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RAPID COMMUNICATIONS

CELLULAR PHARMACOLOGY OF 2',3'-DIDEHYDRO-2',3'-DIDEOXYTHYMIDINE (D4T) IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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2',3'-Didehydro-2',3'-dideoxythymidine (D4T) is a nucleoside analog which has been shown in several in vitro systems to have good activity against HIV, the causative agent of AIDS [1-5]. It has low toxicity to human bone marrow cells in vitro [5] and to murine bone marrow cells in vivo [6].

Previous studies on its mechanism of action have demonstrated that the triphosphate derivative of D4T is a potent inhibitor of HIV reverse transcriptase with a K_i value of approximately 0.03 μM [5]. The intracellular production of D4T triphosphate has been demonstrated in various human cells in culture, including H-9, MT-4, CEM and macrophage/monocytes [1,2,4]. A major target for HIV in vivo is the CD4+ T lymphocyte in which the virus replication causes cytopathic effects, resulting in depletion of this cell population [7]. Therefore, it was of interest to determine whether phosphorylation of D4T to the triphosphate does occur in human peripheral blood mononuclear cells in which lymphocytes are enriched. Since the intracellular half-life of the triphosphate derivative of a nucleoside analog may be more relevant to the persistence of antiviral activity than the serum elimination half-life of the parent nucleoside in drug-treated patients, the decay time of the D4T triphosphate in these cells was measured after removal of drug from medium.

MATERIALS AND METHODS

Chemicals. [^{3}H]D4T (20 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). All other chemicals and reagents were of the highest analytical grade available.

Cells. Human peripheral blood mononuclear cells (PBMC) obtained from whole blood of healthy HIV and hepatitis B virus-seronegative volunteers were separated either by sedimentation of heparinized fresh whole blood in a LeucoPREP cell separation tube at 23° or by single-step Ficoll-Hypaque discontinuous gradient centrifugation. The mononuclear cell layer was collected and washed with cold phosphate-buffered solution (PBS). The cell pellet was then resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% sodium pyruvate, 1% non-essential amino acids and 1% Penicillin-Streptomycin-Fungizone at a concentration of 1 x 10^6 cells/mL. For experiments performed with phytohemagglutinin (PHA)-stimulated PBMC, PHA was added to the cultures at a final concentration of 5 μg/mL. All cultures were incubated at 37° under 5% CO_2 for specified time periods.

To examine the activation of D4T in stimulated PBMC and its degree of variation among different individuals, cultures were prepared by preincubation with PHA for 48 - 72 hr. Following removal of PHA from culture, drug was then added with fresh medium and incubated for time periods up to 48 hr. In resting PBMC, drug was added to the freshly prepared culture and incubated for 24 hr.

After incubation with 2, 10 or 25 μM [^{3}H]D4T, cells were pelleted by centrifugation and washed with cold PBS, and then pellets were extracted twice at -20° with 1 mL of cold 60% methanol. Following removal of

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the solvent under vacuum, the residues were dissolved in deionized water and analyzed using a Whatman Partisil-10 SAX HPLC column (Whatman, Inc., Clifton, NJ) as stationary phase and a 55-min linear gradient of potassium phosphate buffer (pH 3.5) from 15 to 700 mM. Radioactivity was measured after fractionation and addition of scintillation fluid with a Beckman LS 6800 scintillation counter.

RESULTS

Human PBMC were exposed to 2 μM D4T either with or without a 72-hr preincubation in medium containing PHA (Fig. 1). D4T was not phosphorylated in resting cells (not PHA-stimulated) even when a specific activity as high as 20 Ci/mmol was used. In contrast, when PBMC were stimulated by PHA, D4T was activated to its mono-, di- and triphosphates. As previously shown in other cell lines [1,2,4], the concentrations of these phosphorylated forms were in the same order of magnitude.

With higher concentrations of D4T (10 and 25 μM), the time course of activation was studied (Table 1). Formation of the phosphorylated forms was rapid and an equilibrium was reached between 2 and 6 hr after initiation of the experiment. The concentration of 5'-phosphorylated D4T metabolites was increased from 2 to 10 μM of the parent drug. However, minimal differences were observed following incubation of either 10 or 25 μM D4T. This was probably due to variation in the degree of phosphorylation among different individuals, which is displayed in the large standard deviations.

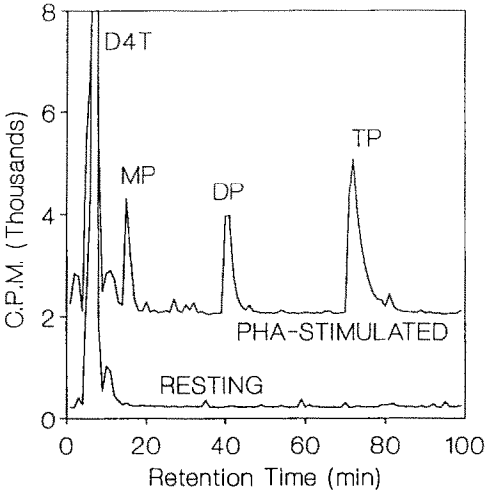

Fig. 1. HPLC chromatogram of intracellular radioactivity after exposure of resting or PHA-stimulated PBMC with 2 μM [3H]D4T. Elution position of standard D4T-monophosphate (MP), D4T-diphosphate (DP) and D4T-triphosphate (TP) was monitored at 254 nm.

To determine the intracellular half-life of D4T nucleotides, cells were exposed to 10 μM labeled D4T for 24 hr, after which cells were washed and incubation was continued in drug-free medium. At various times, cells were then processed to quantitate the phosphorylated metabolite levels. The tri- and diphosphate forms decayed with half-lives of approximately 220 and 140 min, respectively, whereas the monophosphate derivative was eliminated by a two-phase process with an initial half-life of approximately 26 min, followed by a longer half-life of about 85 min after 1 hr (Fig. 2).

Table 1. Concentrations of D4T metabolites in PBM cells: Effects of time and concentration

D4T concentration (μM)		Metabolite concentration (pmol/10^6 cells)			
		Time of Exposure (hr)			
		2	6	24	48
	D4T-MP			0.05 ± 0.04	
2	D4T-DP			0.04 ± 0.02	
(N=5)	D4T-TP			0.16 ± 0.13	
	D4T-MP	0.3 ± 0.03	0.3 ± 0.04	0.5 ± 0.1	0.2 ± 0.1
10	D4T-DP	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.04
(N=3)	D4T-TP	0.2 ± 0.3	0.5 ± 0.3	0.5 ± 0.2	0.4 ± 0.1
	D4T-MP	0.7 ± 0.3	0.9 ± 0.4	0.8 ± 0.2	0.8 ± 0.3
25	D4T-DP	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.05
(N=3)	D4T-TP	0.4 ± 0.2	0.7 ± 0.5	0.6 ± 0.2	0.7 ± 0.3

Values are means ± SD.

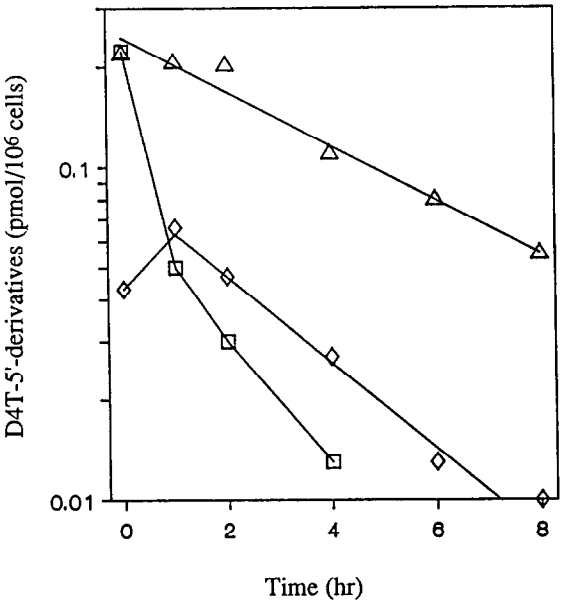

Fig. 2. Decay of D4T-5'-phosphate derivatives in drug-free medium. PBMC were exposed for 24 hr to 10 μM labeled D4T. At zero time, cells were washed and suspended in fresh medium. At specified times, cells were processed, and D4T-monophosphate (□), D4T-diphosphate (◊) and D4T-triphosphate (Δ) were quantitated by HPLC analysis. Values are the mean of at least three experiments with cells from different donors.

DISCUSSION

The present study describes the phosphorylation of D4T by human PBM cells, stimulated or not, by

PHA. In the absence of PHA stimulation, the phosphorylated metabolites of D4T were not detected (Fig. 1). This is consistent with previous studies suggesting that thymidine kinase activates D4T [8] since this enzyme is not expressed in resting PBM cells and is only turned on when cells are stimulated to divide.

After incubation of PHA-stimulated PBMC for 6 hr or more with a clinically achievable concentration of 10 μM D4T, an intracellular triphosphate content of about 0.5 pmol/10^6 cells was achieved. Assuming a mean cellular volume of 1 pL [9], this represents a value of 0.5 μM which is 10-fold higher than the K_i value reported for inhibition of HIV reverse transcriptase.

To maintain antiviral concentrations of 3'-azido-3'-deoxythymidine (AZT) in serum in spite of its short half-life (approximately 1 hr), this drug is administered every 4 hr. However, it has been suggested previously that the intracellular half-life of the active triphosphate derivative should be an important determinant for frequency of dose administration. For example, this has led to the use of only twice-a-day administration of 2',3'-dideoxyinosine (ddI) which has a triphosphate half-life of approximately 12-24 hr in human T-cell lines§.

The estimated half-life of D4T-triphosphate (3.5 hr) in human PBM cells would suggest that infrequent dosing may be appropriate for this compound. Indeed, this dosing strategy is only available because of the apparent dose proportional formation of the triphosphate derivative, a situation which has not been observed with AZT metabolism in human PBMC (Sommadossi et al., unpublished data).

Much discussion has been centered around the issue of measuring the active nucleoside triphosphate concentration in vivo in an attempt to determine therapeutic levels. For example, this approach has been achieved in quantifying ara-C triphosphate in patients with leukemia [10]. This appears to be an exceptional case both because of the extensive metabolism of ara-C (2 μM of parent drug leads to an intracellular production of 300 μM ara-CTP) and the greater number of cells present in this disease state. Following oral administration of 250-280 mg (1.1 to 2.25 mmol) of D4T to patients, a maximum serum concentration of 15-20 μM is achieved. Our best estimates suggest that at exposure concentrations of 10 μM D4T, 10^6 activated PBM cells will produce approximately 0.5 pmol D4T-TP. If one assumes that 2 x 10^6 PBMC are present in 1 mL of blood and only 1 in 10,000 is replicating, 100 mL of blood will contain 2 x 10^4 replicating cells which could produce approximately 0.01 pmol D4T-TP. Thus, even with tritiated compound at 20 Ci/mmol, this would only represent 400 dpm, and would require a dose of approximately 1 mmol (i.e. 20 Ci), which is clearly not feasible.

This study on the cellular pharmacology of D4T in human PBMC provides important information for the development of guidelines for optimal dosage for this anti-HIV agent in patients.

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REFERENCES

1. August EM, Marongiu ME, Lin TS and Prusoff WH. Initial studies on the cellular pharmacology of 3'-deoxythymidine-2-ene (d4T): A potent and selective inhibitor of immunodeficiency virus. *Biochem Pharmacol* **37**: 4419-4422, 1988.

2. Baba M, Pauwels R, De Clercq E, Desmyter J and Vandeputte M. Both 2',3'-dideoxythymidine and its 2',3'-unsaturated derivative (2',3'-dideoxythymidine) are potent and selective inhibitors of human immunodeficiency virus in vitro. *Biochem Biophys Res Commun* **142**: 128-134, 1987.

3. Lin TS, Schinazi RF and Prusoff WH. Potent and selective in vitro activity of 3'-deoxythymidine-2'-ene (3'-deoxy-2',3'-didehydrothymidine) against human immunodeficiency virus. *Biochem Pharmacol* **36**: 2713-2718, 1987.

§Hartman NR, Yarchoan R, Ahluwalia GS, Kelley JA, Broder S and Jones DG. Cellular and clinical pharmacokinetics of 2',3'-dideoxyadenosine and 2',3'-dideoxyinosine. In: *Vth International Conference on AIDS: The Scientific and Social Challenge, Montreal, Canada, 4-9 June 1989*, p. 397. International Development Research Center, Ontario, Canada.

4. Perno CF, Yarchoan R, Cooney DA, Hartman NR, Webb DS, Hao Z, Mitsuya H, Johns DG and Broder S. Replication of human immunodeficiency virus in monocytes: Granulocyte/macrophage colony-stimulating factor (GM-CSF) potentiates viral production yet enhances the antiviral effect mediated by 3'-azido-2',3'-dideoxy-thymidine (AZT) and other dideoxynucleoside congeners of thymidine. *J Exp Med* **169**: 933-951, 1989.

5. Mansuri MM, Starrett JE, Ghazzouli I, Hitchcock MJM, Sterzycki RZ, Brankovan V, Lin TS, August EM, Prusoff WH, Sommadossi JP and Martin JC. 1-(2,3-Dideoxy-ß-D-glycero-pent-2-enofuranosyl) thymine (d4T): A highly potent and selective anti-HIV agent. *J Med Chem* **32**: 461-466, 1989.

6. Mansuri MM, Hitchcock MJM, Buroker RA, Bregman CL, Ghazzouli I, Desiderio JV, Starrett JE, Sterzycki RZ and Martin JC. A comparison of in vitro biological properties and mouse toxicity of three thymidine analogs (D4T, FddT, and AZT) active against HIV. *Antimicrob Agents Chemother.* In Press.

7. Barre-Sinoussi F, Chermann JC, Rey F, Nygeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W and Montagnier L. Isolation of a T-lymphotropic retrovirus from a patient for acquired immune deficiency syndrome (AIDS). *Science* **220**: 868-871, 1983.

8. Ho HT and Hitchcock MJM. Cellular pharmacology of 2',3'-dideoxy-2',3'-didehydro-thymidine, a nucleoside analog active against human immunodeficiency virus. *Antimicrob Agent Chemother* **33**: 844-849, 1989.

9. Hao Z, Cooney DA, Hartman NR, Perno CF, Fridland A, DeVico AL, Sarngadhearan S, Broder S and Jones DG. Factors determining the activity of 2',3'-dideoxynucleosides in suppressing human immunodeficiency virus in vitro. *Mol Pharmacol* **34**: 431-435, 1988.

10. Plunkett W, Liliemark JO, Adams TM, Novak B, Estey E, Kantarjian H and Keating MJ. Saturation of 1-ß-D-arabinofuranosylcytosine 5'-triphosphate accumulation in leukemia cells during high-dose 1-ß-D-arabinofuranosylcytosine therapy. *Cancer Res* **47**: 3005-3011, 1987.