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Antiretrovirus Specificity and Intracellular Metabolism of 2′,3′-Didehydro-2′,3′-dideoxythymidine (Stavudine) and Its 5′-Monophosphate Triester Prodrug So324

J. BALZARINI, H. EGBERINK, K. HARTMANN, D. CAHARD, T. VAHLENKAMP, H. THORMAR, E. DE CLERCQ, and C. MCGUIGAN

Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium (J.B., E.D.C.), Department of Infectious Diseases and Immunology, Institute of Virology, NL-3584 CL Utrecht, The Netherlands (H.E., T.V.), I. Medizinische Tierklinik, D-80539 Munich, Germany (K.H.), Institute of Biology, University of Iceland, 108 Reykjavik, Iceland (H.T.), and Welsh School of Pharmacy, University of Wales Cardiff, Cardiff CF1 3XF, UK (D.C., C.M.)

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SUMMARY

2′,3′-Didehydro-2′,3′-dideoxythymidine (d4T) and its lipophilic 5′-monophosphate triester prodrug, So324, were evaluated for their antiretroviral and metabolic properties in four different animal species cell lines. The antiretrovirus activity of So324 was ~4-10-fold greater than that of d4T against human immunodeficiency virus types 1 and 2 and simian immunodeficiency virus in human T lymphocyte CEM and MT-4 cells and against feline immunodeficiency virus in feline Crandell kidney cells, 50-fold greater against visna virus in sheep choroid plexus cells, but 5-fold inferior against murine (Moloney) sarcoma virus in murine embryo fibroblast (C3H) cells. Although the administration of both d4T and So324 resulted in the formation of the 5′-monophosphate (d4T-MP), 5′-diphosphate, and 5′-triphosphate in the different cell lines, a new d4T metabolite markedly accumulated in So324-treated cells and exceeded d4T-TP levels by 13–242-fold depending on the cell line used. This metabolite could be identified as alaninyl d4T-MP. Alaninyl d4T-MP may be considered to be an intracellular depot form of d4T and/or d4T-MP, which may account for the superior antiretroviral activity of the lipophilic d4T-MP triester So324 compared with d4T.

Many of the nucleoside analogues that are endowed with activity against HIV [i.e., 2′,3′-dideoxyinosine (didanosine), ddA, ddC (zalcitabine), and d4T (stavudine)] are poorly converted to their active metabolite (i.e., their TP forms). In most cases, the first activation step is catalyzed by nucleoside kinases (i.e., thymidine kinase for d4T, 2′-deoxycytidine kinase for ddC and ddA, and adenosine kinase for ddA) or 5′-nucleotidase (i.e., for 2′,3′-dideoxyinosine) (1–6). A variety of masked nucleoside MP analogs have been synthesized in attempts to deliver the MP form directly into the target cells (7–14). Recently, we synthesized a series of lipophilic phosphoramidate triester prodrugs of 3′-azido-2′,3′-dideoxythymidine-MP and d4T-MP. We found that simple phenylalkylamine phosphate derivatives of either 3′-azido-2′,3′-dideoxythymidine or d4T inhibit HIV replication at cytotoxic concentrations without antiviral selectivity (14). However, when the alkylamino substituent was replaced by the amino acid L-alanine in d4T-MP and 3′-azido-2′,3′-dideoxythymidine-MP, marked antiviral activity at nontoxic concentrations was observed. Furthermore, the masked alaninyl d4T-MP prodrug (designated So324) retained full anti-HIV activity in the thymidine kinase-deficient CEM/TK$^-$ cells, whereas the parent compound d4T showed a markedly decreased antiviral efficacy in the CEM/TK$^-$ cells (14). These observations strongly suggest that the phosphotriester prodrug efficiently released the phosphorylated metabolite d4T-MP into the HIV-infected cells.

In the current study, we investigated the antiretrovirus activity of So324 and its parent compound d4T against six different retroviruses in cell lines derived from four different animal species. Striking differences were found in the conversion of d4T and So324 to their antivirally active metabolites. A novel metabolite (alaninyl d4T-MP) accumulated to a

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ABBREVIATIONS: d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; FIV, feline immunodeficiency virus; CrFK, feline Crandell kidney; SCP, sheep choroid plexus; MSV, Moloney murine sarcoma virus; MP, 5′-monophosphate; DP, 5′-diphosphate; TP, 5′-triphosphate; ddA, 2′,3′-dideoxyadenosine; ddC, 2′,3′-dideoxycytidine; RT, reverse transcriptase; $CCID_{50}$, 50% cell culture infective dose.

marked extent in So324-treated cell cultures. This metabolite may play a role as an (intracellular) depot form for the generation of d4T and/or d4T-MP.

Materials and Methods

Compounds. The synthesis and chemical characterization of d4T, the neutral alaninyl d4T-MP triester derivative So324, and alaninyl d4T-MP is described elsewhere (Fig. 1) (15).

Cells. Human T lymphocyte CEM and cervix carcinoma HeLa cells were obtained from American Type Culture Collection (Rockville, MD) and cultivated as described previously (16). MT-4 cells were a kind gift from Dr. N. Yamamoto (Nagoya Memorial Hospital, Nagoya, Japan). CrFK cells, low-passage SCP cells, and murine embryo fibroblast C3H/3T3 cells were maintained according to previously described procedures (16–19).

Viruses. HIV-1 (strain III_B) was obtained from the culture supernatant of persistently infected H9 cells and kindly provided by Dr. R. C. Gallo (National Cancer Institute, Bethesda, MD) (20). HIV-2 (strain ROD) was provided by Dr. L. Montagnier (Pasteur Institute, Paris, France) (21), and SIV (strain MAC251) was originally isolated by Daniel *et al.* (22) and obtained from Dr. H. Schellekens (then of the TNO Primate Center, Rijswijk, The Netherlands). MSV was prepared from tumors obtained in 10-day-old NMRI mice that had been inoculated intramuscularly with the virus when they were 2–3 days old (19). FIV (strain FIV-UT113) was prepared from the supernatant of chronically FIV-infected CrFK cell cultures (17, 23). Visna virus (strain K796) was isolated from visna virus-infected Icelandic sheep (18).

Radiolabeled compounds. [*methyl*-^{3}H]d4T (specific radioactivity, 20 Ci/mmol) and [*methyl*-^{3}H]So324 (specific radioactivity, 18.6 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA).

Antiretroviral assays. The procedures for measuring anti-HIV and anti-SIV activity in CEM and MT-4 cells, anti-FIV activity in CrFK cells, anti-visna virus activity in SCP cells, and anti-MSV activity in C3H cells have been described previously (16–18). Briefly, 4×10^5 CEM or MT-4 cells/ml were infected with HIV-1, HIV-2, and SIV at ~100 $CCID_{50}$/ml of cell suspension. Then, 100 μl of the infected cell suspension was transferred to microtiter plate wells and mixed with 100 μl of the appropriate dilutions of the test compounds (i.e., 100, 20, 4, 0.8, 0.16, 0.03, and 0.006 μM). After 4 days, giant cell formation was recorded microscopically in the virus-infected CEM cell cultures. After 5 days, cell destruction was recorded in the MT-4 cell cultures by trypan blue dye exclusion. The 50% effective concentration (EC_{50}) was defined as the compound concentration required to reduce by 50% the number of giant cells (CEM) or the number of living cells (MT-4) in the virus-infected cell cultures.

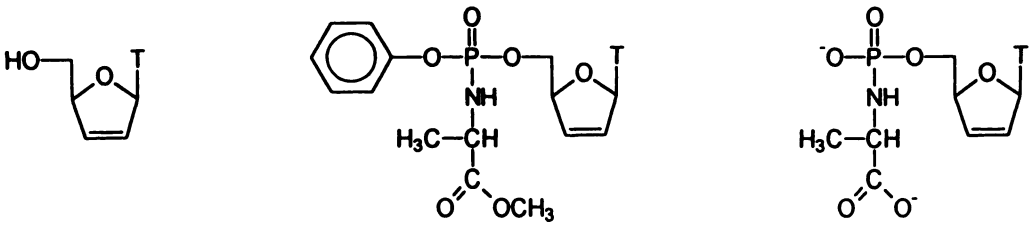
For the anti-FIV assays, 10^5 CrFK cells were seeded onto 24-well tissue culture plates. Cells were cultured with 2 ml of culture medium/well containing 2.5% of fetal calf serum in the presence of various drug concentrations. The assays were carried out in triplicate. After a 1-hr incubation period at 37°, cells were infected with FIV. Virus was left in contact with the cultures for 1 day, after which the medium was removed and new medium containing the appropriate drug concentrations was added. After 6 days, the presence of FIV p24 antigen was examined by an antigen capture assay.

For the anti-visna virus assays, 6×10^3 SCP cells/well of 96-well microtiter plates were grown in 100 μl of growth medium, and the cell monolayers were used for the experiments after 4–5 days, when they were confluent. One hundred microliters of serial 5-fold dilutions of the test compounds was added to the monolayer cells in duplicate. They were then infected with 10 μl of virus at 0.05 $CCID_{50}$. The cultures were fixed in ethanol after 6 days' incubation at 37°, stained with 2% Giemsa solution, and examined for multinucleated syncytia, which represent the hallmark of visna virus cytopathicity.

In the anti-MSV assays, confluent C3H cell cultures in 48-well microtiter plates were infected with 100 $CCID_{50}$ of MSV. After 1-hr exposure, the virus was removed, and the cell monolayers were exposed to 1 ml of the appropriate serial 5-fold dilutions of the test compounds. At day 6 after infection, virus-induced cell transformation was recorded microscopically. The EC_{50} was defined as the compound concentration required to inhibit virus-induced cell transformation by 50%.

Metabolism of [*methyl*-^{3}H]d4T and [*methyl*-^{3}H]So324 in CEM, CrFK, SCP, C3H, and HeLa cell cultures. CEM and MT-4 cells were seeded at 4×10^5 cells/ml in 5-ml culture bottles (25 cm^2), 6×10^5 SCP and C3H cells were seeded in 5-ml culture bottles (25 cm^2), and 6×10^5 CrFK and HeLa cells were seeded in Petri dishes (60-mm diameter). The radiolabeled test compounds d4T and So324 were added to the cell cultures either immediately after seeding (CEM) or 1–2 days after seeding (CrFK, SCP, C3H, HeLa) at a concentration of 1 μM. The radioactive input was ~5 μCi/ml of culture medium. At several time points (i.e., 6, 24, and 48 hr), cell cultures were carefully washed with cold culture medium (without serum) to remove extracellular radiolabel. Then, nucleotide pools were extracted in the presence of 60% methanol and quantified by high performance liquid chromatography on an anion exchange Partisphere SAX-10 column (Whatman, Clifton, NJ). Separation was performed using a gradient system from 0.007 M $(NH_4)H_2PO_4$, pH 3.8, to 0.25 M $(NH_4)H_2PO_4$ plus 0.5 M KCl, pH 4.5, as described previously (2). The different fractions of the eluate were assayed for radioactivity in a scintillation counter (model 2200; Packard, Meriden, CT).

RT assays. The inhibitory effects of the test compounds on the RT activity were evaluated. The sources of the RTs were derived from the virus particles (visna virus, FIV, and MLV) or recombinant enzyme (expressed in *Escherichia coli*) (HIV-1). The reaction mixture (50 μl) contained 50 mM Tris·HCl, pH 7.8, 5 mM dithiothreitol, 300 mM glutathione, 500 μM EDTA, 150 mM KCl, 5 mM $MgCl_2$, 1.25 μg of bovine serum albumin, a fixed concentration of the radiolabeled substrate [*methyl*-^{3}H]d4T-TP (specific radioactivity, 49 Ci/mmol), a fixed concentration of the template/primer poly(A)·oligo(dT) (0.015 mM), 0.06% Triton X-100, 10 μl of inhibitor solution (containing various concentrations of the test compounds), and 1 μl of the RT preparation. The reaction mixtures were incubated at 37° for 30 min, at which time 100 μl of calf thymus DNA (150 μg/ml), 2 ml of $Na_4P_2O_7$ (0.1 M in 1 M HCl), and 2 ml of trichloroacetic acid (10% v/v) were added. The solutions were kept on ice for 30 min, after which the acid-insoluble material was washed and analyzed for radioactivity.



d4T So 324 alaninyl d4T-MP

Fig. 1. Structures of d4T, So324, and alaninyl d4T-MP.

Results

Antiretrovirus activity of d4T and its d4T-MP triester prodrug So324. d4T and its d4T-MP triester prodrug So324 (Fig. 1) were evaluated for their antiretroviral activity in cell culture. HIV-1 (strain III_B), HIV-2 (strain ROD) and SIV (strain MAC256) were evaluated in CEM and MT-4 cell cultures, FIV (strain UT113) in CrFK cells, visna virus (strain K796) in SCP cells, and MSV in murine embryo fibroblast C3H cells (Table 1). So324 proved invariably to be more inhibitory than d4T to the replication of all retroviruses, except for MSV. Its 50% effective concentration (EC_{50}) was in the range of 0.04–0.80 μM for HIV-1, HIV-2, SIV, FIV, and visna virus, whereas its 50% inhibitory effect on MSV-induced transformation of C3H cells required a 20–200-fold higher EC_{50} (15 μM) (Table 1). So324 proved to be 4–10-fold more inhibitory to HIV-1, HIV-2, SIV, and FIV in CEM, MT-4, and CrFK cells; 50-fold more inhibitory to visna virus in SCP cells; but 5-fold less inhibitory than d4T against MSV in C3H cells (Table 1). So324 proved to be markedly less cytostatic to MT-4 cells than d4T (≥25-fold) and also less inhibitory to CrFK and C3H cell proliferation than d4T, although to a minor extent. However, there was no main difference in the antiproliferative activities of So324 and d4T against CEM cell cultures (Table 1). Thus, the cytostatic effects of both compounds was highly dependent on the cell lines evaluated.

Metabolism of d4T and its d4T-MP triester prodrug So324. The metabolism of d4T and So324 was examined in the five cell lines that had been used to evaluate the antiretrovirus activity of the test compounds. In all cell lines, d4T was metabolized to its corresponding MP, DP, and TP derivatives (Table 2). A typical high performance liquid chromatogram revealing the different metabolites of d4T in MT-4, HeLa, and SCP cell cultures is shown in Fig. 2A. The extent of the eventual conversion of d4T to its active metabolite d4T-TP varied markedly from one cell line to another (Table 2). The human (CEM, MT-4, and HeLa) and murine (C3H) cell cultures efficiently converted d4T to d4T-TP. Within 24 hr of incubation, the levels of d4T-TP reached 288-2251 pmol/10^9 cells. In contrast, the CrFK and SCP cell cultures converted d4T to its TP at a 20–150-fold and a 45–300-fold lower rate, respectively, than the human and murine cell cultures (Table 2).

When the metabolism of the d4T-MP triester prodrug So324 was investigated in the same cell lines, d4T-TP was most extensively formed in the human CEM, MT-4, and HeLa cell cultures (959–3207 pmol/10^9 cells) compared with the C3H and SCP cell cultures (177 and 338 pmol/10^9 cells, respectively). In striking contrast, CrFK cells converted So324 very poorly to d4T-TP (15 pmol/10^9 cells) (Table 2). It should be noted that the d4T-TP levels that originated from So324 in CEM, MT-4, and HeLa cells were 1.5–3-fold higher than when the cells were exposed to an equimolar d4T concentration. In contrast, C3H cells converted d4T 3-fold more efficiently to d4T-TP than did So324, whereas CrFK cells proved to be equally inefficient in converting d4T or So324 to d4T-TP. Surprisingly, SCP cell cultures converted So324 to d4T-TP with ~50-fold higher efficiency than when they converted d4T to d4T-TP (Table 2). A typical high performance liquid chromatogram revealing the different metabolites of So324 in MT-4, HeLa, and SCP cell cultures is shown in Fig. 2B.

A new metabolite, identified as the alaninyl derivative of d4T-MP, accumulated in all cell lines exposed to So324. The intracellular concentrations of this new metabolite varied markedly from one cell line to another but invariably reached much higher levels than the corresponding d4T-MP, d4T-DP, or d4T-TP metabolites, regardless of the incubation time (i.e., 6, 24, or 48 hr) of the cell cultures with So324. The levels reached by alaninyl d4T-MP were ~15-, ~7-, ~242-, ~67-, ~13-, and ~22-fold higher than those of d4T-TP in CEM, MT-4, CrFK, SCP, C3H, and HeLa cell cultures after a 24-hr incubation period, respectively. The lowest levels of alaninyl d4T-MP were generated in CrFK and C3H cells (2151–3630 pmol/10^9 cells); they were 8–10-fold higher in HeLa, SCP, and CEM cells. There was no obvious correlation between the extent of d4T-TP formation from d4T or So324 and the formation of alaninyl d4T-MP from So324. Also, the time at which intracellular peak levels of alaninyl d4T-MP were recorded differed from one cell line to another. The highest alaninyl d4T-MP concentrations were obtained after 24 hr in CEM and MT-4 cells, whereas the highest alaninyl d4T-MP levels were already noted after 6 hr of incubation of the monolayer SCP and C3H cell cultures with So324 (Table 2).

Activity of d4T derivatives against RT. The inhibitory effects of d4T, d4T-MP, d4T-TP, alaninyl d4T-MP, and the d4T-MP triester prodrug So324 were examined for their activity against the four RTs derived from HIV-1, FIV, visna virus, and murine leukemia virus (Table 3). For all RTs, poly(rA)·oligo(dT) was used as the template/primer, and [*methyl*-^{3}H]dTTP was used as the radiolabeled substrate. At a concentration of 50–100 μM, d4T, d4T-MP, alaninyl d4T-MP, and So324 were not inhibitory to any of the RTs tested. In contrast, d4T-TP was exquisitely inhibitory to HIV-1 RT and visna virus RT (IC_{50} = 0.007–0.02 μM). The MLV RT was 7–20-fold less sensitive to the inhibitory effect of d4T-TP,

TABLE 1

Antiretrovirus effect of d4T and So324 in different cell lines

Cell cultures were infected with several retroviruses and exposed to serial 5-fold dilutions of d4T or So324 for 4 days (HIV-1 and HIV-2 in CEM), 5 days (HIV-1, HIV-2, and SIV in MT-4), or 6 days (FIV in CrFK, visna in SCP, and MSV in C3H/3T3), after which virus-induced cytopathicity was recorded.

Cell line	Virus	Compound	EC_{50}[a]	CC_{50}[b]
			μM	
CEM	HIV-1	d4T	0.80	213
		So324	0.18	129
CEM	HIV-2	d4T	0.78	213
		So324	0.20	129
MT-4	HIV-1	d4T	0.65	4
		So324	0.07	>100
MT-4	HIV-2	d4T	0.77	4
		So324	0.07	>100
MT-4	SIV	d4T	0.11	4
		So324	0.04	>100
CrFK	FIV	d4T	4.0	193
		So324	0.80	>500
SCP	Visna	d4T	13	
		So324	0.26	
C3H	MSV	d4T	2.6	86
		So324	15	128

[a] 50% effective concentration or compound concentration required to cause a 50% inhibition of HIV-1-, HIV-2-, SIV-, or visna virus-induced cytopathic effect, MSV-induced transformation, or FIV-induced p24 antigen expression.

[b] 50% cytostatic concentration or compound concentration required to cause a 50% inhibition of cell proliferation.

TABLE 2

Intracellular pool levels of different d4T metabolites after incubation of different cell lines with 1 μM [*methyl*-^{3}H]d4T and 1 μM [*methyl*-^{3}H]So324 for different time periods

Approximately 4 × 10^5 CEM and MT-4 cells/ml, 6 × 10^5 SCP and C3H cells/25-cm^2 culture bottles, or 6 × 10^5 CrFK and HeLa cells/28.3-cm^2 Petri dishes were seeded and exposed to 1 μM radiolabeled (5 μCi) d4T or So324 for 6, 24, and/or 48 hr. Extracellular label was then removed at the indicated time points, and nucleotide pools were extracted in the presence of 60% methanol and quantified by high performance liquid chromatography on an anion exchange Partisil SAX-10 column.

Cell culture	Incubation time	d4T and/or So324	d4T-MP	d4T-DP	d4T-TP	Alaninyl d4T-MP
				pmol/10^9 cells[a]		
CEM + d4T (1 μM)	6	2,155	250	26	255	
	24	2,500	298	75	600	
	48[b]	1,074	233	121	444	
+ So324 (1 μM)	6	738	496	65	602	16,349
	24	1,662	510	194	1,449	21,804
	48	489	357	96	647	10,286
MT-4 + d4T (1 μM)	6	729	782	129	2,713	
	24	814	477	116	2,251	
+ So324 (1 μM)	6	769	423	92	1,191	13,598
	24	1,038	461	209	3,207	19,773
CrFK + d4T (1 μM)	6	N.D.[c]	N.D.	N.D.	N.D.	
	24	3,120	189	83	14	
+ So324 (1 μM)	6	N.D.[c]	N.D.	N.D.	N.D.	N.D.
	24	390	43	23	15	3,630
SCP + d4T (1 μM)	6	4,841	141		8	
	24	1,859	40	9	6.5	
	48	6,368	126	47	17	
+ So324 (1 μM)	6	5,321	951	350	810	31,296
	24	1,810	809	470	338	22,788
	48	2,801	814	568	625	22,875
C3H + d4T (1 μM)	6	2,221	30	42	121	
	24	4,610	136	141	451	
	48	2,838	124	100	73	
+ So324 (1 μM)	6	605	75	74	274	2,292
	24	691	78	62	177	2,151
	48	921	131	67	152	1,823
HeLa + d4T (1 μM)	6	N.D.[c]	N.D.	N.D.		
	24	5,562	303	133	288	
+ So324 (1 μM)	6	N.D.[c]	N.D.	N.D.	N.D.	N.D.
	24	4,338	391	572	959	21,060

[a] Data are the mean of two or three independent experiments.
[b] Data taken from one experiment.
[c] N.D., not determined.

whereas d4T-TP inhibited FIV RT with an IC_{50} value of 0.065 μM.

Discussion

The d4T-MP triester prodrug So324 seemed to be a potent inhibitor of several retroviruses, including the lentiviruses HIV-1, HIV-2, SIV, FIV, and visna virus and the oncovirus MSV. It proved to be more inhibitory to lentiviruses than the parent compound d4T. The most striking difference between the antiviral potency of d4T and that of So324 was observed in visna virus-infected SCP cell cultures, where So324 was 50-fold more efficient than d4T. Interestingly, d4T-TP levels that originated from So324 were also 50-fold higher in SCP cells than those that originated from d4T. Also, there seemed to be a close correlation between the d4T-TP levels generated in So324- and d4T-exposed cell cultures and the antiretroviral effects of these test compounds. For example, in CEM (and HeLa) cell cultures, So324 generated ~3-fold higher d4T-TP levels than did d4T, and accordingly, So324 was 4-fold more inhibitory to HIV-1 replication than was d4T in CEM cells. Also, d4T-TP levels in C3H cell cultures generated by So324 were 3-fold lower than those generated by d4T, and So324 proved to be 6-fold less inhibitory than d4T to MSV in C3H cells.

Several findings suggest that the accumulation of alaninyl d4T-MP may play a role in the eventual superior antiretroviral action of the So324 prodrug. For example, in the CrFK cell cultures, equally (very) low levels of d4T-TP appeared after exposure to either d4T or So324, although FIV was inhibited to a 5-fold greater extent by So324 than d4T. Lower levels of d4T-MP and d4T-DP were generated after exposure to So324 compared with d4T; therefore, if So324 induced a higher anti-FIV response in CrFK cells, this could accounted for only by the formation of alaninyl d4T-MP in So324-treated cells, presumably acting as a depot form for the release of the d4T-TP precursor (presumably d4T and/or d4T-MP). Indeed, this novel metabolite exceeded the d4T-TP levels in the CrFK cells by >200-fold. However, to what extent alaninyl d4T-MP versus d4T-TP plays a role in the eventual antiretrovirus activity of So324 may vary depending on the virus/cell culture system. So324 is 10-fold more inhibitory to HIV-1 and HIV-2 replication in MT-4 cells than d4T, whereas d4T-TP levels only differ by ~2-fold. This may point to a contribution of alaninyl d4T-MP to the eventual anti-HIV activity of So324 in MT-4 cell cultures. So324 is approximately equally active against visna virus in SCP cells and HIV-1 activity in CEM cells (EC_{50} = 0.26 and 0.18 μM, respectively). However, alaninyl d4T-MP levels are only 15-fold higher than d4T-TP levels in CEM cells but 67-fold higher than d4T-TP levels in SCP cells. This points to a

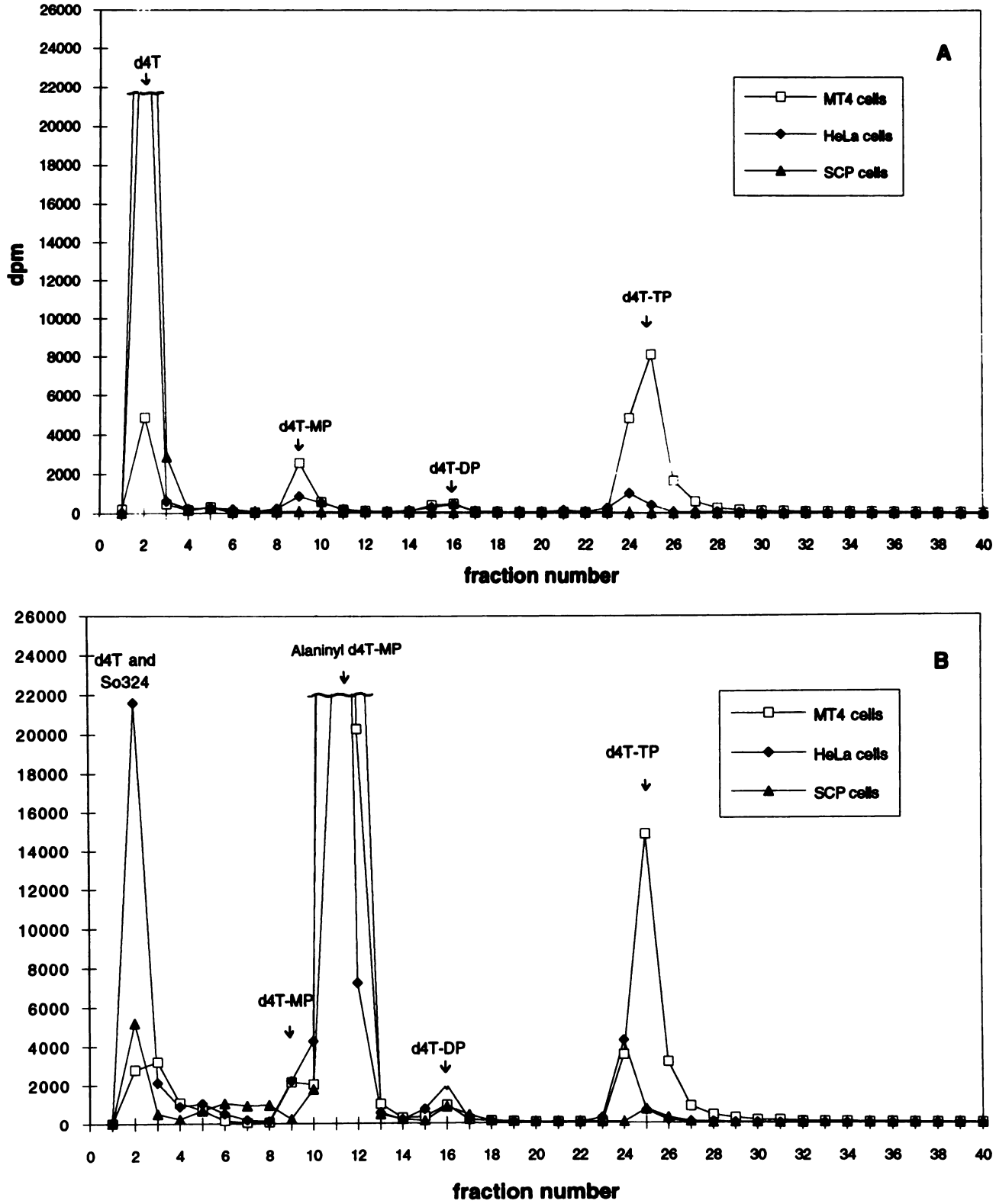


Fig. 2. High performance liquid chromatogram of [^{3}H]d4T (A) and [^{3}H]So324 (B) metabolism in MT-4, HeLa, and SCP cell cultures. The radiolabeled metabolites were separated on an anion exchange Partisil SAX column. The radioactivity was quantified for each of the 40 fractions per gradient in a scintillation counter and expressed as disintegrations per minute (dpm).

comparatively greater importance of the accumulation of alaninyl d4T-MP in the activity of So324 against visna virus than HIV.

Another finding that points to a potential role of alaninyl d4T-MP in the eventual antiretroviral activity of So324 is our observation that alaninyl d4T-MP, when exposed to HIV-infected CEM cell cultures, exerts an antiviral efficacy that was only 5-fold lower than that of So324 (EC_{50} = ~1 μM).

TABLE 3
Activity of d4T derivatives against RT

Compound	IC_{50}[a]			
	HIV-1[b]	FIV[b]	MLV[b]	Visna[b]
	μM			
d4T	>100	>100	>100	>100
d4T-MP	>100	>100	>100	>100
d4T-TP	0.007	0.065	0.15	0.02
Alaninyl d4T-MP	>100	>50	>50	>50
So324	>100	>100	>100	>100

[a] 50% inhibitory concentration, or compound concentration required to inhibit the RT-catalysed polymerization reaction by 50%. The template/primer consisted of poly(rA)$^+$ · oligo(dT), and the radiolabeled substrate was 2 μM [CH_3-^{3}H]dTTP, as described in Materials and Methods.
[b] The RT source for FIV, MLV, and visna virus was particle derived. The HIV-1 RT was recombinant enzyme, expressed in E. coli.

Most likely, alaninyl d4T-MP is taken up by the cells at a much lower efficiency than So324 because of its more polar character compared with its highly lipophilic prodrug So324. Thus, the novel metabolite alaninyl d4T-MP may act as an intracellular and/or extracellular depot form of d4T and/or d4T-MP.

The differences in the levels of d4T-TP and alaninyl d4T-MP generated by So324 and d4T in the different cell lines reflect marked differences in the uptake and/or metabolism of So324 and d4T from one cell line to another. This is obvious based on a comparison of the intracellular levels of d4T-MP, d4T-DP, and d4T-TP in CEM, MT-4, CrFK, SCP, C3H, and HeLa cells (Table 2). The lowest levels of d4T-MP, after exposure to d4T, were noted in SCP cells, which is indicative of the poor d4T kinase activity in these cells. In CrFK cells, d4T-MP was formed to a similar extent as in CEM, HeLa, and C3H cells, but it was less efficiently converted to d4T-TP. This points to a poor metabolic conversion of d4T-MP in CrFK cells by the thymidylate kinase and/or nucleoside diphosphate kinase.

When So324 metabolism was compared in the different cell lines, even more striking differences were noted. The amounts of alaninyl d4T-MP and d4T-TP that were formed from So324 varied from one cell line to another. The ratio of alaninyl d4T-MP to d4T-TP was highest in CrFK cells, as was the ratio of alaninyl d4T-MP to the total amount of d4T-MP plus d4T-DP plus d4T-TP (Table 4). Thus, the metabolic conversion of the triester d4T-MP prodrug So324 to its metabolites (i.e., alaninyl d4T-MP and the phosphorylated d4T products) strongly depends on the cell species. The pathway or pathways of intracellular metabolism of So324, as well as the enzymes that account for the metabolism of So324 to alaninyl d4T-MP, d4T-MP, d4T-DP, and d4T-TP, are not yet clarified. Most likely, the first metabolic conversion of So324 consists of the hydrolysis of the phenyl on the phosphate moiety (metabolite A) or the methyl on the alaninyl moiety (metabolite B). The next step obligatorily releases the remaining ester group from metabolite A or B to form alaninyl d4T-MP. The eventual formation of d4T-MP may occur through two different pathways: either hydrolysis of the phosphoramidate bond from alaninyl d4T-MP or the phosphoramidate hydrolysis of metabolite A. The contribution of both pathways in the formation of d4T-MP (and, eventually, d4T-TP) is unclear and the subject of further investigation.

TABLE 4
Ratios of alaninyl d4T-MP and other d4T metabolites in 24-hr So324-exposed cell cultures

Cell cultures were incubated with 1 μM [*methyl*-^{3}H]So324 for 24 hr. Then, the ratios of the formation of alaninyl d4T-MP versus d4T-TP (first column), alaninyl d4T-MP versus d4T-MP (second column), and alaninyl d4T-MP versus the sum of d4T-MP, d4T-DP, and d4T-TP (third column) were calculated.

Cell culture	Alaninyl d4T-MP/d4T-TP	Alaninyl d4T-MP/d4T-MP	Alaninyl d4T-MP/ (d4T-MP + d4T-DP + d4T-TP)
CEM	15	52	10
MT-4	6	52	5.0
CrFK	242	85	45
SCP	67	28	14
C3H	13	28	6.7
HeLa	22	54	11

In conclusion, we have shown that alaninyl d4T-MP, as well as d4T-MP, d4T-DP, and d4T-TP, are formed intracellularly from the triester d4T-MP prodrug So324 and that these metabolites are formed in amounts that vary considerably from one cell species to another. The eventual antiretrovirus activity of So324 can be attributed to the formation of d4T-TP and alaninyl d4T-MP, with the latter metabolite acting as a depot form for d4T, d4T-MP, or both.

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Send reprint requests to: Dr. J. Balzarini, Katholieke Universiteit Leuven/Rega Institute, Minderbroedersstraat 10, B-3000 Leuven, Belgium. E-mail: jan.balzarini@rega.kuleuven.ac.be