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Effects of Drugs on 2′,3′-Dideoxy-2′,3′-Didehydrothymidine Phosphorylation In Vitro

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Drugs commonly administered to patients infected with the human immunodeficiency virus (HIV) have been studied for their propensity to alter the intracellular phosphorylation of the anti-HIV nucleoside analog stavudine (2′,3′-dideoxy-2′,3′-didehydrothymidine; d4T) in peripheral blood mononuclear cells (PBMCs) and U937 cells in vitro. PBMCs isolated from the blood of healthy volunteers were stimulated by the mitogen phytohemagglutinin (10 μg/ml) for 72 h. Stimulated PBMCs (3×10^6 cells/plate) were then incubated with [^{3}H]d4T (0.65 μCi; 3 μM) and either acyclovir, dapsone, ddC, ddI, fluconazole, foscarnet, ganciclovir, itraconazole, lobucavir, ranitidine, ribavirin, rifampin, sorivudine, sulfamethoxazole, trimethoprim, lamivudine (3TC), zidovudine, or thymidine (30 and 300 μM) for 24 h. Doxorubicin and drugs showing some evidence of inhibition were also studied at 0.3 and 3 μM. Cells were extracted overnight with 60% methanol prior to analysis by radiometric high-performance liquid chromatography. Additional data for nine of the drugs were obtained by incubation with [^{3}H]d4T in U937 cells for 24 h. The effect of d4T (0.2 to 20 μM) on zidovudine (0.65 μCi; 0.018 μCi) phosphorylation was also studied. Zidovudine significantly reduced d4T total phosphates in PBMCs and U937 cells (in PBMCs to 33% [$P < 0.001$] and 17% [$P < 0.001$] of that in control cells at 3 and 30 μM, respectively). A small reduction in zidovudine phosphorylation was seen with d4T but only at d4T:zidovudine ratios of 100 and 1,000. Of the other compounds screened, only thymidine, ribavirin, and doxorubicin produced inhibition of d4T phosphorylation in both PBMCs and U937 cells. However, doxorubicin was cytotoxic at 3 μM. The decrease in d4T phosphorylation in the presence of ribavirin is consistent with previous findings with zidovudine. Although ddC significantly inhibited the phosphorylation of d4T in PBMCs, this was not seen in U937 cells, and it is probable that the findings in PBMCs are related to mitochondrial toxicity [based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide cytotoxicity assay]. The only drugs screened which may interfere with d4T phosphorylation at clinically relevant concentrations were zidovudine, ribavirin, and doxorubicin.

Stavudine (2′,3′-dideoxy-2′,3′-didehydrothymidine; d4T) is an antiretroviral thymidine analog licensed for use in patients with advanced human immunodeficiency virus (HIV) infection unable to tolerate or who no longer benefit from other nucleoside analog therapy (9, 23). d4T has in vitro activity against HIV type 1 similar to that of zidovudine (4, 5), and in common with other nucleoside analogs, d4T undergoes intracellular phosphorylation, via monophosphate and diphosphate, to the active triphosphate anabolite which inhibits HIV reverse transcriptase and terminates the elongating DNA chain (4, 5, 16, 21, 24, 32). The ratio of intracellular concentrations of d4T phosphate anabolites is approximately equimolar, in contrast to those of zidovudine, for which a buildup of the monophosphate is seen (16, 32). There is no alteration of intracellular endogenous nucleotide pools with d4T, which may be important in relation to the fact that d4T is less toxic than zidovudine to bone marrow cells (6, 25).

Recent studies have demonstrated that combination therapy with nucleoside analogs produces greater and more sustained benefit than monotherapy in the treatment of HIV (13). Combination anti-HIV therapy coupled with the treatment of opportunistic infections can result in a number of drugs being administered to HIV-infected patients and hence the potential for drug-drug interactions.

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Since intracellular phosphorylation of nucleoside analogs defines their efficacy and to some degree their toxicity, it is important to investigate if drugs commonly administered to HIV-positive patients alter anabolite formation. Interference with the phosphorylation process will be a potential cause of reduced antiviral effect. We have previously shown the effect of various drugs on zidovudine and ddC phosphorylation in vitro (17, 28–30). The aim of the present work was to evaluate the effects of drugs on d4T phosphorylation by using an in vitro assay system in order to highlight any potential problems in drug combinations. A range of drugs used to treat HIV-positive patients was studied.

MATERIALS AND METHODS

Chemicals. Lymphoprep was purchased from Nycomed Pharma AS, Oslo, Norway. Penicillin-streptomycin and L-glutamine were obtained from Northumbria Biologicals, Alnwick, United Kingdom. Fetal calf serum was acquired from Sera Lab, Sussex, United Kingdom. Mono-poly resolving medium and ribavirin were obtained from ICN Biomedicals Inc., Costa Mesa, Calif. d4T, [^{3}H]d4T, lobucavir, and sorivudine were gifts from Bristol-Myers Squibb, Wallingford, Conn. Doxorubicin was obtained from Farmitalia Carlo Erba Ltd., St. Albans, United Kingdom. Acyclovir, ganciclovir, foscarnet, fluconazole, and itraconazole were gifts from Wellcome Research Laboratories, Beckenham, United Kingdom; Roche Products Ltd., Welwyn Garden City, United Kingdom; Astra Pharmaceuticals, King's Langley, United Kingdom; Pfizer Central Research, Sandwich, United Kingdom; and Janssen Research Foundation, Beerse, Belgium, respectively. All other drugs and chemicals were purchased from Sigma Chemical Company Ltd., Poole, United Kingdom.

Isolation of peripheral blood mononuclear cells (PBMCs). Fresh heparinized venous blood (10 ml) from healthy volunteers was carefully layered onto Lymphoprep (5 ml) and centrifuged (693 × *g*, 22 min, 4°C), and the central band corresponding to mononuclear cells was removed and added to fresh RPMI

medium. The resulting cell suspension was centrifuged (693 × *g*, 5 min, 4°C), the supernatant was discarded, and the cell pellet was resuspended in growth medium (RPMI medium supplemented with fetal calf serum [10%], L-glutamine [2 mM], and penicillin [5,000 U ml^{-1}]-streptomycin [5,000 μg ml^{-1}]).

Stimulation of cells with mitogen. Cells were seeded in 5-cm-diameter petri dishes (3×10^6 cells/plate). The mitogen phytohemagglutinin (PHA; 10 μg ml^{-1}) was added, and the total volume was made up to 4 ml with RPMI growth medium. Cells were cultured at 37°C in a humidified incubator gassed with 5% CO_2 for 72 h.

Incubation of PBMCs with [^{3}H]d4T. In initial studies cells stimulated with PHA for 72 h were incubated with [^{3}H]d4T (0.65 μCi; 3 μM) for 3 to 36 h. Maximal d4T phosphorylation was observed following 24 h of incubation, and this incubation time was used in subsequent experiments.

For interaction studies, stimulated PBMCs were incubated with [^{3}H]d4T (0.65 μCi; 3 μM) and either acyclovir, dapsone, ddC, ddI, fluconazole, fluoxetine, foscarnet, ganciclovir, itraconazole, lobucavir, ranitidine, ribavirin, rifampin, sorivudine (a novel antiherpetic nucleoside analog), sulfamethoxazole, trimethoprim, lamivudine (3TC), zidovudine, or thymidine for 24 h at 37°C in a humidified incubator gassed with 5% CO_2. All compounds except doxorubicin were studied at 30 and 300 μM; doxorubicin was studied at 0.3 and 3 μM. Previous work had shown that doxorubicin is cytotoxic at concentrations above 3 μM (17). Experiments were performed on five separate occasions in triplicate. Any drugs potentially interacting at 30 and 300 μM were also investigated at 0.3 and 3 μM.

Incubation of PBMCs with [^{3}H]zidovudine. Following preincubation, stimulated PBMCs were incubated with [^{3}H]zidovudine (0.65 μCi; 0.018 μM) and d4T (0.2 to 20 μM) for 5 h at 37°C in a humidified incubator gassed with 5% CO_2. Five separate experiments were performed in triplicate.

Cell collection and extraction. Petri dishes were scraped clean of cells, and the cell suspensions were transferred to 7-ml tubes. Aliquots were removed and cell viability was measured by a trypan blue exclusion assay. The remaining cell suspensions were centrifuged (2,772 × *g*; 5 min; 4°C) and the supernatant was discarded. The resulting cell pellets were washed in 500 μl of phosphate-buffered saline and recentrifuged (2,772 × *g*, 5 min, 4°C), and the supernatant was removed. A total of 500 μl of 60% methanol was then added to the cells, which were vortexed and extracted overnight at 4°C. After extraction, samples were centrifuged to remove cellular debris, and the methanol extracts of each set of plates were pooled. The radioactivity in a 50-μl aliquot of the extract was counted to determine the total intracellular radioactivity. The 60% methanol was then evaporated under a stream of nitrogen. Samples were stored at −20°C until analysis by high-performance liquid chromatography (HPLC).

U937 cell incubations. The U937 human monocytoid cell line was propagated in RPMI medium supplemented with 10% fetal calf serum and L-glutamine (2 mM) at 37°C in a humidified incubator gassed with 5% CO_2. Cells were maintained as a cell suspension (10^5 to 10^6 cells) at 37°C in a humidified incubator gassed with 5% CO_2, and doubling times were approximately 24 h.

Cells were incubated with [^{3}H]d4T (0.65 μCi; 3 μM) for 24 h in the presence and absence of ddC, doxorubicin, foscarnet, lobucavir, ribavirin, rifampin, sulfamethoxazole, 3TC, zidovudine, and thymidine (0.3 to 300 μM).

To examine the effect of d4T on zidovudine phosphorylation, U937 cells were incubated with [^{3}H]zidovudine (0.65 μCi; 0.018 μM) and d4T (0.2 to 20 μM) for 3 h at 37°C. Cell viability was assessed by trypan blue exclusion. All experiments were performed in triplicate in six-well multiplates. Following incubation the cells were harvested, washed, and extracted as described above.

Toxicity studies. Cell viability at the termination of an interaction study was assessed by the method of neutral red uptake. In the neutral red uptake assay, the dye (50 μg ml^{-1}) was added to cell incubations 3 h before the end of the interaction experiments (i.e., at 21 h). Cells were harvested by centrifugation, followed by two washes with phosphate-buffered saline (500 μl) to remove excess neutral red. Destaining solution (1% glacial acetic acid and 50% ethanol; 500 μl) was then added to release the dye taken up by the cell lysosomes. Absorbances were read at 540 nm. ddC toxicity in PBMCs was also assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay measuring formazan formation (15). A total of 3×10^5 cells maintained in 100 μl of RPMI growth medium were stimulated with PHA (10 μg ml^{-1}) for 72 h in 96-well multiplates. Incubations with ddC (0.3 to 300 μM) were carried out for 24 h, with 25 μl of MTT (5 mg ml^{-1}) being added at 22 h. The incubation was terminated by the addition of 100 μl of MTT lysing buffer (20% [wt/vol] sodium dodecyl sulfate in 50% [vol/vol] dimethylformamide and water [pH 4.7]). Absorbances were read at 540 nm.

HPLC analysis of d4T phosphates. Cell extracts were reconstituted in 50 μl of 50 mM phosphate buffer (pH 3.8) prior to HPLC analysis. d4T and its phosphate metabolites were separated by HPLC on an anion-exchange column (Partisil 10-SAX; 25 cm by 4.6 mm) eluted with a gradient from 15 mM ammonium dihydrogen phosphate (pH 3.8)–7% methanol (buffer A) to 480 mM ammonium dihydrogen phosphate (pH 3.8)–7% methanol (buffer B).

The gradient ran between 0 and 11 min with buffer A at a flow rate of 0.75 ml min^{-1}. At between 11 and 18 min, the linear gradient changed from 100% buffer A to 100% buffer B, and at 18 min the flow rate changed to 1.5 ml min^{-1}. Buffer B then ran isocratically from 18 to 35 min. The column was reequilibrated at between 35 and 37 min by running a linear gradient of 100% buffer B to 100% buffer A. Buffer A then ran from 37 to 45 min at a flow rate of 1.5 ml min^{-1}.

The eluent was collected in insert vials at 30-s intervals. Scintillation fluid (3 ml) was added to the collected fractions, the radioactivity of which was then counted. d4T and its triphosphate were identified by cochromatography with authentic standards. d4T monophosphate (d4TMP) and d4T diphosphate (d4TDP) were assigned according to their elution order by cochromatography with the hydrolyzed d4T triphosphate (d4TTP) standard. Peaks corresponding to d4T, d4TMP, d4TDP, and d4TTP eluted after 5 to 8, 18 to 20, 22 to 24, and 33 to 36 min, respectively. Quantification of parent drug and phosphates was determined from the radioactive counts that were obtained. The limit of quantification was 0.02 pmol/10^6 cells. The coefficient of variation for repeated analysis of phosphorylated d4T anabolites extracted from the same sample was less than 10%.

HPLC analysis of zidovudine phosphates. HPLC analysis of zidovudine and its phosphates was similar to that described above for separation of d4T and its phosphates and was performed with a Partisil 10-SAX column eluted with a gradient from 10 mM ammonium dihydrogen phosphate (pH 3.8)–7% methanol to 400 mM ammonium dihydrogen phosphate (pH 3.8)–7% methanol. Phosphates were identified by their retention times (17, 30) and were quantified by on-line radiometric detection (FLO-ONE/Beta Series A200; Radiomatic Packard).

Statistical methods. Phosphorylation, neutral red assay, and MTT assay data were analyzed by analysis of variance (ANOVA), followed by a modified *t* test (Bonferroni's correction).

RESULTS

PHA stimulation (72 h of preincubation at a concentration of 10 μg ml^{-1}) was essential for d4T phosphorylation in PBMCs (Fig. 1). A similar intracellular phosphate profile was seen in U937 cells following a 24-h incubation. Previous studies have indicated that optimal conditions for zidovudine phosphorylation were 72 h of PHA stimulation with 5 h of incubation (17). There was considerable inter- and intraindividual variability in d4T phosphate formation in stimulated PBMCs.

Zidovudine (0.3 μM) significantly decreased d4T (3 μM) phosphorylation in PBMCs (59% of control total phosphates; $P < 0.01$). d4T phosphorylation was further reduced at zidovudine/d4T concentration ratios of 1 and 10 (Fig. 2A; Table 1). Marked inhibition was also seen in U937 cells (at 3 μM zidovudine, 28% of control total d4T phosphates remained; $P < 0.01$) (Table 1). In contrast, d4T had no effect on zidovudine phosphorylation in PBMCs at equimolar concentrations or a d4T:zidovudine concentration ratio of 10 (Fig. 2B). Increasing the d4T:zidovudine concentration ratio to 100 and 1,000 produced a small but significant reduction in zidovudine phosphorylation in PBMCs (Fig. 2B; Table 1) and U937 cells (at 20 μM zidovudine, 79% of control total d4T phosphates; $P < 0.05$).

The endogenous nucleoside thymidine inhibited the formation of d4T phosphate anabolites in PBMCs at concentrations of 30 μM (25% of control total phosphates; $P < 0.001$) and 300 μM (3% of control total phosphates; $P < 0.001$) (Fig. 3).

Figure 3 shows the effect of 18 drugs (and thymidine) on d4T phosphorylation in PBMCs. The ratios of the concentrations of the interacting drug to the concentration of d4T were initially 10 and 100 (i.e., d4T at 3 μM and the other drugs at 30 and 300 μM). At 30 μM, a significant decrease in d4T phosphorylation was seen in the presence of ddC and lobucavir; inhibitory trends were also seen with fluoxetine, ribavirin, and rifampin. At 300 μM, a significant decrease in d4T phosphorylation was evident not only with ddC and lobucavir but also with fluconazole, fluoxetine, rifampin, and sulfamethoxazole. However, there was cell toxicity, as judged by the neutral red uptake assay with by the last four compounds.

The following drugs at 30 or 300 μM had no significant inhibitory effects in PBMCs: acyclovir, dapsone, ddI, foscarnet, ganciclovir, itraconazole, ranitidine, sorivudine, 3TC, and trimethoprim. Drugs showing some evidence of inhibition at 30 or 300 μM were also studied at 0.3 and 3 μM (i.e., values closer to the concentrations of many of the drugs in plasma; Table 2). In addition to zidovudine, only doxorubicin significantly re-

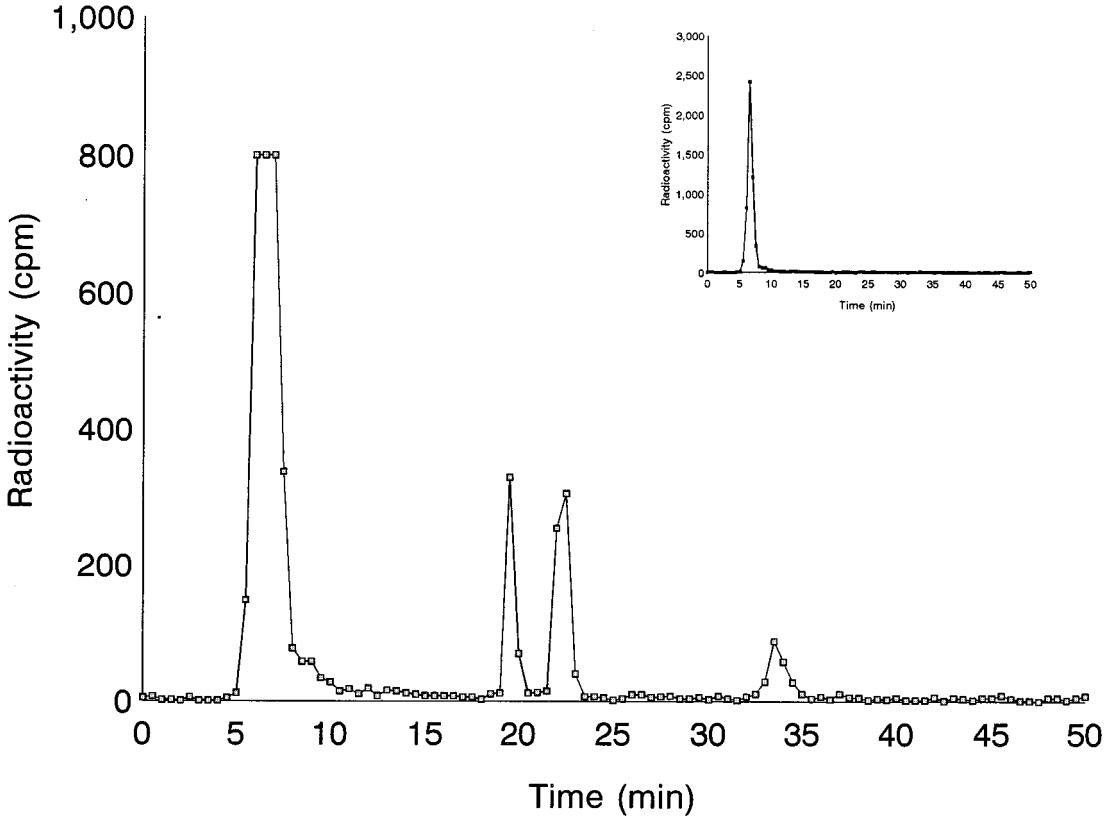

FIG. 1. Radiochromatogram showing detection of d4T and phosphates after 24 h of incubation of PHA-stimulated PBMCs with [^{3}H]d4T (0.65 μCi; 3 μM). A total of 9×10^6 cells were stimulated. (Inset) Similar radiochromatogram after incubation with acid phosphatase (60 U for 12 h).

duced d4T phosphorylation at 3 μM, but this was related to a cytotoxic effect.

Nine of the drugs were studied with U937 cells. Data for the drugs tested at 3 and 30 μM are presented in Table 2. There was significant inhibition with zidovudine, doxorubicin, and ribavirin. However, as with PBMCs, doxorubicin was cytotoxic. ddC and lobucavir did not inhibit d4T phosphorylation in the cell line, unlike in PBMCs. An additional study was then performed with ddC in PBMCs, which revealed cytotoxicity based on the MTT (but not neutral red) assay.

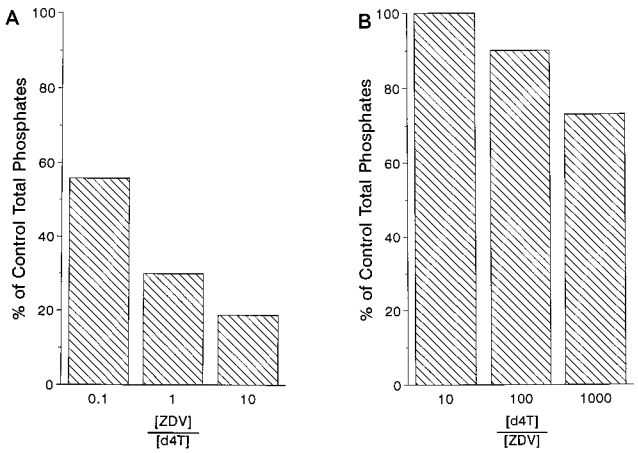

FIG. 2. Interaction of zidovudine (ZDV) and d4T in PBMCs. Each bar represents mean data from five separate experiments. (A) d4T phosphorylation. [^{3}H]d4T (0.65 μCi; 3 μM) was incubated with zidovudine for 24 h. (B) Zidovudine phosphorylation. [^{3}H]zidovudine (0.65 μCi; 0.018 μM) was incubated with d4T for 5 h.

DISCUSSION

The enzymes responsible for the phosphorylation of d4T have yet to be fully elucidated. The results obtained in studies with resting PBMCs showing that d4T phosphates were not detected are in agreement with previous findings (33). This suggests that mitochondrial thymidine kinase 2, which is expressed in resting cells, is not responsible for the first phosphorylation step. After stimulation with PHA, d4T phosphates were detected, suggesting that the enzyme(s) responsible is upregulated by this T-cell mitogen. Thus, cellular thymidine kinase 1, which is upregulated by PHA (to a variable extent), is at least partially responsible for initial d4T phosphorylation.

Results of the interaction of zidovudine and d4T show that d4T has no significant effect on zidovudine phosphorylation in PBMCs at concentrations which are orders of magnitude greater than that of zidovudine. It is clear that the enzyme responsible for initial phosphorylation of zidovudine will only be inhibited by d4T at very high concentrations. In contrast, a significant decrease in d4T phosphates was seen with a zidovudine concentration of 0.3 μM (i.e., 10-fold less than that of d4T). At equimolar concentrations of the drugs, d4T phosphorylation was reduced to less than 35% of that for the controls. The reduction in d4T phosphorylation with zidovudine (30 μM) is similar to that seen with thymidine.

The data obtained in this study are in good agreement with those from previous studies showing that thymidine kinase has

TABLE 1. Interaction between zidovudine and d4T phosphorylation in PBMCs

Studied effect and conditions	pmol/10^6 cells[a]								% Control	
	d4T	d4TMP	d4TDP	d4TTP	ZDV	ZDVMP	ZDVDP	ZDVTP	Total phosphates	Total phosphates	Neutral red uptake
Effect of zidovudine on d4T phosphorylation											
Control	68.44 ± 21.56	2.07 ± 1.44	2.90 ± 2.12	0.83 ± 0.86					5.79 ± 3.24		
Zidovudine at 0.3 μM	62.67 ± 14.32	1.57 ± 1.40**	1.31 ± 1.22*	0.33 ± 0.35*					3.23 ± 2.65**	58.9 ± 31.0	NS
Zidovudine at 3 μM	64.10 ± 14.67	1.13 ± 1.08**	0.55 ± 0.37**	0.05 ± 0.08**					1.73 ± 1.29***	33.0 ± 18.3	NS
Zidovudine at 30 μM	66.63 ± 17.09	0.83 ± 1.42***	0.21 ± 0.25***	ND***					1.09 ± 1.39***	16.8 ± 19.5	92.0 ± 10.2
Effect of d4T on zidovudine phosphorylation											
Control					0.39 ± 0.26	2.81 ± 1.10	0.38 ± 0.10	0.28 ± 0.15	3.48 ± 1.03		
d4T at 0.2 μM					0.33 ± 0.30	2.75 ± 0.69	0.42 ± 0.14	0.25 ± 0.09	3.42 ± 0.60	101.9 ± 16.3	103.4 ± 7.9
d4T at 2 μM					0.31 ± 0.11	2.51 ± 0.62	0.36 ± 0.09	0.27 ± 0.05	3.13 ± 0.67*	84.5 ± 20.7	106.6 ± 5.2
d4T at 20 μM					0.28 ± 0.15	2.16 ± 0.49**	0.27 ± 0.05	0.19 ± 0.04	2.62 ± 0.53***	77.8 ± 12.8	104.0 ± 8.9

[a] Results are expressed as the mean ± standard deviation, with $n = 6$ for zidovudine and d4T anabolite formation and $n = 4$ for toxicity experiments. NS, not studied; ND, not detected. Data were analyzed by ANOVA followed by modified t test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ZDV, zidovudine; ZDVMP, zidovudine monophosphate; ZDVDP, zidovudine diphosphate; ZDVTP, zidovudine triphosphate.

a 600-fold lower affinity for d4T than for thymidine and zidovudine (K_m for thymidine, 6.6 μM; K_i for d4T, 4,600 μM; K_i for zidovudine, 7.6 μM) (16). The potent effect of zidovudine on d4T phosphorylation suggests a higher affinity of zidovudine for thymidine kinase. This would also explain the lack of inhibition by d4T on zidovudine phosphorylation until a concentration ratio of d4T:zidovudine of 1,000 is achieved. However, the data in Table 1 also imply an effect on the conversion of d4TMP to d4TDP. This could be due to the fact that the accumulated zidovudine monophosphate is competing for thymidylate kinase and hence is inhibiting d4TDP formation. The clinical implications for zidovudine-d4T combination therapy may be reduced levels of d4TTP. Indeed, this combination has been shown to be antagonistic in vitro against a zidovudine-resistant viral isolate (22). This may be explained by the inhibition of d4T phosphorylation when the zidovudine was added.

There was considerable variation in intracellular phosphorylation following stimulation with PHA, and this must be taken into account when interpreting the data. There is also the problem of cell-directed toxicity. To exclude the possibility that inhibition of phosphorylation was caused by cell toxicity, neutral red uptake (by lysosomes) and trypan blue exclusion (measuring cell membrane dysfunction) were studied. U937 cells were also used to substantiate the data obtained with PBMCs. The majority of drugs tested by the in vitro approach had no inhibitory effects on phosphorylation (Fig. 3; Table 2).

The potential interaction of anti-HIV nucleoside analogs on d4T phosphorylation is important in terms of combination therapy. As outlined above, there was a clear interaction with zidovudine. No interaction was seen with ddI or 3TC, as anticipated from different phosphorylation pathways (1, 11), but ddC in PBMCs had a significant inhibitory effect. Since inhibition of phosphorylation was not present in the U937 cells with ddC, we suspect that the findings obtained with PBMCs are a result of cell toxicity. Although no significant reduction in neutral red uptake into lysosomes was seen, mitochondrial toxicity, as measured by the MTT assay, was demonstrated. Previous studies have found mitochondrial toxicity with ddC (8, 12). Cell toxicity linked with high concentrations of ddC (relative to the concentration in plasma) used in this study suggest that the in vitro findings obtained with PBMCs will not be clinically relevant.

The widespread use of antiviral, antifungal, and antimicrobial agents for prophylaxis and treatment of opportunistic infections in immunocompromised patients gives an enormous potential for drug-drug interactions. At the phosphorylation level, drug interactions may have implications for the efficacy of d4T in vivo.

Ribavirin has previously been shown to antagonize the phosphorylation of zidovudine (3, 31), and the mechanism of action is thought to involve a ribavirin-induced increase in nucleoside triphosphate, leading to an inhibition of thymidine kinase (31). Hence, in the present study there is reduced conversion of d4T to d4TMP.

Lobucavir, an antiviral agent in development, significantly reduced d4T phosphate formation in PBMCs, but the effect was not seen in U937 cells. At present we are unable to explain this finding. Apart from doxorubicin, none of the other antiviral agents tested (e.g., acyclovir and ganciclovir, which are phosphorylated but by virus-encoded enzymes) and none of the other drugs tested altered d4T phosphorylation.

In conclusion, we have shown that zidovudine is a potent inhibitor of d4T phosphorylation in vitro; this may have implications for combination therapy in patients. The only other drugs which may interfere with d4T phosphorylation at clini-

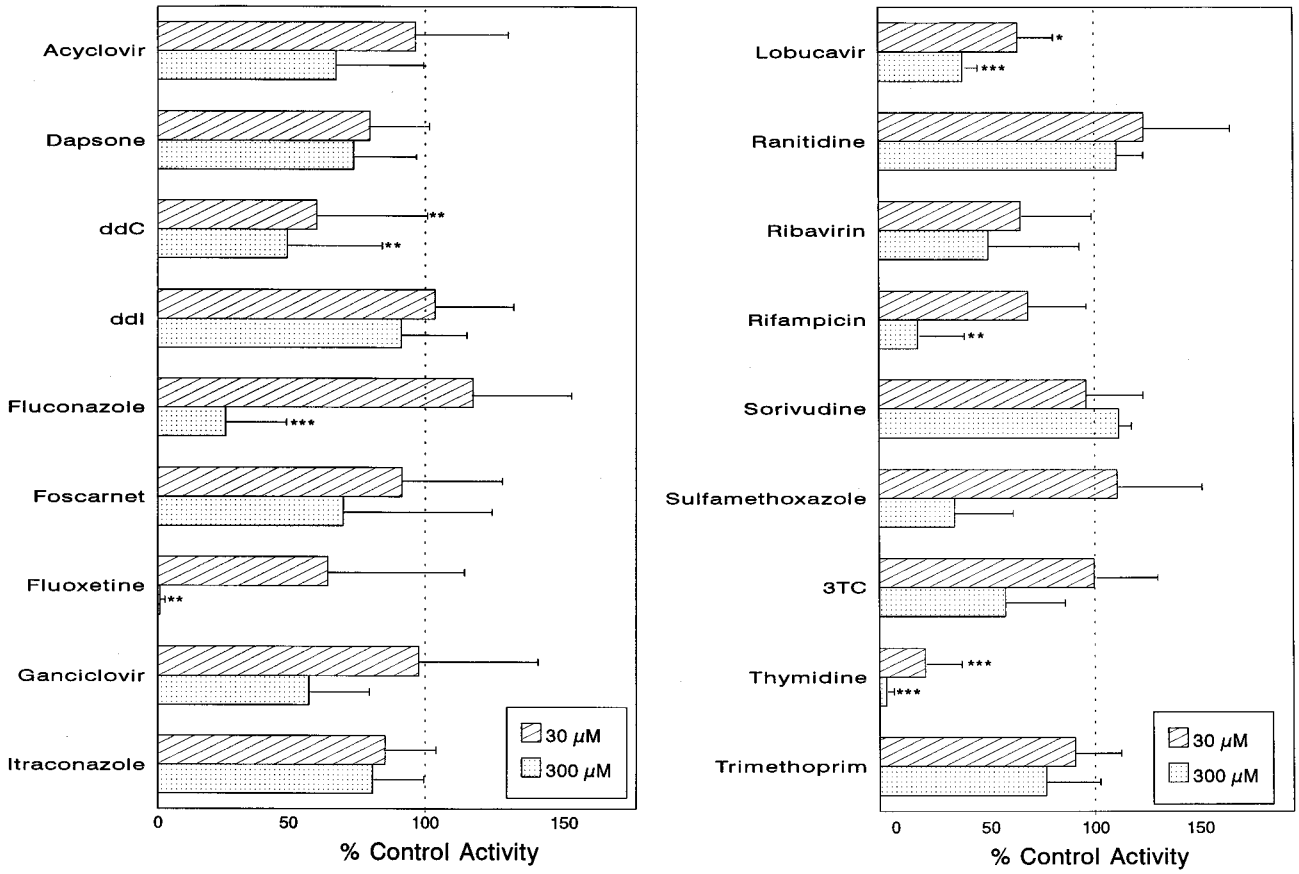

FIG. 3. Effects of various compounds on total d4T phosphorylation in PHA-stimulated PBMCs. Each bar represents the mean ± standard deviation ($n = 5$). Data were analyzed by ANOVA followed by a modified t test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

cally relevant concentrations are ribavirin and doxorubicin. The in vitro approach has limitations, and there is always a danger of trying to overinterpret the data. However, it is evident that many drugs commonly used by HIV-positive patients have no effect on phosphorylation, even at concentrations far in excess of the concentrations achievable in plasma. When one drug interferes with the phosphorylation of another one, it is important to bear in mind (i) any cytotoxic effect and (ii) the fact that the antiviral effect of d4T is dependent on the ratio of the d4TTP concentration to the endogenous thymidine

TABLE 2. Drug interactions with d4T phosphorylation in PBMCs and U937 cells[a]

Drug	PBMCs			U937 cells			Peak concn in plasma (μM)
	Mean % inhibition of d4T phosphorylation		NR toxicity (%)	Mean % inhibition of d4T phosphorylation		NR toxicity (%)	
	3 μM	30 μM		3 μM	30 μM		
Zidovudine	67***	83***	8	72***	73***	0	3 (7)[b]
Thymidine	0	75***	12	31*	61**	0	NA
ddC[c]	19	40**	0 (19**)	0	10	0	0.12 (18)
Doxorubicin	51**	NS	39*	68**	68**	76***	1.5 (14)
Lobucavir	24	33*	3	3	0	0	2.5
Ribavirin	19	31	0	25*	54**	0	6 (20)
Rifampin	11	28	7	0	0	0	8.5 (10)
3TC	0	0	6	0	21	0	10 (27)
Sulfamethoxazole	0	0	0	0	0	0	276 (19)
Foscarnet	0	0	0	0	0	0	600 (26)
Fluoxetine	0	22	21	NS	NS	NS	0.1 (2)

[a] Values are means from $n \geq 5$ separate experiments for d4T phosphorylation and $n \geq 4$ separate experiments for neutral red (NR) toxicity assay. The values for neutral red toxicity are for a drug concentration of 30 μM, except for doxorubicin in PBMCs, which is for 3 μM. NA, not applicable; NS, not studied. Data were analyzed by ANOVA followed by a modified t test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

[b] Values in parentheses are reference numbers.

[c] ddC toxicity in PBMCs was also determined by the MTT assay ($n = 6$).

triphosphate concentration. Therefore, any change in d4TTP will only have relevance to the efficacy of the drug if this ratio is altered.

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