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Comparison of In Vitro Biological Properties and Mouse Toxicities of Three Thymidine Analogs Active against Human Immunodeficiency Virus

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Three analogs of thymidine, D4T [2',3'-didehydro-2',3'-dideoxythymidine; 1-(2,3-dideoxy- β -D-glycero-pent-2-enofuranosyl)thymine], FddT (3'-fluoro-3'-deoxythymidine), and AZT (3'-azido-3'-deoxythymidine), were compared in biological tests designed to assess their potential utility as anti-human immunodeficiency virus (HIV) agents. The in vitro potencies of these compounds against HIV infection in CEM cells were measured, with FddT and AZT being more potent than D4T. The cytotoxicities of D4T, FddT, and AZT for CEM cells were comparable. The triphosphates of these three derivatives inhibited purified HIV reverse transcriptase, and their affinities for this polymerase were found to be 1 or 2 orders of magnitude greater than that for the normal substrate, dTTP. D4T was less toxic than FddT or AZT for cultured human and mouse bone marrow cells (granulocyte-macrophage CFU). The three compounds had similar toxicities for human progenitor erythrocyte burst-forming units. In a 30-day mouse toxicity study, AZT and FddT produced a similar spectrum of hematopoietic toxicities. These toxic effects occurred at much lower doses of FddT than of AZT. At the higher doses of FddT, a significant incidence of lethality occurred. By contrast, D4T was considerably less toxic than both AZT and FddT in this study. The dose-limiting toxicity of D4T in mice was hepatotoxicity. The very different phosphorylation patterns of D4T, its lower toxicity, and its comparable potency relative to FddT and AZT suggest that the potential of D4T as an anti-HIV agent should be further explored.

Acquired immunodeficiency syndrome (AIDS) arises from infection by human immunodeficiency virus (HIV) (5, 24). HIV infection results in immunosuppression, rendering the patient susceptible to opportunistic infections. These opportunistic infections lead to a high incidence of morbidity and mortality. Research on potential anti-HIV agents has focused to a large extent on analogs of deoxyribonucleosides. 3'-Azido-3'-deoxythymidine (AZT; Fig. 1), an analog of thymidine (Fig. 1), has shown good activity against HIV in infected cells in vitro (22). AZT has also exhibited beneficial results in clinical trials (9) and is currently the only drug approved for the treatment of AIDS.

The mechanism of action of nucleoside analogs requires that host kinases phosphorylate the nucleoside substrate to the corresponding nucleoside mono-, di-, and triphosphates. It is the triphosphate which is responsible for the antiviral effect, either as an inhibitor of virus-specified reverse transcriptase (RT) or as a terminator of the growing viral DNA chain (29). However, the same triphosphate can also serve as a substrate for host DNA polymerases and thereby be incorporated into host DNA. The incorporation of the drug into host DNA is a potential source of toxicity. For good selectivity, the nucleoside triphosphate should be a good substrate or inhibitor of virus-specified RT but a poor substrate or inhibitor of host DNA polymerases.

While AZT is an efficacious drug, toxicity problems are associated with AZT therapy. The most serious toxicity is

for bone marrow progenitor cells (28), and this toxicity can lead to anemia and neutropenia (25). The initial phosphorylation product, AZT monophosphate, has a high affinity for thymidylate kinase, but the V_{max} of AZT monophosphate for this enzyme is very low (10). This V_{max} results in an excessive accumulation of AZT monophosphate and a consequent saturation of the thymidylate kinase enzyme. Therefore, dTMP phosphorylation is inhibited, leading to a reduction in the levels of dTTP required for normal host cell DNA synthesis. The decreased levels of dTTP have been postulated to be one of the factors associated with the toxicity of AZT (10, 31).

The search for other potent but less toxic agents than AZT remains an important goal. Further impetus has recently been given by the isolation of AZT-resistant variants of HIV from patients on prolonged AZT therapy (17). Several other structurally related thymidine analogs have been reported. Two of these analogs, 2',3'-didehydro-2',3'-dideoxythymidine (D4T; Fig. 1) and 3'-fluoro-3'-deoxythymidine (FddT; Fig. 1), are very potent inhibitors of HIV replication in vitro (2, 6, 13, 14, 19-21, 29a).

The lack of a good and predictive animal infection model for AIDS requires that any potential clinical candidate for AIDS therapy be examined in a broader range of biochemical and toxicity studies than has been done for previously studied antiviral agents. In this study, the in vitro potencies of D4T, FddT, and AZT against both murine leukemia virus and HIV (lymphadenopathy-associated virus strain) were compared. The data confirmed and extended the observa-

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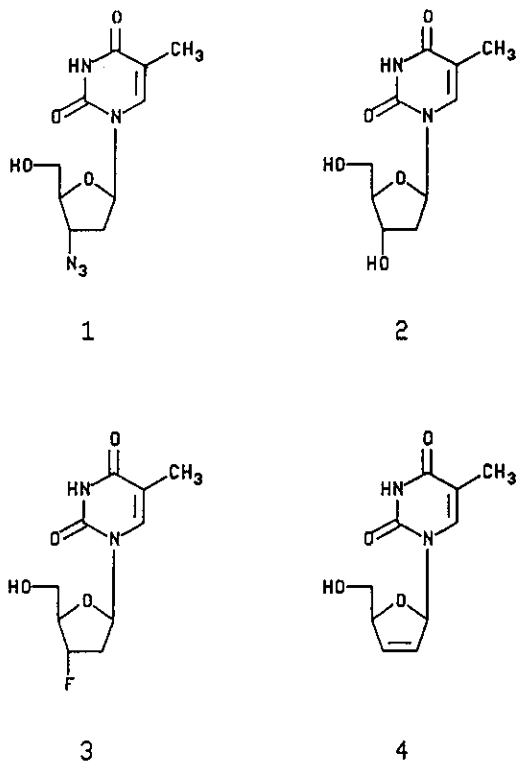


FIG. 1. Thymidine analogs. 1, AZT; 2, thymidine; 3, FddT; 4, D4T.

tions of biological activity reported elsewhere (2, 6, 13, 14, 19–21, 29a). In addition, the results of a 30-day mouse toxicity study comparing D4T, FddT, and AZT are outlined. These observations provide insight into parameters which may be important in the evaluation of potential candidates for clinical trials of therapies for AIDS.

MATERIALS AND METHODS

D4T, FddT, and AZT and their corresponding 5'-triphosphates (D4T-TP, FddT-TP, and AZT-TP) were synthesized as described elsewhere (11, 20, 29a). Purified HIV type 1 RT produced in *Escherichia coli* by recombinant methods was obtained from K. Moelling (12).

Antiviral activity. The activity of the drugs against Moloney murine leukemia virus (M-MuLV) was assessed by the standard XC assay (18, 26). SC-1 cells were infected with M-MuLV for 1 h at 37°C. Different concentrations of test compounds were added and incubated for 5 days. The medium was removed, and the cell monolayer was UV irradiated and overlaid with XC cells. After a further 4-day incubation, the cells were stained and syncytia were counted.

The activity of the compounds against HIV was determined as described previously (20). CEM cells were infected with HIV type 1 (lymphadenopathy-associated virus strain) and incubated at 37°C with different concentrations of drugs. After 8 days, the presence of p24 antigen in the supernatant was measured by an enzyme-linked immunosorbent antigen capture assay.

Cytotoxicity assay. The cytotoxicity of the compounds was determined by incubating different concentrations of the three analogs with the cells for 5 days and then assaying for

TABLE 1. In vitro anti M-MuLV and anti HIV (lymphadenopathy-associated virus) activities and cellular toxicities of D4T, FddT, and AZT

Compound	50% Effective dose (μM) for:		50% Inhibitory dose (μM)
	MuLV	HIV	
D4T	2.5	0.33	90
FddT	0.023	0.007–0.1	51–73
AZT	0.1	0.16–0.45	54

proliferation by measuring radiolabeled thymidine incorporation (30). Cytotoxicity for bone marrow cells was determined as previously described (28).

RT assay. For the RT assay, poly(rA) · oligo(dT) was used as the template · primer pair and [^3H]dTTP was used as the substrate (7). K_m and K_i values were derived with a statistical analysis software program which produces the best fit of the equation $v = (V_{\max} \cdot S)/(S + \{K_m[1 + (I/K_i)]\})$ to the measured initial velocities from a number of substrate and inhibitor combinations. In the equation, v is defined as the initial velocity of the reaction, S is the substrate concentration, and I is the inhibitor concentration.

Mouse toxicity. Groups of 10 male CD-1 mice were given D4T, FddT, or AZT orally by gavage at single doses of 100, 250, 500, or 1,000 mg/kg per day for 30 days. Dosing suspensions were prepared in 1% sodium carboxymethyl cellulose, which was administered alone to one group of 10 control mice. At day 26, blood from five mice for each group was taken for measurement of hematological and clinical chemistry parameters. Normal ranges of values for each clinicopathological parameter were established by nonparametric estimation of the central 95% of all historical control values determined in our laboratory, with the elimination of the high 2.5% and the low 2.5% of the values.

At the conclusion of the 30-day dosing period, mice were sacrificed. Tissues from these mice and from mice which died during the course of the study were collected for histopathological evaluation.

RESULTS AND DISCUSSION

The in vitro activities of D4T, FddT, and AZT were determined against both M-MuLV and HIV. The 50% effective doses of D4T and FddT against M-MuLV were 2.5 and 0.023 μM , respectively, as compared with 0.1 μM for AZT (Table 1). This potent activity of AZT in murine cells was not unexpected, since the phosphorylation of AZT in mouse cells is not blocked at the monophosphate level (see below); consequently, higher concentrations of the corresponding triphosphate are achieved (4).

In a p24 antigen capture assay, D4T, FddT, and AZT all showed a significant antiviral effect in HIV-infected CEM cells (Table 1), with FddT being the most potent. The cytotoxicities of the three compounds in uninfected cells were also determined (Table 1). The concentrations of D4T, FddT, and AZT required to inhibit cell proliferation by 50% were similar (90, 51, and 54 μM , respectively [20, 28]). The anti-HIV activity and cellular toxicity of D4T, FddT, and AZT have also been reported in other cell lines (2, 13, 14, 16, 19, 21).

To determine the mechanism of action of D4T and FddT as compared with that of AZT, we prepared D4T-TP, FddT-TP, and AZT-TP (20, 28). The inhibition of RT by D4T-TP, FddT-TP, and AZT-TP was determined by measuring the incorporation of labeled dTTP into an oligo(dT)-

TABLE 2. Inhibition of HIV RT by the nucleoside triphosphates

Compound	K_i (μM)	K_m/K_i^a
D4T-TP	0.012	400
FddT-TP	0.047	100
AZT-TP	0.018	300

^a Determined with an average K_m of 5 μM for dTTP.

primed poly(rA) template. The results, expressed as K_i s, were 0.012, 0.047, and 0.018 μM , respectively (Table 2). A K_i of 0.05 μM was measured for ddTTP under these conditions. While the K_i s for AZT-TP and D4T-TP were comparable to other published data (8, 20, 21), the K_i for FddT-TP was significantly higher than that reported by other groups (2, 6, 8). The K_i s for these three analogs were significantly lower than the K_m measured for dTTP (2.9 to 6.8 μM), suggesting that D4T-TP, FddT-TP, and AZT-TP are very efficient inhibitors of RT.

The intracellular production of triphosphates of these three compounds in tissue culture cells has also been reported (10, 15, 16). As discussed earlier, the metabolism of AZT in human cells leads to an accumulation of AZT monophosphate (see above). FddT, with an extract from carcinoma cells, is also readily phosphorylated to FddT monophosphate; however, FddT monophosphate accumulates and the subsequent phosphorylation of FddT diphosphate and then to FddT-TP is slow (16). The phosphorylation pattern of FddT, therefore, appears to be qualitatively very similar to that of AZT (10). By contrast, the metabolic phosphorylation of D4T in human cells is very different. In this case, D4T conversion to D4T monophosphate is the rate-limiting step (15) and phosphorylation of D4T monophosphate to D4T diphosphate and D4T-TP occurs readily. Consequently, no accumulation of D4T monophosphate is found (1, 3, 15, 23).

Since the major clinical toxicity observed for AZT is myelosuppression (25), it is important to ascertain the toxicities of other potential anti-HIV agents for normal human hematopoietic progenitor cells. The effect of D4T, FddT, and AZT on cultured granulocyte-macrophage CFU (CFU-GM) and erythrocyte burst-forming units (BFU-E) was measured (28) (Table 3). The 50% inhibitory dose of D4T for CFU-GM was 100 μM , while those of FddT and AZT were 10 and 1 μM , respectively (20). The three agents were also tested for their effects on the growth of bone marrow BFU-E; the 50% inhibitory doses were 10 μM for D4T, 1 μM for FddT, and 6.7 μM for AZT (20). While AZT and FddT were more toxic for CFU-GM cells than was D4T, the three analogs appeared to have similar toxicities for BFU-E cells. The selective toxicity of the nucleoside analogs for bone marrow cells may result from the fact that these cells divide more rapidly (28).

The toxicities of the three agents in an in vitro murine

TABLE 3. In vitro human and murine bone marrow cell toxicities of D4T, FddT, and AZT

Compound	50% Inhibitory dose (μM)		
	Human		Murine CFU-GM
	CFU-GM	BFU-E	
D4T	100.0	10.0	11.2
FddT	10.0	1.0	0.5
AZT	1.0	6.7	1.5

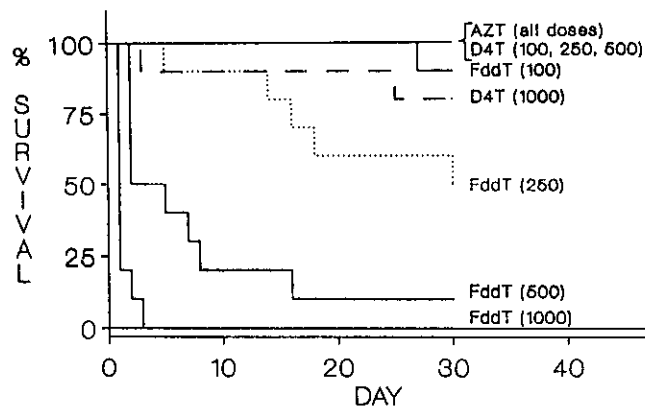


FIG. 2. Survival seen with D4T, FddT, and AZT in a 30-day toxicity study in mice (doses [in milligrams per kilogram] are shown in parentheses).

hematopoietic assay were also determined. In the murine CFU-GM assay, as in the human CFU-GM assay, D4T was less toxic than was either FddT or AZT (Table 3).

The CFU-GM assay accurately predicts the granulocytopenia observed with AZT in humans, but the intact-mouse model does not (see below). By contrast, the intact-mouse model appears to demonstrate a significant difference between D4T and AZT with respect to erythropoietic toxicity, but the human BFU-E assay does not. It will be of interest to determine the clinical toxicities of D4T to decide which of these laboratory tests more closely predicts the clinical situation.

The oral bioavailabilities of D4T and AZT were also determined independently (27). The results showed that D4T was rapidly absorbed, with an oral bioavailability of 98% in mice. In addition, D4T, unlike AZT, was not conjugated with glucuronic acid in either dogs or monkeys. The maximum concentration of D4T in plasma, after oral administration of a 25-mg/kg dose, was 23 $\mu\text{g}/\text{ml}$ after 5 min. The half-lives of D4T and AZT were 17 and 24 min, respectively.

A 30-day mouse toxicity study was undertaken to assess the in vivo toxicity of D4T and FddT in comparison with that of AZT. The mortality seen during the study is shown in Fig. 2. The highest mortality was seen with FddT. In the group given 1,000 mg of FddT per kg per day, 8 of 10 mice died on day 1 and all the mice in the group were dead by day 3. In the group given 500 mg of FddT per kg per day, 9 of 10 mice died between days 1 and 16; at a dose of 250 mg/kg per day, 5 of 10 mice died during the study; and at the lowest dose, 100 mg/kg per day, 1 of 10 mice died on day 27. In contrast to the mortality results with FddT, no deaths were observed in any of the AZT dose groups. Although there were two deaths in mice receiving D4T at 1,000 mg/kg per day (days 3 and 25), these mice did not show clinical signs of toxicity.

The results of the hematological testing of mice dosed with these compounds are given in Table 4. In the FddT group, tested mice displayed significant changes in erythrocyte parameters (erythrocytopenia, anemia, and reticulocytopenia), leukocyte numbers (leukopenia, lymphopenia, and neutropenia), and platelet numbers (thrombocytopenia). By contrast, AZT had a profound effect on the number of erythrocytes and a small effect on the number of leukocytes. Both erythrocytopenia and increased mean corpuscular volume were observed at all doses, and anemia was seen in all the mice tested at 1,000 mg/kg per day. With D4T, no significant changes in erythrocyte or leukocyte parameters

TABLE 4. Subacute toxicities of D4T, FddT, and AZT in mice

Compound	Dose (mg/kg)	% ^a With:							
		Anemia ^b	Erythrocytopenia ^b	Increased mean corpuscular vol	Reticulocytopenia	Thrombocytopenia	Leukopenia	Absolute neutropenia	Absolute lymphopenia
Control		0	20	20	0	0	0	0	0
D4T	100	20	20	0	0	20	0	0	20
	250	0	0	0	0	0	20	0	20
	500	0	0	0	0	20	20	0	0
	1,000	0	20	60	0	0	20	0	20
FddT	100	100	100	0	100	40	100	80	100
	250	100	100	0	100a	20	100	100a	75a
	500	100b	100b	0	100b	0	100b	100b	100b
	1,000	D ^c	D	D	D	D	D	D	D
AZT	100	0	80	40	25a	20	0	0	0
	250	20	100	100	0	0	20	0	20
	500	0	100	100	0	0	20	20	0
	1,000	100	100	60	20	0	40	20	40

^a Results represent the percentage of findings out of the normal range for blood samples taken at day 26 from five mice in most cases. In some dose groups, low survival rates or the inadequacy of a sample reduced the sample number to 4 (a) or 1 (b).

^b Anemia is defined as concurrent decreases in erythrocyte count numbers, hemoglobin concentration, and hematocrit. Erythrocytopenia is defined as a decrease in erythrocyte numbers alone.

^c D, All 10 mice were dead at the time of sampling.

were seen at the lower dose levels. At a dose of 1,000 mg/kg per day, D4T caused an increase in the mean corpuscular volume and some erythrocytopenia was also seen.

Blood urea nitrogen and alanine aminotransferase were also determined for 10 mice per group (when available) as indicators of kidney and liver toxicity. Elevated blood urea nitrogen levels were only seen in the last survivor of the group given 500 mg of FddT per kg per day. Elevated alanine aminotransferase levels were not seen with either AZT or FddT or the two lower doses of D4T (100 and 250 mg/kg per day). However, at 500 and 1,000 mg of D4T per kg per day, three of nine and two of eight mice, respectively, had elevated alanine aminotransferase levels.

The histopathological findings with AZT included lymphoid depletion, reticuloendothelial hyperplasia in spleen and thymus, and bone marrow hypocellularity. These changes are consistent with the lymphopenia, neutropenia, and anemia observed in peripheral blood. These toxicities correlate well with the dose-limiting toxicities observed during a clinical evaluation of AZT therapy (25).

With FddT, the most prominent histopathological changes were also thymic and splenic lymphoid depletion and bone marrow hypocellularity. These findings occurred frequently in the low-dose groups (100 and 250 mg/kg). The lower incidences of these changes at the higher doses were due to the high incidence of early mortality, which precluded lesion development. Thus, the toxicities of FddT in mice appear to be the same as those of AZT but occur at lower doses.

For D4T, gross pathological findings of small yellowish tan foci on the liver in 1 of 10 mice given 500 mg/kg per day and 3 of 10 mice given 1,000 mg/kg per day supported the drug-related changes in alanine aminotransferase levels. These were characterized microscopically as multifocal collections of giant cells, which were often mineralized. Thus, the dose-limiting toxicity of D4T in mice appears to be hepatotoxicity.

The results discussed above suggest that the biochemical and toxicological properties of FddT and AZT are similar. FddT, like AZT, accumulates at the monophosphate level. Although more potent than AZT in vitro, FddT was more

toxic than AZT, showing a hematological toxicity similar to that of AZT but at much lower doses. FddT also resulted in a significant mortality at the higher doses tested. These factors, taken together, suggest that FddT would offer little advantage over AZT. The hematological toxicities produced by AZT in the mouse toxicity study are consistent with the toxicities seen clinically (25). By contrast, the biochemical and toxicological properties of D4T are significantly different from those of AZT. The process of activation to the triphosphate shows very different kinetics and specificities for the various enzymes involved. D4T displays both a lower toxicity and a different dose-limiting toxicity endpoint than does AZT in mice. These data, taken together with the recent finding that AZT-resistant HIV strains remain susceptible to D4T in an in vitro assay (17), suggest that D4T has the potential to offer therapeutic advantages over AZT in patients with AIDS.

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