (AZT), with the monophosphate metabolite as the major metabolite, in contrast to that for d4T, with relatively poor formation of total metabolites (7). The antiviral activity of 4'-Ed4T in cells persisted even 48 h after drug removal from culture in contrast to AZT and d4T (14). In this study, we found that no efflux of 4'-Ed4TMP and less efficient efflux of 4'-Ed4T nucleoside from cells after drug removal might be one of the biochemical determinants for longer retention of 4'-Ed4T metabolites and its persistent antiviral activity 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 zidovudine (AZT) were purchased from Sigma-Aldrich Corp. (St. Loius, 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'-^{3}H]4'-Ed4T and [*methyl*-^{3}H]AZT, were purchased from Moravek 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 (NIH) and was contributed by Robert Gallo. The cells were grown and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/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). Cells were cultured at 37 ^{o}C in the presence of a humidified 5% CO_2 atmosphere.

Analysis of intracellular metabolites of 4'-Ed4T. To evaluate the intracellular 4'-Ed4T metabolites, CEM cells (1 x 10^{6} cells/ml) were incubated with various concentrations (2, 5, 10 µM) of [^{3}H]4'-Ed4T (250 mCi/mmol) in RPMI 1640 medium supplemented with 10% dialyzed FBS, 100 units/ml penicillin G, and 100 µg/ml streptomycin (experimental medium) at 37 ^{o}C in 5% CO_2 for 12 h. At different time intervals (2, 4, 8, and 12 h), aliquots of the cells were harvested by centrifugation and washed three times in ice-cold PBS containing 20 µM dipyridamole. The cell pellets were extracted with 70% (v/v) cold methanol. The methanol-soluble extracts were brought to dryness in a Speedvac centrifuge connected to a Labconco lyophilizer (Labconco corp., Kansas City, MO). The samples were dissolved in water and analyzed with high-performance liquid chromatography (HPLC) as described below.

To evaluate the retention of the radioactive metabolites of 4'-Ed4T, CEM cells (1 x 10^6 cells/ml) were treated with various concentrations (2, 5, 10 μM) of [^{3}H]4'-Ed4T (250 mCi/mmol) for 15 h as previously described (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 anaolog metabolites, CEM cells (1 x 10^6 cells/ml) were pre-incubated with 1 μM (500 mCi/mmol) of [^{3}H]AZT or [^{3}H]4'-Ed4T in medium comprised of RPMI 1640 with 10% dialyzed FBS and 100 units/ml penicillin G, and 100 μg/ml streptomycin (experimental medium) at 37 ^{o}C in 5% CO_2 for 15 h. The cells were then washed with ice-cold phosphate-buffered saline (PBS) and resuspended in fresh experimental medium without drugs. At different time intervals (0, 30, 60 and 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% (v/v) cold methanol. The media were first concentrated with a Speedvac centrifuge connected to a Labconco lyophilizer and then extracted with 70% (v/v) 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 with DE-81 disc (Whatman, Clifton, NJ) assay.

To investigate the effect of the amount of intracellular monophosphate metabolites on their efflux, CEM cells were cultured with various concentrations of [^{3}H]AZT or [^{3}H]4'-Ed4T with double the radiospecificity (1000 mCi/mmol). After 15 h, the cells were then washed with ice-cold phosphate-buffered saline (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 using DE-81 disc assay and HPLC analysis 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 [^{3}H]AZT or [^{3}H]4'-Ed4T (500 mCi/mmol). After 15 h, the cells were then washed with ice-cold phosphate-buffered saline (PBS) and resuspended in fresh experimental medium with different concentrations of dipyridamole. The cells and media were harvested after 2 h of incubation and extracted as described above. The metabolites in the extracts were determined with 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 total metabolites measurement. The discs were washed by different methods, three times with 95% ethanol for 5 min to remove the nucleoside, and subsequently dried, or washed three times with washing solutions (1 mM ammonium formate plus 4 M formic acid) for 5 min, once with 95% ethanol for 3 min to remove the nucleoside and monophosphate (MP), and subsequently dried. For both methods, the improvement in the detection efficiency of ^{3}H-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 quality of DE-81 disc assay were confirmed by measuring the same extract with 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 [^{3}H]AZT or [^{3}H]4'-Ed4T were used at a standard. All data showed are representative of three independent experiments. The amounts of intracellular and extracellular metabolites were expressed as pmols/10^6 cells/ml.

High-performance liquid chromatography (HPLC). The HPLC analysis was performed according to previous reports (3, 14). A 50-μl aliquot of each extract was auto-injected into the HPLC column. The metabolites in the soluble fraction were

analyzed by HPLC (Shimatzu, Braintree, MA) connected to radiometric detector (flow scintillation analyzer 150TR; Packard) using a Partisil SAX column (Whatmann, Clifton, NJ). The nucleotides were eluted by a gradient of H_2O to 300 mM potassium phosphate buffer at a flow rate of 1.5 ml/min. the nucleoside and nucleotide peaks were determined using a diode array detector with a channel set at 265 nm, the λ_{max} 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 for 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 RKO cells (human colorectal carcinoma cell line) were grown in RPMI 1640 medium with 10% (tetracycline-free) fetal bovine serum (Clontech, Mountain View, CA) and were transfected with pcDNA6/TR vector. Permanent cell lines containing pcDNA6/TR were selected by 5 µg/ml blasticidin. In the down-regulated MRP4 cell lines, a shRNA sequence for down-regulation of MRP4 was designed by using the software provided by Invitrogen. The complementary DNA oligonucleotide MRP4 (5'-GCACAGAAGCCTTCTTTAACAgagaTGTTAAAGAAGGCTTCTGTGC -3') (SENSE-loop-ANTISENSE) was cloned into pENTR/H1/TO to express shRNA. Clones were selected by using 400 µg/ml of 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 AZTMP was examined by down-regulating the level of MRP4 expression in RKO cells. The RKO/shMRP4 cells were seeded at 5 x 10^5 cells per culture dish in the presence of 10 ng/ml of doxcycline for the shRNA induction. After 72 h of induction, 5 µM (500 mCi/mmol) of [^{3}H]4'-Ed4T or 2 µM (500 mCi/mmol) of [^{3}H]AZT were added to the cells and left for

16 h. The cells were then washed with ice-cold phosphate-buffered saline (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 extracts were determined with DE-81 disc assay.

The impact of dipyridamole on drug uptake. The dipyridamole, which is inhibitor of equilibrative nucleoside transporters, was used in drug uptake studies (2, 11). A total of 2 x 10^6 CEM cells were incubated with various concentration of dipyridamole at 37 ^oC for 15 min prior to the uptake assays. [^{3}H]thymidine, [^{3}H]AZT and [^{3}H]4'-Ed4T (50 mCi/mmol) was 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 radioactivity was determined in a liquid scintillation counter.

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 intracellular metabolites of 4'-Ed4T with different concentration of [^{3}H]4'-Ed4T in CEM cells at different time intervals by using HPLC. The identities of the peaks of the radiolabeled nucleotides of 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 μM [^{3}H]4'-Ed4T, the MP, DP and TP metabolites of 4'-Ed4T accumulated in the cells in a time-dependent manner and reached plateau at about 8 to 12 h (Figure 1A, 1B, and 1C). When the cells were exposed to higher concentrations (5 and 10 μM) of [^{3}H]4'-Ed4T, the amounts of total metabolites and 4'-Ed4TMP increased at all time point (Figure 1A and data not shown). While 4'-Ed4TDP and 4'-Ed4TTP increased disproportionately in comparison with that 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 Figure 1D, 1E, and 1F. The intracellular level of 4'-Ed4TTP persisted much longer than those of 4'-Ed4TMP and 4'-Ed4TDP for all concentrations of 4'-Ed4T pre-treatment. When CEM cells were pretreated with 2 μM [^{3}H]4'-Ed4T, the intracellular levels of 4'-Ed4TMP, DP, and TP at 8 h after 4'-Ed4T removal had remained, with 3%, 18% and 59%, respectively, of the values at 0 h. While at 24 h after drug removal, intracellular levels of 4'-Ed4TMP, DP, and TP were 1%, 18% and 21%, respectively. The data showed that the half-life ($t_{1/2}$) values of 4'-Ed4TMP, DP, and TP were 1.4, 2.4 and 9.7 h, respectively. These results indicate that longer retention of intracellular 4'-Ed4TTP might contribute to the

persistent antiviral activity after drug removal (Fig. 1D, 1E, 1F). When the cells were pre-treated with higher concentrations (5 and 10 μM) of [^{3}H]4'-Ed4T, the retention time of all three intracellular metabolites slightly increased. However these increases were not statistically significant.

The efflux profile of 4'-Ed4T in CEM cells. The profile of 4'-Ed4T metabolites was qualitatively similar to that of zidovudine (AZT), with the monophosphate metabolites as the major metabolite (7). Though the total intracellular metabolites of 4'-Ed4T were only about one third of those of AZT, the antiviral activity of 4'-Ed4T in cells persisted much longer after drug removal from cell culture in comparison with 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 drug removal from cell culture, both 4'-Ed4T and AZT effluxed out of cells in a temperature- and time-dependent manner (Fig. 2A and 2B). When the component of effluents was examined, the nucleoside was found to be the major effluent in the medium for both 4'-Ed4T and AZT. In addition, AZT monophosphate (MP) metabolite also effluxed into 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 cultivation, there was about 35.3 % of AZT effluxed out of cells, while only 9.3 % of 4'-Ed4T was found in the medium. Moreover, even after 120 minutes, there was only 39.4 % of 4'-Ed4T was effluxed out of cells (Fig. 2C).

To further qualitatively confirm the efflux profile of 4'-Ed4T and AZT, the medium extract was further concentrated and analyzed by using HPLC method. As shown in Figure 3A and 3C, according to the retention time, the nucleosides for both 4'-Ed4T and AZT were effluxed into the medium, while only AZTMP but not 4'-Ed4TMP was detected in the medium. The 4'-Ed4TMP peak in the figure 3C might come from contaminated or dead cell lysis during the harvesting of medium, since there was no difference between the peak from 0h background and 2 h efflux (Figure 3C

inset). Since there was a 3-fold more AZTMP than 4'-Ed4TMP accumulation in the CEM cells when they exposed to the same concentration of drugs, we further examined whether the efflux of AZTMP but not 4'-Ed4TMP was due to the higher amount of AZTMP in the cells. When CEM cells were exposed to 0.1 μM of AZT, they accumulated less AZTMP (5.7 pmols/10^6 cells) than the 4'-Ed4TMP (9.0 pmols/10^6 cells) accumulated in the cells exposed to 1 μM of 4'-Ed4T. Under those conditions, the efflux of AZTMP still could be clearly detected in the medium (Figure 3B), which indicates that the lack of efflux of 4'-Ed4TMP from cells was not due to the lower intracellular concentration of 4'-Ed4TMP.

The efflux mechanism of 4'-Ed4T and AZT. To further characterize the efflux mechanism of 4'-Ed4T or AZT, the effect of dipyridamole, an inhibitor of equilibrative nucleoside transporters (ENTs), on the efflux of 4'-Ed4T or AZT was examined in CEM cells. As shown in Figure 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 μM, dipyridamole almost completely abolished AZTMP efflux.

The ENTs are bi-directional nucleoside transporters, which are sensitive to the inhibitory effect of dipyridamole (15). In order to confirm whether the efflux of 4'-Ed4T or AZT nucleoside was due to the ENTs, the effect of dipyridamole on the uptake of 4'-Ed4T or AZT was examined. As shown in figure 4C, the uptake of thymidine was inhibited by dipyridamole in a dose-dependent manner; while up to 100 μM of dipyridamole did not show significant inhibitory effect on the uptake of 4'-Ed4T or AZT. This result indicates that the efflux of 4'-Ed4T or AZT nucleoside might be due to unknown dipyridamole-sensitive nucleoside transporter(s) but not those defined bi-directional 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). We checked the expression of those transporters in CEM cells by using RT-PCR method. In accordance with the previous report, the expression of MRP4/ABCC4 but not 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 or AZTMP in cells. The impact of MRP4 expression on the efflux of 4'-Ed4TMP or AZTMP was examined by down-regulating the level of MRP4 expression with doxcycline in a stable Tet-On RKO/shMRP4 cells. RKO/shMRP4 cells were cultured with 10 ng/ml of doxcycline for 72 h. Total cell extracts were prepared and analyzed by Western blotting with monoclonal MRP4 antibody, using actin as an internal control. As shown in Fig. 5A, the expression of MRP4 was down-regulated to undetectable level in the presence of 10 ng/ml of doxcycline. Though the down-regulation of MRP4 expression completely abolished the efflux of AZTMP into medium in RKO/shMRP4 cells, it did not show any impact on efflux of 4'-Ed4TMP (Fig. 5B). The intracellular AZTMP level also slightly increased when the MRP4 was down-regulated in those cells. In addition, the down-regulation 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 mtDNA synthesis in cell culture than its progenitor d4T (13, 24). Furthermore, 4'-Ed4T has superior persistence of antiviral activity even after drug removal in comparison with other RT inhibitors, including d4T and AZT (14). Considering that less-frequent dosing could enhance treatment adherence (5, 21), we examined whether the pharmacokinetics of 4'-Ed4T potentially supported a once- or twice-a-day regimen by determining the profiles of anabolic phosphorylation of 4'-Ed4T in CEM cells. Consistent with our previous study (14), the present data from CEM cell line showed that 4'-Ed4T efficiently underwent cellular uptake into the cells and was readily phosphorylated to 4'-Ed4TMP, 4'-Ed4TDP, and 4'-Ed4TTP in a time- and concentration-dependent manner (Fig. 1A, 1B, and 1C). The amounts of 4'-Ed4TMP and 4'-Ed4TDP reached a plateau in 8 to 12 hours, while the amount of 4'-Ed4TTP still slightly increased between 8 to 12 hours. The longer-scale study on the profile of intracellular 4'-Ed4TTP in the future is needed. Most importantly, 4'-Ed4TTP, the active metabolite of 4'-Ed4T, persisted significantly longer (with a half life ($t_{1/2}$) as 8.0 to 9.7 h) than 4'-Ed4TDP ($t_{1/2}$ as 2.4 to 5.1 h) and 4'-Ed4TMP ($t_{1/2}$ as 1.4 to 2.4 h) after drug removal. It was reported that the intracellular $t_{1/2}$ of the triphosphate forms of d4T, AZT, ddC, ABC, 3TC, ddI, and TDF 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 half-lives of these FDA-approved drugs, 4'-Ed4TTP's intracellular $t_{1/2}$ was in the middle range, and these results suggest its favorable intracellular pharmacokinetics and the possibility for less-frequent dosing in clinical studies.

The profile of phosphorylated metabolites of 4'-Ed4T in T-lymphoid cells is more similar to that of AZT than to its progenitor d4T, with the monophosphate as its major

metabolite in the cells (7). Interestingly, the cells exposed to 4'-Ed4T accumulated about 3 fold less total metabolites than the cells exposed to AZT, while 4'-Ed4T showed much longer persistence of antiviral activity than AZT after drug removal (14). Nakata et al. reported that CEM cells exposed to AZT, not only the intracellular levels of the accumulated AZTMP but also that of AZTDP and AZTTP rapidly declined (12). The $t_{1/2}$ values of AZTMP, AZTDP, and AZTTP were 1.4, 1.6, and 2.8 h in CEM cells, respectively. The intracellular half-lives of all three 4'-Ed4T phosphates persisted longer than those of AZT phosphates in CEM cells, especially the intracellular $t_{1/2}$ of the active metabolite, 4'-Ed4TTP, was much greater, at 8.0 to 9.7 h, 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 they undergo further phosphorylation to replenish the critical concentration of 4'-Ed4TTP. The data also suggest that 4'-Ed4TTP might be difficult to catabolized intracellularly than AZTTP. Once formed, 4'-Ed4TTP may remain relatively stable and active in cells to inhibit HIV-1 replication. The relative longer half-life and accumulation of 4'-Ed4TTP in cells could result in higher cytotoxicity. Even though, 4'-Ed4T showed much less cytotoxicity than d4T and AZT in cell culture studies, that 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 in 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 nucleos(t)ide 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 MRP/ABCC or BCRP/ABCG2 proteins (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 CEM cells showed that both 4'-Ed4T and AZT were mainly effluxed out of cells in their nucleoside form, while the efflux of 4'-Ed4T is very slow and less efficient in the comparison with AZT (Fig. 2C). Our data suggest that nucleoside carrier-mediated transport may contribute to the efflux of 4'-Ed4T and AZT based on 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 2B). Second, the down-regulation of MRP4 completely abolished AZTMP efflux but did not showed any effect on the efflux of 4'-Ed4T or AZT nucleoside further suggested the 4'-Ed4T or AZT nucleoside detected in the medium was not due to the dephosphorylation of their MP form. Third, though the ENTs have bi-directional transport activity, no effect of nucleoside transporter inhibitor dipyridamole on the uptake of 4'-Ed4T or AZT into CEM cells suggested that efflux of 4'-Ed4T or AZT nucleoside might not be due to ENTs but other unknown nucleoside transporter(s). Fourth, the different effect of dipyridamole on the efflux of 4'-Ed4T or AZT nucleoside also suggests that there might be two different nucleoside transporters contributing to their efflux (Fig. 4C). The lack of persistence of AZT metabolites may also due to the efflux of AZTMP out of cells by MRP4 (Fig. 4 and 5). Unlike AZTMP, the accumulated 4'-Ed4TMP can not be efflux out of CEM cells by MRP4. These results suggest that no 4'-Ed4TMP efflux and less efficient efflux of 4'-Ed4T nucleoside from cells after drug removal might be one of the biochemical determinants for longer retention of 4'-Ed4TTP and its persistent antiviral activity.

In conclusion, the novel 4'-ethynylthymidine analog, 4'-Ed4T, has the

characteristic of intracellular metabolites similar to AZT in terms of uptake but differs from AZT in terms of its efflux. Together with its potent anti-HIV-1 activity, lack of cross resistant with other anti-HIV-1 thymidine nucleoside analogs, and low toxicity, 4'-Ed4T may be a promising candidate for HIV-1 chemotherapy.

Acknowledgements

The authors thank Dr. Rong Hu, Wing Lam and Ginger E. Dutschman for helpful suggestions. We thank Dr. Elijah Paintsil and Ginger E. Dutschman for the 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

1. **Cheng, Y. C., and W. H. Prusoff.** 1974. A new rapid assay for measuring deoxycytidylate- and deoxythymidylate-kinase activities. Anal Biochem **60:**545-50.
2. **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.
3. **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-beta-L(-)-5-fluorocytidine and its activity in combination with clinically approved anti-human immunodeficiency virus beta-D(+) nucleoside analogs in vitro. Antimicrob Agents Chemother **42:**1799-804.
4. **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:**1640-6.
5. **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:**1529-37.
6. **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'-didehydro-3'-deoxy-4'-ethynylthymidine. Bioorg Med Chem Lett **13:**3775-7.
7. **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:**1687-93.

8. **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:**40847-57.

9. **Kakuda, T. N.** 2000. Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. Clin Ther **22:**685-708.

10. **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:**1624-31.

11. **Liu, S. H., K. L. Grove, and Y. C. Cheng.** 1998. Unique metabolism of a novel antiviral L-nucleoside analog, 2'-fluoro-5-methyl-beta-L-arabinofuranosyluracil: a substrate for both thymidine kinase and deoxycytidine kinase. Antimicrob Agents Chemother **42:**833-9.

12. **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:**2701-8.

13. **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'-didehydro-3'-deoxy-4'-ethynylthymidine in vitro. Antimicrob Agents Chemother **49:**3355-60.

14. **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'-didehydro-3'-deoxy-4'-ethynylthymidine, a novel thymidine analog. Antimicrob Agents Chemother **51:**3870-9.

15. **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:**151-64.

16. **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.

17. **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:**749-58.

18. **Pomerantz, R. J., and D. L. Horn.** 2003. Twenty years of therapy for HIV-1 infection. Nat Med **9:**867-73.

19. **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(isopropyloxymethylcarbonyl)PMPA. Antimicrob Agents Chemother **42:**612-7.

20. **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:**1048-51.

21. **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:**422-7.

22. **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.

23. **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:**1363-70.

24. **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. Antiviral Res **73:**185-91.

25. **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:**251-65.

Figure legends

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 [^{3}H]4'-Ed4T. At different time intervals, the cells were harvested and extracted with 70% cold methanol. The extracts were subjected to HPLC analyses. The detected radioactivity was calculated as picomoles/10^6 cells for 4'-Ed4TMP (A), 4'-Ed4TDP (B), 4'-Ed4TTP(C). Data points represent mean ± SD of triplicate determinations. (D to F) Intracellular persistence of 4'-Ed4T radioactive metabolites in CEM cells. CEM cells were incubated with various concentrations of [^{3}H]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 detected radioactivity was calculated as picomoles/10^6 cells for 4'-Ed4TMP (D), 4'-Ed4TDP (E), 4'-Ed4TTP (F). Data points represent mean ± SD of triplicate determinations.

Fig. 2. Time- and temperature-dependent efflux of 4'-Ed4T (A) or AZT (B) and/or their metabolites from CEM cells. CEM cells were pre-incubated with 1 μM 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 with DE-81 disc assay. Extracellular 4'-Ed4T (A) or AZT (B) and/or their metabolites in the media was shown. (C) The efflux ratio of 4'-Ed4T or AZT and/or their metabolites was shown as percentages of total metabolites.

Fig. 3. Efflux of 4'-Ed4T or AZT and/or their metabolites from CEM cells. CEM cells were pre-incubated with 1 μM (A) or 0.1 μM (B) of [^{3}H]AZT , or 1 μM 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 insert in each figure was the enlargement of the area for MP peak.

Fig. 4. The effect of nucleoside inhibitor dipyridamole on the efflux and influx of 4'-Ed4T or AZT in CEM cells. For the efflux study, CEM cells were cultured with 2 μM of [^{3}H]AZT and [^{3}H]4'-Ed4T. After 15 h, the cells were washed and resuspended in fresh medium with different concentrations of dipyridamole. The cells and media were harvested after 2 h of incubation and extracted with 70% cold methanol. The metabolites in the extracts were determined with DE-81 disc assay. (A) The effect of dipyridamole on the efflux of 4'-Ed4T or AZT. (B) The effect of dipyridamole on the efflux of AZT nucleoside and its MP metabolite. (C) The effect of dipyridamole on the influx of thymidine, 4'-Ed4T, or AZT in CEM cells. For the influx study, CEM cells were incubated with various concentration of dipyridamole at 37 ^{o}C for 15 min prior to the uptake assays. [^{3}H]thymidine, [^{3}H]AZT and [^{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 μM dipyridamole. The cells were washed with the same buffer and solubilized with 1% Sarkosyl. The radioactivity was determined in a liquid scintillation counter.

Fig. 5. The effect of down-regulation 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-induced RKO/shMRP4 cells were pre-treated with 5 μM of [^{3}H]4'-Ed4T or 2 μM of [^{3}H]AZT for 16 h. The cells were then washed with ice-cold PBS and resuspended in fresh medium. The cells and media extract were prepared and subjected to DE-81 disc assay. The detected radioactivity was calculated as picomoles of total intracellular or extracellular MP metabolites for 4'-Ed4T or AZT.

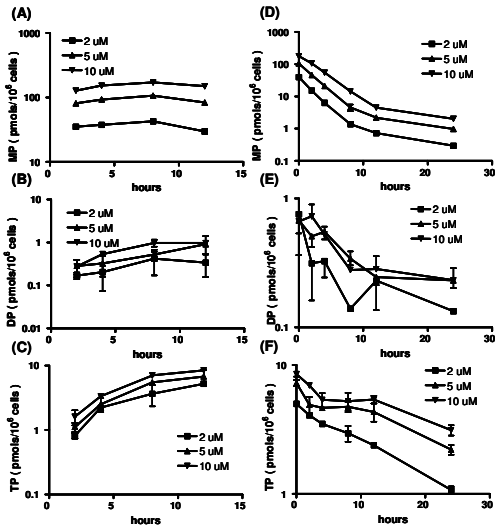

Fig. 1. Wang et al.

Fig. 2. Wang et al.

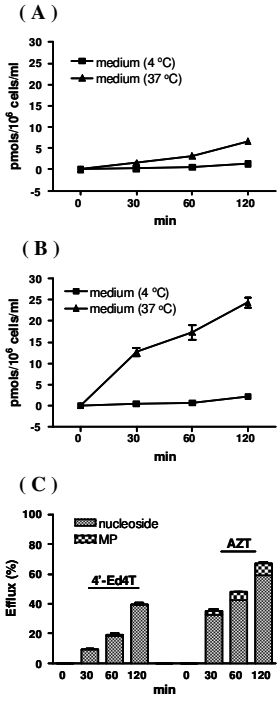

Fig. 3. Wang et al.

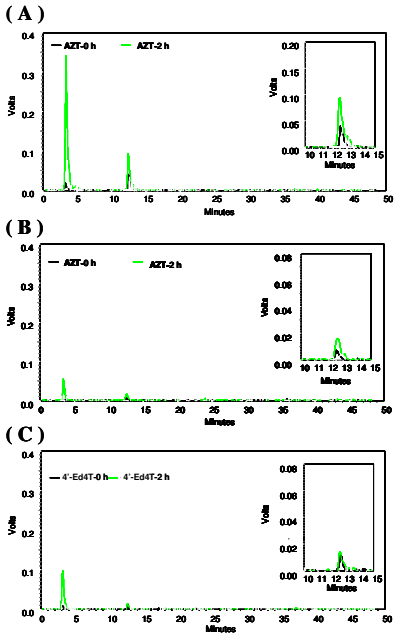

Fig. 4. Wang et al.

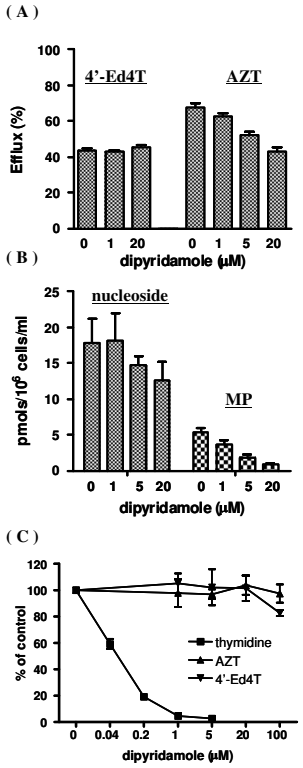

Fig. 5. Wang et al.