Biochemical Pharmacology, Vol. 37, No. 23, pp. 4419–4422, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
© 1988. Pergamon Press plc

RAPID COMMUNICATION

INITIAL STUDIES ON THE CELLULAR PHARMACOLOGY OF 3'-DEOXYTHYMIDIN-2'-ENE (d4T): A POTENT AND SELECTIVE INHIBITOR OF HUMAN IMMUNODEFICIENCY VIRUS

E. Michael August, Maria Elena Marongiu, Tai-Shun Lin and William H. Prusoff

Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510, U.S.A.

(Accepted 13 September 1988)

Acquired immunodeficiency syndrome (AIDS) is generally accepted to be a consequence of infection with the retrovirus designated as human immunodeficiency virus (HIV-1) [1–3]. Recent reports have shown 3'-deoxythymidin-2'-ene (d4T) to be a potent and selective inhibitor of HIV-replication and of cytopathic effects in a variety of mammalian cells [4–7]. The present report concerns our initial studies of the cellular pharmacology of d4T in the human lymphoid cell line H9.

The cytotoxic effect of d4T was assessed by measuring the inhibition of cell proliferation. The IC_{50}, which is defined as the concentration of a compound that reduces the cell number in a treated culture by 50%, depends on the number of cells seeded at time 0 (Fig. 1). When the initial cell concentration was 1×10^4, 5×10^4 and 1×10^5/ml, the IC_{50} values were 35, 250 and 500 μM respectively. The effects of natural pyrimidine nucleosides on the cytotoxicity of d4T were determined by simultaneous exposure of uninfected H9 cells to 250 μM d4T (IC_{50}) plus increasing concentrations of thymidine or 2'-deoxyuridine (37.5–150 μM) and 2'-deoxycytidine, uridine, or cytidine (37.5–1000 μM). None of the pyrimidine nucleosides tested, at subtoxic concentrations, prevented the toxic effect of d4T.

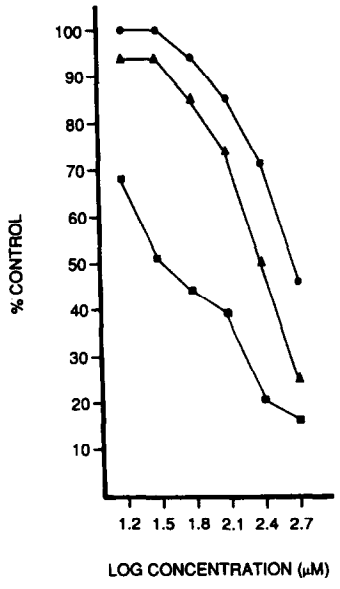

Fig. 1. Effect of d4T on H9 cell proliferation. Cells were suspended in growth medium at three different initial concentrations: 1×10^4 (■), 5×10^4 (▲), and 1×10^5 (●) cells/ml, and cultured for 72 hr with various concentrations of d4T. Cell number was determined with a model ZM Coulter Counter/model 256 Channelyzer.

Anabolic phosphorylation is of critical importance in the activation of nucleoside analogs. Therefore, we first determined the ability of uninfected H9 cells to phosphorylate d4T. H9 cells (1 x 10^5/ml) were incubated with 25 μM [methyl-^{3}H]d4T (Moravek Biochemicals, sp. act. 20 Ci/mmol, >99% radiochemical purity) for 24 hr, and a 60% methanol extract was prepared. The methanol-soluble fraction was resolved by anion exchange HPLC (Fig. 2). Unphosphorylated nucleoside accounted for ca. 80% of total soluble radioactivity, with the remaining 20% distributed among the 5'-mono-, di-, and triphosphates of d4T (d4TMP, d4TDP, d4TTP respectively). Reanalysis of unphosphorylated material on C-18 reverse phase HPLC revealed that 96% of the radioactivity was present as d4T.

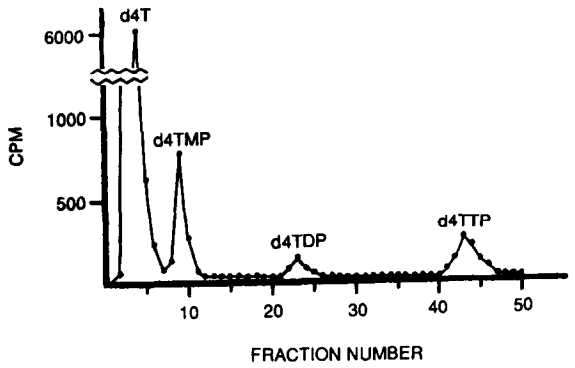

Fig. 2. Anion exchange HPLC profile of the 60% methanol-soluble extract of H9 cells exposed to 25 μM [^{3}H]d4T (250 cpm/pmol) for 24 hr. These and subsequent analyses employed a Partisil-SAX Radial-PAK column (Waters). A linear gradient of 0-90% 0.4 M $(NH_4)_2HPO_4$ (pH 5.0) into 0.01 M $(NH_4)_2HPO_4$ (pH 4.0) was developed over 45 min at 2.0 ml/min. Retention times: d4T, 4 min; d4TMP, 9 min; d4TDP, 23 min; d4TTP, 43 min. Peaks were identified by comparison of retention times with unlabeled standards (d4T, d4TMP, d4TTP) or as the 5'-phosphates of d4T by reanalysis on C-18 Radial-Pak column after enzymatic digestion with alkaline phosphatase and venom phosphodiesterase to the parent nucleoside. The reversed phase system employed a 0-20% linear gradient of acetonitrile into 0.1 M ammonium acetate (pH 5.5) developed over 50 min at 3.0 ml/min. Unlabeled d4T, d4TMP, and d4TTP were synthesized as described [4,8].

To further investigate the metabolism of d4T, H9 cells were incubated with 25 μM [^{3}H]d4T for 72 hr. At 2, 6, 12, 24, 48, and 72 hr a 60%-methanol extract was prepared from 10^7 cells and subjected to anion exchange HPLC as described (Fig. 2). The methanol-insoluble pellet was hydrolyzed with 0.3 N NaOH for 1 hr at 37°, and the DNA was reprecipitated by the addition of 5% trichloroacetic acid. The resulting supernatant was designated as the "alkaline labile" fraction, and the pellet was dissolved in dimethyl sulfoxide and designated the "alkaline stable" fraction.

The distribution of radioactivity among the soluble nucleotides of d4T, as well as the "insoluble" macromolecular fractions, appears in Fig. 3, A and B. d4T was rapidly phosphorylated in H9 cells to the mono-, di-, and triphosphates (Fig. 3A) with the maximal levels of d4TMP and d4TTP accumulating within 6 hr. These levels rapidly declined, but remained stable from 24 to 72 hr. Radioactivity from [^{3}H]d4T appeared in the alkaline stable ("DNA") as well as the alkaline labile ("RNA") fractions (Fig. 3B), suggesting incorporation into both low and high polymeric DNA. The inability to detect [^{3}H]thymidine metabolites in the soluble extracts suggests that d4T is not converted to thymidine.

The absence of a 3'-hydroxyl in d4T may be presumed to cause DNA chain termination when incorporated into a nascent DNA strand. The presence of radioactivity in the alkaline labile ("RNA") fraction after [^{3}H]d4T treatment (Fig. 3B) suggests that terminal

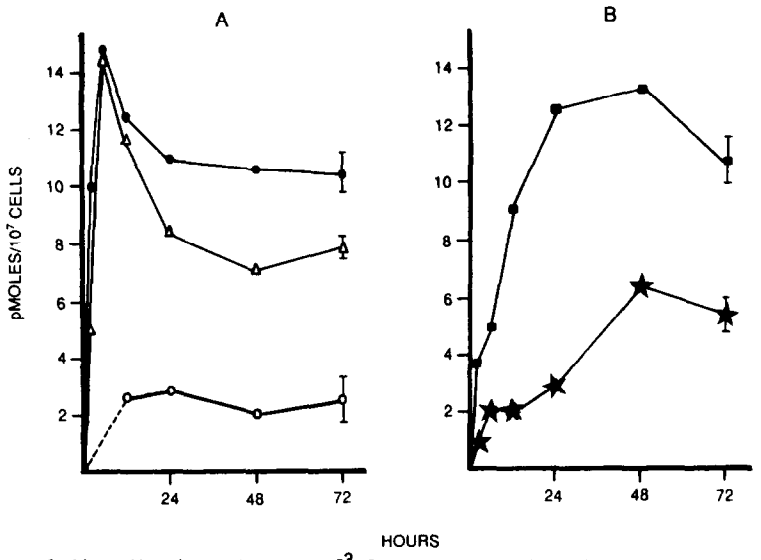

Fig. 3. Uptake and distribution of 25 μM [^{3}H]d4T (250 cpm/pmol) into 60% methanol-soluble (A) and -insoluble (B) fractions of H9 cells. (A) Conversion of d4T into d4TMP (●), d4TDP (○, dotted portion of line not determined), and d4TTP (△). (B) Distribution of radioactivity into alkaline stable (■) and alkaline labile (★) fractions. Each point is the average of two to three determinations ± SD.

incorporation of d4T results in the formation of truncated DNA strands, the shortest of which are not TCA-precipitable and thus remain soluble in the alkaline labile fraction.

Using an average cell volume of 10.5 μl/10^7 cells (SD = 0.70 μl/10^7 cells, N = 15), the cellular concentration of d4TMP in the plateau region of Fig. 3A was 1.0 μM, while the concentration of d4TTP was 0.7 μM. Since the triphosphate analog is the penultimate antiviral species, the maintenance of this concentration of d4TTP for at least 48 hr was significant, in light of the recently reported K_i of d4TTP for HIV-reverse transcriptase of 0.032 μM [9].

The uptake of [^{3}H]d4T into H9 cells was dependent upon the concentration in the medium. After a 24-hr exposure of H9 cells to a range of [^{3}H]d4T concentrations (25-250 μM), the accumulation of radioactivity in the methanol-soluble fraction (Fig. 4A) appeared to be saturable at approximately 1600 pmol/10^7 cells. The relative distributions of radioactive d4T into nucleoside, mono-, di- and triphosphate pools were similar regardless of the concentration employed (data not shown). Exposure of H9 cells to [^{3}H]d4T (250 μM) for 24 hr produced a concentration of d4TTP of 2.7 μM, which was 80-fold greater than the K_i of d4TTP for HIV-reverse transcriptase. The effect of d4TTP on the activity of cellular DNA polymerases is currently under study.

The uptake of [^{3}H]d4T into methanol-insoluble fractions was likewise concentration dependent (Fig. 4B), with d4T accumulating in both the alkaline stable and alkaline labile fractions. As shown previously (Fig. 3B), a significant amount of radioactivity was present in the alkaline labile fraction, suggesting chain termination. To ascertain whether the presence of radioactivity derived from [^{3}H]d4T in the alkaline labile fraction was a result of chain termination or poor separation of RNA and DNA, H9 cells were incubated with non-radioactive d4T (0-250 μM) and [^{3}H]thymidine (0.2 μCi/ml, ICN Radiochemicals) for 24 hr and fractionated as above. In the absence of d4T, less than 0.01% of total methanol-insoluble radioactivity (RNA and DNA) was present in the alkaline labile fraction (RNA and truncated DNA), indicating satisfactory resolution of RNA and DNA by the methods employed.

Furthermore, we observed a d4T-concentration-dependent increase in the amount of radioactivity present in the alkaline labile fraction (from <0.01 to 3.5% over the range of 0-250 μM d4T), further indicating the synthesis of short DNA fragments in the presence of d4T.

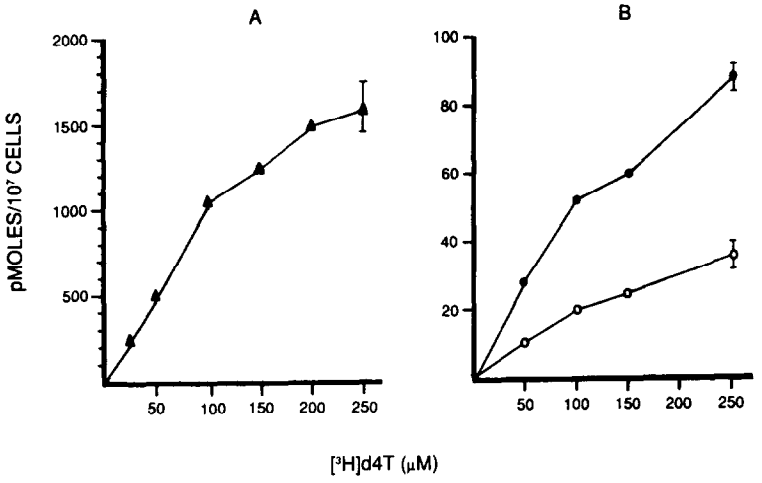

Fig. 4. Concentration dependence of uptake and distribution of [^{3}H]d4T (50 cpm/pmol) in H9 cells. Cells were incubated with the indicated concentration of [^{3}H]d4T for 24 hr and fractionated as described. (A) Total 60% methanol-soluble radioactivity (▲); (B) 60% methanol-insoluble radioactivity in alkaline stable (●) and alkaline labile (○) fractions. Points represent the average of two determinations ± SD.

We have shown that the 2',3'-unsaturated analog of thymidine (d4T), a potent inhibitor of HIV-replication in vitro (EC_{50} = 0.009-4.1 μM, [4-7]), is relatively non-toxic to the uninfected human T-cell line H9 (IC_{50} = 250 μM). The toxicity of d4T was dependent upon the concentration of H9 cells and, at an inhibitory dose of d4T for replication of these cells, exogenous pyrimidine ribo- and deoxyribonucleosides did not prevent cellular toxicity. Furthermore, we report that d4T was phosphorylated by cellular enzymes to the mono-, di-, and triphosphates and was ultimately incorporated into the DNA of growing cells. A significant amount of radioactivity appeared in the alkaline labile fraction of cells treated with [^{3}H]d4T, suggesting terminal addition of d4T to DNA and resultant chain termination.

Acknowledgements: Supported by U.S.P.H.S Grants CA-05262 and CA-44094 from the National Cancer Institute and a grant from the Bristol-Myers Co. The authors wish to thank E. Birks for excellent technical assistance and R. Kirck and S. Granata for assistance in the preparation of this manuscript.

REFERENCES

1. Barre-Sinoussi F, Cherman JC, Rey R, Nugeyre MT, Chamaret S, Gruest C, Dauget C, Axler-Blin C, Rouzioux C, Rosenbaum W and Montagnier L, Science 220: 868-871, 1983.
2. Broder S and Gallo RC, N Engl J Med 311: 1292-1297, 1984.
3. Broder S and Gallo RC, Ann Rev Immunol 3: 321-336, 1985.
4. Lin T-S, Schinazi RF and Prusoff WH, Biochem Pharmacol 36: 2713-2718, 1987.
5. Baba M, Pauwels R, Herdewijn P, DeClercq E, Desmyter J and Vandeputte M, Biochem Biophys Res Commun 142: 128-134, 1987.
6. Balzarini J, Kang G-J, Dalal M, Herdewijn P, DeClercq E, Broder S and Johns DG, Mol Pharmacol 32: 162-167, 1987.
7. Hamamoto Y, Nakashima H, Matsui T, Matsuda A, Ueda T and Yamamoto N, Antimicrob Agents Chemother 31: 907-910, 1987.
8. Ruth JL and Cheng Y-C, Mol Pharmacol 20: 415-422, 1981.
9. Mansuri MM, Starrett JE, Ghazzouli I, Hitchcock MJ, Sterzycki R, Branckovan V, Lin T-S, August EM, Prusoff WH, Sommadossi JP and Martin JC, J Med Chem, in press.