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Mechanism of anti-HIV action of masked alaninyl d4T-MP derivatives

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ABSTRACT **So324 is a 2′,3′-dideoxy-2′,3′-didehydrothymidine-5′-monophosphate (d4T-MP) prodrug containing at the phosphate moiety a phenyl group and the methylester of alanine linked to the phosphate through a phosphoramidate linkage. So324 has anti-HIV activity in human CEM, MT4, and monocyte/macrophage cells that is superior to that of d4T. In contrast to d4T, So324 is also able to inhibit HIV replication in thymidine kinase-deficient CEM cells. After uptake of So324 by intact human lymphocytes, d4T-MP is released and subsequently converted intracellularly to d4T-TP. In addition, accumulation of substantial amounts of a novel d4T derivative has been found. This d4T metabolite has been characterized as alaninyl d4T-MP. The latter metabolite accumulates at ≈13- to 200-fold higher levels than d4T-TP depending the experimental conditions. Alaninyl d4T-MP should be considered as an intra- and/or extracellular depot form of d4T and/or d4T-MP. These findings may explain the superior anti-retroviral activity of So324 over d4T in cell culture.**

2′,3′-Dideoxynucleoside (ddN) analogues are potent inhibitors of HIV replication in cell culture. To date, five ddN analogues have been formally licensed for treatment of HIV-infected individuals [(*i*) zidovudine (azidothymidine; AZT), (*ii*) zalcitabine (dideoxycytidine; ddC), (*iii*) didanosine (dideoxyinosine; ddI), (*iv*) stavudine (didehydrodideoxythymidine; d4T), and (*v*) lamivudine (3′-thia-2′,3′-dideoxycytidine; 3TC)]. The ddN analogues have to be converted to their corresponding 5′-triphosphate derivatives to act as inhibitors of the retroviral reverse transcriptase. However, the rate and extent of phosphorylation of the ddN analogues to their active 5′-triphosphate metabolite may be insufficient. In particular, the first activation (phosphorylation) step may be rate-limiting in that most ddN analogues show poor affinity for nucleoside kinases [i.e., thymidine kinase (TK), 2′-deoxycytidine kinase, adenosine kinase] or other cellular enzymes (i.e., 5′-nucleotidase) that play a crucial role in the conversion of the ddN analogues to their 5′-monophosphates (1–6). In efforts to circumvent the dependence of ddN analogues on activation by the nucleoside kinases, prodrugs of the 5′-monophosphate forms of several nucleoside analogues have been prepared. The phosphoramidate derivatives of azidothymidine and fluorothymidine (FLT), two good substrates for cytosol TK, have been found to exhibit marked antiviral activity in cell culture (7, 8). We have recently focused on masked nucleoside phosphoramidate derivatives of nucleoside analogues [i.e., d4T and dideoxyuridine (ddU)] that have relatively poor affinity for cytosol TK (2, 9–11). In this study, we revealed that the phosphoramidate derivative of d4T, designated So324, not only proved able to deliver intracellularly the free 5′-monophosphate of d4T (d4T-MP) [leading to pronounced levels of the corresponding active d4T 5′-triphosphate (d4T-TP)], but also released a phosphoramidate derivative of d4T-MP. This latter metabolite markedly accumulates into the cells, and appears to act as an intra- and/or extracellular depot form of d4T and/or d4T-MP.

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MATERIALS AND METHODS

Cells. MT-4 cells (an immortalized helper/inducer T cell clone) were a kind gift from N. Yamamoto (Tokyo Medical and Dental University). CEM cells were derived from the American Type Culture Collection. Peripheral blood lymphocyte (PBL) cells were obtained from a healthy HIV-seronegative donor. TK-deficient CEM cells were a kind gift of S. Erikson (Swedish University of Agricultural Sciences, Uppsala, Sweden) and A. Karlsson (Karolinska Institute, Stockholm, Sweden)

Viruses. HIV-1 (strain III_B) was obtained from the culture supernatant of persistently infected H9 cells and kindly provided by R. C. Gallo (National Cancer Institute, Bethesda) (12). The monocytotropic strain of HIV-1 (HIV-1/Ba-L) was also kindly provided by R. C. Gallo and grown in primary macrophages. HIV-2 (strain ROD) was provided by L. Montagnier (Pasteur Institute, Paris) (13). Moloney murine sarcoma virus (MSV) was prepared from tumors obtained in 10-day-old NMRI mice that were inoculated intramuscularly with the virus when they were 3 days old (14).

Compounds. The synthesis of 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T) and So324 is described elsewhere (15). The structures are depicted in Fig. 1.

Radiochemicals. [*methyl*-^{3}H]d4T (specific radioactivity, 20 Ci/mmol; 1 Ci = 37 GBq) 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 activity in MT-4, CEM, and monocyte/macrophage (M/M) cells and anti-MSV activity in C3H/3T3 cell cultures have been described previously (16, 17).

Metabolism of [*methyl*-^{3}H]d4T and [*methyl*-^{3}H]So324 in Human CEM, MT-4, PBL and M/M Cells. CEM, MT-4, activated PBL and M/M cells were seeded at 4×10^5, 4×10^5, 2×10^6, and 2×10^5 cells/ml, respectively, and incubated with varying concentrations of the radiolabeled test compounds. At different time intervals, cells were centrifuged, washed twice with cold PBS, and precipitated with cold methanol (66%).

Abbreviations: d4T, didehydrodideoxythymidine; d4T-MP, -DP, -TP, d4T 5′-monophosphate, -diphosphate, -triphosphate; ddN, 2′,3′-dideoxynucleoside; PBL, peripherial blood lymphocyte; MSV, Moloney murine sarcoma virus; M/M, monocyte/macrophage; TK, thymidine kinase.

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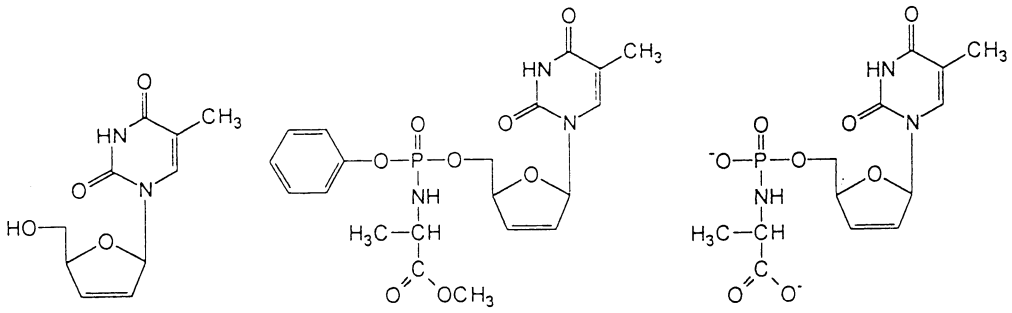

FIG. 1. Structural formula of d4T, So324, and alaninyl d4T-MP (metabolite X).

After standing in ice-water for 10 min, the cell extracts were centrifuged in an Eppendorf centrifuge and analyzed on HPLC using a Partisil SAX-10 radial compression column. A linear gradient of 0.007 M ammonium dihydrogen phosphate (pH 3.8) (buffer B) to 0.25 M ammonium dihydrogen phosphate + 0.50 M KCl (pH 4.5) (buffer C) was used [i.e., 0.2 min 90% buffer A (H_2O) + 10% buffer B; 5.8 min linear gradient to 100% buffer B; 10 min linear gradient to 70% buffer C + 30% buffer B; 6 min linear gradient to 100% buffer C; 22 min 100% buffer C; 3 min linear gradient to 100% buffer B; and 5 min equilibration at 100% buffer B]. The different fractions of the eluate were assayed for radioactivity in a toluene-based scintillant. Metabolism of [*methyl*-^{3}H]d4T and [*methyl*-^{3}H]So324 was also examined in TK-deficient CEM/TK$^-$ cells. The procedure was essentially the same as described for the wild-type CEM cells.

Intracellular Retention of [*methyl*-^{3}H]So324 and its Metabolites in CEM Cells After Removal of the Drug from the Culture Medium. CEM cells were seeded at 4×10^5 cells per ml and incubated with 0.2 and 25 μM [*methyl*-^{3}H]So324 for 20 hr. Then, extracellular (radiolabeled) drug was removed by centrifugation of the cells and washing the cell cultures three times with warm culture medium. At 0, 6, 12, and 24 hr after removal of [*methyl*-^{3}H]So324, cell extracts were prepared and radiolabeled metabolites determined by HPLC. From these experiments, the extracellular medium of the drug-exposed CEM cell cultures was also examined for the presence of [*methyl*-^{3}H]So324 metabolites after the radiolabeled parent compound was removed from the extracellular medium.

Treatment of Drug-Exposed CEM Cell Extracts with Alkaline Phosphatase and Phosphodiesterase. CEM cell cultures were exposed to [*methyl*-^{3}H]So324 or [*methyl*-^{3}H]d4T for 20 hr as described. Then, cell extracts were prepared, treated with alkaline phosphatase and Crotalus durissus phosphodiesterase (1.5 units/ml), and analyzed by HPLC on an anion exchange Partisil SAX-10 column using the same solvent gradient as described above.

Treatment of [*methyl*-^{3}H]So324 with Hog Liver Esterase. [*Methyl*-^{3}H]So324 was exposed to 20 units/ml of hog liver esterase (Boehringer Mannheim) in a buffer containing 25 mM Tris·HCl, pH 7.6, and 10 mM $MgCl_2$. The reaction was performed at 37°C. At several time points (i.e., 1, 3, 24, 48, 72, ... hr), aliquots were taken and analyzed by HPLC on an anion exchange Partisil SAX-10 column. Separation was performed using the same gradient system as described for the analysis of the drug-treated cell extracts.

Synthesis and Characterization of Synthetic Alaninyl d4T-MP. So324 (0.25 mmol) was dissolved in a 1:1 mixture of triethylamine/water (8 ml). After 3 hr at room temperature, the triethylamine phase was removed and the aqueous phase evaporated under high vacuum at ambient temperature. The resulting crude product was purified on silica using the chromatotron with the mixture $CHCl_3$/MeOH/H_2O/NH_4OH (120:70:10:1) as eluent. Pooling and freeze-drying of appropriate fractions gave the pure compound at a yield of 54%. The compound was characterized as follows: δp (D_2O) 7.63; δ_H 1.12 (d, ^{3}H, J = 6.9Hz, Ala-Me), 1.73 (s, ^{3}H, 5-Me), 3.42 (m, 1H, Ala-CH), 3.83 (m, 2H, H5′), 4.93 (m, 1H, H4′), 5.80 (m, 1H, H2′), 6.34 (m, 1H, H3′), 6.78 (m, 1H, H1′), 7.45 (s, 1H, H6); δ_C (D_2O) 11.80 (5-Me), 19.65 (Ala-Me), 50.21 (Ala-CH), 65.32 (C5′), 86.26 (C4′), 90.38 (C1′), 111.57 (C5), 125.19 (C2′), 134.86 (C3′), 138.67 (C6), 152.57 (C2), 166.92 (C4), 179.30 (Ala-CO_2H); *m/z* FAB 398 (MNa^+, 17%), 376 (MH^+, 6); HPLC reverse phase 3.15 min, on a reverse phase C-18 column with an elution system being isocratic from 0 to 10 min using a mixture of H_2O (82%)/acetonitrile (18%) and then linear from 10 to 30 min using a gradient from H_2O (82%)/acetonitrile (18%) to H_2O (20%)/acetonitrile (80%). The flow rate was 1 ml/min (15).

RESULTS

Antiretroviral Activity of d4T and So324. Both d4T and So324 proved to be potent and highly selective inhibitors of HIV replication in CEM and MT-4 cells (Table 1). d4T showed an EC_{50} (50% effective concentration) of 0.78 to 0.80 μM against HIV-1 and HIV-2 in CEM cell cultures, whereas So324 was about 4-fold more active (Table 1). In MT-4 cell cultures, the EC_{50}s of d4T and So324 were 0.65 to 0.77 μM and 0.066 to 0.067 μM, respectively. So324 is much less cytotoxic than

Table 1. Inhibitory effects of d4T and So324 on HIV-induced cytopathicity in MT-4 and CEM cells, on MSV-induced transformation of murine C3H/3T3 cells, and on virus production in M/M

	EC_{50}, μM*							CC_{50}, μM
	MT-4 cells		CEM/0		CEM/TK$^-$	M/M	C3H/3T3 cells	MT-4
Compound	HIV-1 (III_B)	HIV-2 (ROD)	HIV-1 (III_B)	HIV-2 (ROD)	HIV-2 (ROD)	HIV-1 (Ba-L)	MSV	
d4T	0.65	0.77	0.80	0.78	25	0.25	2.6	4.0
So324	0.066	0.067	0.18	0.20	0.075	0.02	15	>100

*Compound concentration required to inhibit HIV-induced destruction of MT-4 or giant cell formation in CEM cell cultures or MSV-induced transformation of C3H/3T3 cells by 50%. CEM/TK$^-$ cells are TK-deficient CEM cells.

d4T in MT-4 cells, leading to a markedly increased selectivity index (ratio cytotoxic concentration/antivirally effective concentration) *in vitro*. Interestingly, d4T was 30-fold less effective against HIV-2 in CEM/TK$^-$ cells than in wild-type CEM/0 cells, whereas the antiviral activity of So324 was even enhanced (2- to 3-fold) against HIV-2 in CEM/TK$^-$ cells. These data are suggestive for a highly efficient uptake of So324 and intracellular release of d4T-MP in the intact CEM cells. So324 proved 10-fold more efficient than d4T in inhibiting HIV-1 (Ba-L) in M/M (Table 1). In murine C3H/3T3 cells, d4T was 6-fold more effective in inhibiting MSV-induced cell transformation than So324 (EC_{50}: 15 μM), whereas neither compound was toxic for C3H/3T3 cells at 100 μM (Table 1).

Phosphorylation of [*methyl*-^{3}H]So324 in CEM Cells as a Function of Different Incubation Times. Upon incubation of CEM cells with 0.2 μM So324, d4T and the mono-, di-, and triphosphorylated derivatives of d4T could be detected in the cell extracts. In addition, a new radiolabeled metabolite (designated X) was formed and reached an intracellular level of 0.93 nmol/10^9 cells after 2 hr of incubation, compared with the formation of 0.07 nmol d4T-TP/10^9 CEM cells. After 17 hr, metabolite X reached a peak level of 3.1 nmol per 10^9 cells that is an approximately 13-fold higher level than the level reached by d4T-TP). Continued incubation of the cell cultures (up to 72 hr) with So324 resulted in a progressive decrease of the intracellular levels of the phosphorylated d4T metabolites and of metabolite X (data not shown).

Metabolism of [*methyl*-^{3}H]d4T and [*methyl*-^{3}H]So324 After 24 hr Incubation in CEM, MT-4, PBL and M/M Cell Cultures as a Function of Different Input Concentrations. Intracellular formation of the 5′-mono-, 5′-di-, and 5′-triphosphate derivatives of d4T as well as metabolite X increased with higher input concentrations of So324 (Table 2). A serial (5-fold) increase of the initial (0.2 μM) extracellular concentration of So324 resulted in a concomitant proportional increase of the intracellular amounts of d4T-MP (up to 500 μM initial So324 concentration), whereas the formation of d4T-TP and metabolite X started to level off at initial extracellular So324 concentrations higher than 25 μM; d4T-TP formation leveled off markedly faster than metabolite X formation. As a result, the ratio of the X levels versus d4T-TP levels progressively increased in function of the initial concentration of the So324 prodrug, and reached more than 200-fold at the highest So324 concentration tested (i.e., 500 μM) (Table 2). The concentration-dependent kinetics of So324 metabolism in MT-4 cells were virtually identical to those observed for CEM cells. However, in PBL cells, markedly lower (≈7- to 8-fold) metabolite X levels appeared during the 24-hr incubation period, whereas d4T-MP and d4T-TP levels reached in PBL were comparable with those detected in the CEM and MT-4 cell cultures (Table 2). The metabolism of So324, and the phosphorylation pattern of d4T in human M/M proved very similar to that observed in PBL at an initial So324 concentration of 0.2 μM (Table 2). However, when compared with d4T metabolism in M/M, 4-fold higher d4T-TP levels were obtained when So324 was administered to the M/M cells instead of d4T at equimolar initial (0.2 μM) concentrations of the test compounds. The X metabolite, as in PBL, exceeded the d4T-TP levels in the M/M by ≈2-fold (Table 2).

When d4T metabolism in CEM cells was compared with So324 metabolism at different initial d4T concentrations (i.e., 5, 25, 100, and 500 μM), d4T-MP levels increased proportionally with higher input concentrations (up to 500 μM), whereas d4T-TP levels increased linearly, but at a lower rate than d4T-MP levels (Table 2). The d4T-TP levels generated by d4T were inferior to the d4T-TP levels generated by So324 at the lower initial drug concentrations (i.e., 5–100 μM), but exceeded those generated by So324 at the higher drug concentrations (i.e., 100–500 μM) (Table 2).

Retention of the Intracellular [*methyl*-^{3}H]d4T and Metabolite X Levels Upon Removal of So324 from the Culture Medium, and Appearance of Metabolite X in the Extracellular Medium. CEM cells were incubated with 0.2 or 25 μM [*methyl*-^{3}H]So324 for a 16-hr incubation period, upon which the drug was removed from the extracellular medium. Following withdrawal of [*methyl*-^{3}H]So324, the intracellular levels of metabolite X had decreased by 73–76% at 6 hr, by 94–95% at 12 hr, and by more than 99% at 24 hr, irrespective of the initial (0.2 or 25 μM) So324 concentration. The initial intracellular half-life of metabolite X could be estimated at ≈3 hr, regardless of the initial intracellular concentration of So324. The d4T-TP levels also decreased, albeit at a lower rate than the metabolite X levels (data not shown). The intracellular half-life of d4T-TP could be estimated at ≈6 hr. The decay of total phosphorylated d4T levels coincided with the disappearance of the d4T-TP levels.

Reappearance of d4T metabolites in the extracellular medium was measured after the CEM cells had been loaded with 0.2 or 25 μM [*methyl*-^{3}H]So324 for 16 hr followed by withdrawal of the compound. [*methyl*-^{3}H]d4T appeared quickly in the extracellular medium, the [*methyl*-^{3}H]d4T concentration increased for about 6 hr (10 and 1, 589 nmol per 10^9 cells at an initial So324 concentration of 0.2 and 25 μM, respectively), and then leveled off for up to 24 hr (9.4 and 1, 762 nmol per 10^9 cells at initial So324 concentrations of 0.2 and 25 μM, respectively). Also, significant amounts of radiolabeled metabolite X appeared in the culture medium within 6 hr, and then remained at a constant level for at least 24 hr (5.4 to 6.6

Table 2. Metabolism of [*methyl*-^{3}H]So324 and [*methyl*-^{3}H]d4T after 24 hr incubation in human CEM, MT-4, PBL, and M/M cells

	nmole per 10^9 cells*											
	CEM						MT-4		M/M	PBL		
	Initial drug concentration, μM											
Metabolite	0.2	1.0	5.0	25	100	500	0.2	25	0.2	0.2	1.0	25
	[*methyl*-^{3}H]So324											
So324 + d4T	0.33	2.1	10	39	191	734	0.35	55	0.03	0.09	0.53	11
d4T-MP	0.17	0.48	2.4	21	99	500	0.13	17	0.08	0.11	0.62	16
d4T-DP	0.07	0.22	0.95	3.3	9.4	19	0.09	5.1	0.02	0.02	0.08	0.67
d4T-TP	0.45	1.7	7.8	24	30	41	0.99	24	0.27	0.29	1.2	6.6
X	5.9	25	141	712	2818	8995	5.1	642	0.49	0.77	4.3	88
	[*methyl*-^{3}H]d4T											
d4T			15	69	295	1,386			0.009			
d4T-MP			1.6	8.9	40	191			0.006			
d4T-DP			0.31	1.8	2.0	32			0.004			
d4T-TP			3.1	11	27	88			0.074			

*Data represent the mean of at least 2 to 3 independent experiments.

nmol per 10^6 cells at 0.2 μM So324, and 920–975 nmol per 10^6 cells at 25 μM So324). The levels of metabolite X that appeared in the extracellular medium after loading the cells with So324 and withdrawal of the compound, were proportional with the initial concentrations of So324. No other (i.e., phosphorylated) d4T metabolites were detected in the extracellular medium.

Metabolism of [*methyl*-^{3}H]So324 and [*methyl*-^{3}H]d4T in Wild-Type CEM and TK-Deficient CEM/TK$^-$ Cells. The metabolism of d4T was markedly decreased in the TK-deficient CEM/TK$^-$ cells. Total phosphorylated d4T decreased by at least 17-fold, and d4T-TP levels were below the detection limit (Table 3). In contrast, the levels of phosphorylated d4T metabolites, as well as metabolite X levels, generated by So324, were quite comparable in both CEM/0 and CEM/TK$^-$ cell cultures (Table 3).

Characterization of Metabolite X. The retention times of So324, d4T, d4T-MP, d4T-DP, d4T-TP, and metabolite X after separation on the HPLC anion exchange partisil SAX-10 column were 1, 1, 5, 17, 27, and 11 min, respectively, pointing to a relatively strong polar nature of the metabolite X. Metabolite X eluted very early (within 3 min) on a C-8 reverse phase HPLC column, which again is suggestive of the strong polar nature of the metabolite. When extracts from CEM cells that had been preincubated with [*methyl*-^{3}H]So324 for 24 hr were treated with alkaline phosphatase for 1 hr at 37°C, there was a complete disappearance of d4T-TP, d4T-DP, and d4T-MP levels, whereas the metabolite X levels were virtually unaffected. Treatment of duplicate cell extracts with Crotalus durissus phosphodiesterase for 1 hr at 37°C converted d4T-TP, d4T-DP and a major part (80%) of metabolite X to d4T-MP. Thus, metabolite X proved alkaline phosphatase-resistant but phosphodiesterase-sensitive.

Based on these observations, the formation of alaninyl d4T-MP from So324 was hypothesized. Indeed, chemically synthesized alaninyl d4T-MP coeluted both with hog liver esterase-treated So324 and with the radiolabeled metabolite X that was formed in So324-incubated CEM cells in three different chromatography systems [i.e., Partisphere SAX-10 ($(NH_4)H_2PO_4$/KCl buffer system, including a pH 3.8 to 4.5 gradient), reverse phase C-8 (acetonitrile/H_2O buffer system), and ion-pairing reverse phase C-8 (tetrabutylammoniumhydrogensulfate/$(NH_4)H_2PO_4$, pH 5 buffer system)]. Moreover, HPLC elution times on a Partisphere SAX-10 column proved identical for hog liver esterase-derived X metabolites that were generated from So324 analogues containing different ester groups on the phosphate (i.e., dibromophenyl instead of phenyl) or on the alanine moiety (i.e., benzyl ester instead of methyl ester). The elution times differed from one another for So324 analogues that contained different amino acids (i.e., β-alanine, valine, etc.). Thus, metabolite X derived from So324 could be unambiguously identified as alaninyl d4T-MP.

Table 3. Intracellular metabolites of 0.2 μM [*methyl*-^{3}H]So324 and 0.15 μM [*methyl*-^{3}H]d4T in CEM/0 and CEM/TK$^-$ cells after 20 hr of incubation

Radiolabeled metabolites	nmole per 10^9 cells			
	[*methyl*-^{3}H]So324, 0.2 μM		[*methyl*-^{3}H]d4T, 0.15 μM	
	CEM/0	CEM/TK$^-$	CEM/0	CEM/TK$^-$
d4T + So324	0.31	0.05	0.46	0.27
d4T-MP + d4T-DP + d4T-TP	0.43	0.56	0.17	0.01
d4T-TP	0.25	0.48	0.14	< 0.01
X	5.4	2.5	—	—

Anti-Retrovirus Activity of Alaninyl d4T-MP. Alaninyl d4T-MP (metabolite X) that was chemically prepared from So324 was found to be inhibitory to HIV-1 and HIV-2 replication in CEM cell cultures, with an EC_{50} that was comparable with the EC_{50} of d4T for HIV-1 and HIV-2 in cell culture (EC_{50} d4T and alaninyl d4T-MP: 0.80–0.95 μM, and 0.80–1.0 μM, respectively).

DISCUSSION

So324 is a potent inhibitor of the replication of several retroviruses including HIV-1 and HIV-2, feline immunodeficiency virus (FIV), visna virus, and MSV (18). The potent antiviral activity of So324 in TK-deficient CEM/TK$^-$ cell cultures clearly points to the ability of So324 to cross the cell membrane as an intact molecule, and to release d4T-MP intracellularly. We have demonstrated that So324, unlike d4T, was able to generate virtually similar levels of d4T-MP, d4T-DP, and d4T-TP in CEM/TK$^-$ cells as in wild-type CEM/0 cells. These data should be interpreted as the successful TK bypass by So324. However, in addition to the 5′-mono-, di-, and triphosphates of d4T, an unexpected metabolite was found in the cells incubated with So324. This metabolite was identified as alaninyl d4T-MP, with the alanine linked to d4T-MP through a phosphoramidate bond. Alaninyl d4T-MP reached about 13-fold higher levels in So324-exposed cells than d4T-TP.

So324 may be considered as an attractive new lead compound in the development of anti-HIV agents. First, this class of molecules acts as highly efficient nucleoside kinase by-pass compounds, thus circumventing the first activation (phosphorylation) step that is often the rate-limiting step in the intracellular conversion of nucleoside analogues to their bio-active triphosphate derivatives. Thus, So324 is highly active against HIV replication in TK-deficient cells, which reflects its capability to be effective in cellular environments low in cytosolic TK (i.e., M/M, resting lymphocytes). Second, these molecules generate high levels of an entirely novel type of d4T metabolite, which acts in its own right as an (intra- and/or extracellular) depot form of d4T and/or d4T-MP, and which shows antiviral activity in intact cell systems as well. We propose the metabolic conversion sequence of So324 as depicted in Fig. 2. The relative importance of each pathway to eventually reach the X metabolite and d4T-TP is now under investigation.

From our metabolism data (Table 2), it became evident that at the highest alaninyl d4T-MP levels in the cells (resulting from So324 at initial concentrations of 100 and 500 μM), the flow of d4T-MP to d4T-DP and eventually d4T-TP is markedly retarded, whereas d4T-MP levels tend to accumulate. These observations suggest that thymidylate kinase is inhibited by millimolar levels of alaninyl d4T-MP. This could also explain why at lower initial So324 concentrations (<100 μM) more d4T-TP could be generated intracellularly from So324 than from d4T, whereas at higher initial So324 concentrations (= 100 μM), more d4T-TP was formed intracellularly from d4T than from So324.

It is clear from our studies that So324 may have an interesting mechanism of action. On exposure to the cells, two different metabolites, the d4T 5′-triphosphate and the alaninyl d4T-MP, are generated intracellularly. The unexpected property of So324 to accumulate alaninyl d4T-MP into drug-exposed cells opens an exciting new area for the development of novel, structurally related nucleotide prodrugs.

Conversion of So324 to d4T or the X metabolite within 6 hr of incubation in heat-inactivated fetal calf serum was virtually negligible. When So324 was incubated at 37°C in the presence of human serum, the d4T prodrug was converted to the X metabolite after 2 hr by 5% and after 6 hr by 20%. No appreciable d4T was released during this incubation period. These properties are beneficial for So324 in that it may stay

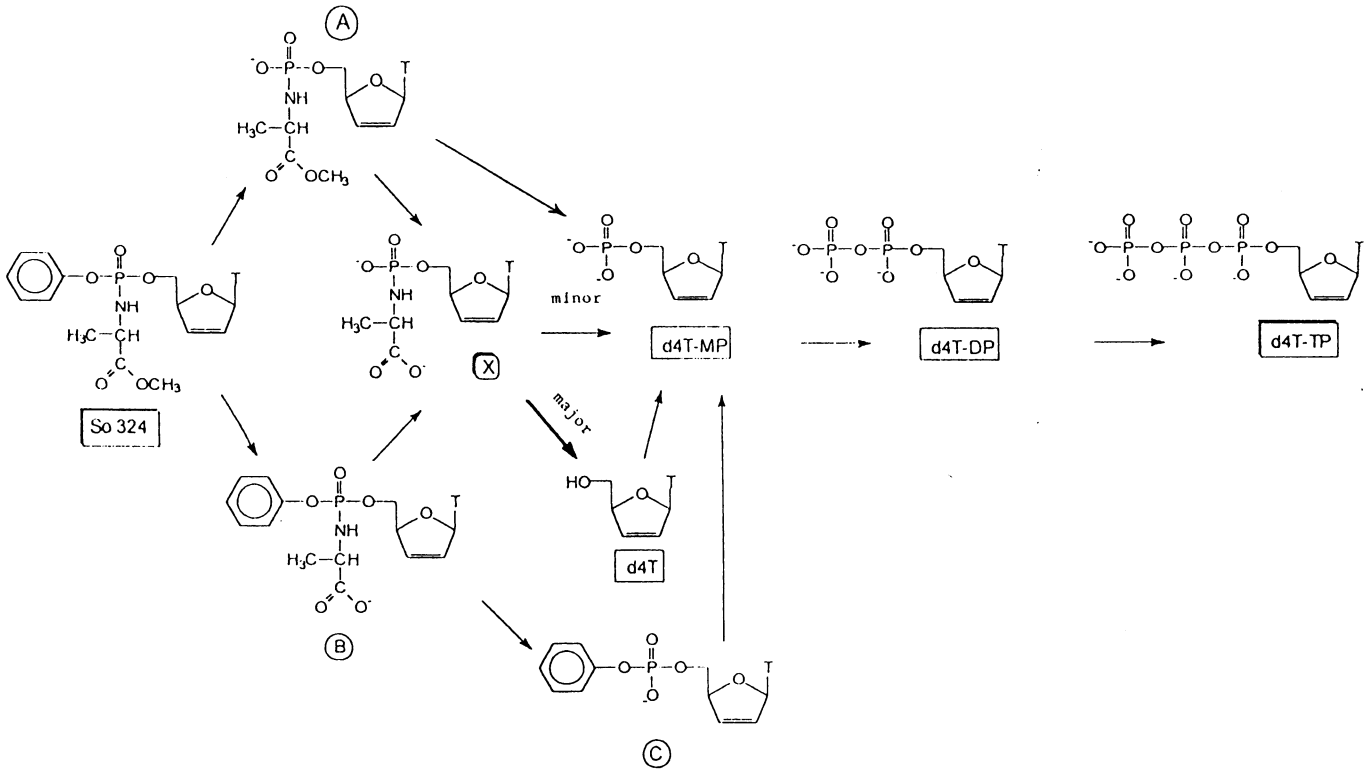

FIG. 2. Proposed scheme of the major pathways of metabolism of [*methyl*-^{3}H]So324. So324 is converted through metabolite A and/or B to alaninyl d4T-MP (metabolite X). Subsequent formation of d4T-MP can occur through three different pathways, either from metabolite A, B, and/or X. Metabolite X is mainly converted to d4T.

virtually intact in human plasma and can be taken up by the target (lymphocyte and M/M) cells from the blood stream in its intact prodrug form.

In conclusion, a novel class of antiretroviral nucleotide prodrugs has been discovered, that was demonstrated to be able to rapidly taken up by the cells and to deliver directly the 5′-monophosphate form of the nucleoside (d4T) into the cells. In addition, a new metabolite originating from the nucleotide prodrug accumulates and may act as an intra- and/or extracellular depot form of d4T and/or d4T-MP. Novel So324 analogues with structural changes in the nucleoside part, the aryl part and the amino acid part of the molecule have been recently synthesized and are now under investigation for their activity against reverse transcriptase and retrovirus replication.

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