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Effects of Antiviral Nucleoside Analogs on Human DNA Polymerases and Mitochondrial DNA Synthesis

J. LOUISE MARTIN,[1] CHADWICK E. BROWN,[1] NICOLE MATTHEWS-DAVIS,[1] AND JOHN E. REARDON[2]*

Divisions of Virology[1] and Experimental Therapy,[2] Burroughs Wellcome Co., Research Triangle Park, North Carolina 27709

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Inhibition constants were determined for 16 nucleoside analog triphosphates against human DNA polymerases α, β, γ, and ε, and 7 nucleoside analogs were examined as inhibitors of mitochondrial DNA synthesis in human Molt-4 cells in culture. The results demonstrate no clear quantitative or qualitative correlation between inhibition of DNA polymerases, particularly mitochondrial DNA polymerase γ, and the inhibition of mitochondrial DNA synthesis in Molt-4 cell culture. Furthermore, the data indicate that inhibition of isolated DNA polymerases may not be predictive of in vitro or in vivo toxicity. Finally, it is not clear whether inhibition of mitochondrial DNA synthesis will be an accurate predictor of the potential in vivo toxicity of antiviral nucleoside analogs.

Many 2′,3′-dideoxyribonucleosides possess antiretroviral activity (47). These nucleoside analogs exert their antiviral effect, after anabolism to the triphosphate form, by acting as alternate substrates for the virally encoded reverse transcriptase (45, 55). Incorporation of the nucleoside analog monophosphate into the viral DNA results in the premature termination of viral DNA synthesis. Four nucleoside analogs, AZT, ddI, ddC, and d4T (see Materials and Methods for definitions of compound abbreviations) have been approved for clinical use in the treatment of human immunodeficiency virus (HIV) infection, and several other nucleoside analogs including FLT, 3TC [(−)SddC], 524W91, and 935U83, were or are in clinical trials. AZT has shown a clinical benefit in the management of HIV infection (21), and ddI, ddC, and d4T have been shown to have a positive effect on surrogate markers associated with HIV disease (31, 35). Anemia and neutropenia are the most common adverse effects associated with AZT therapy (30, 46), and myopathy has been reported to be associated with long-term AZT treatment (13, 37). In contrast, ddI, ddC, and d4T are not significantly myelotoxic; however, all three drugs can cause a painful peripheral neuropathy and, in the case of ddI, pancreatitis (36, 59).

The in vitro and in vivo mechanisms of cytotoxicity of these nucleoside analogs are not well understood. The triphosphate derivatives could serve as substrates for human DNA polymerases, resulting in inhibition of cellular DNA synthesis. Consistent with this, ddC triphosphate is a potent inhibitor of mitochondrial polymerase γ (54), and prolonged exposure of cells to ddC results in decreased mitochondrial DNA synthesis (9). Furthermore, ultrastructural changes and depletion of mitochondrial DNA have been reported in the muscle mitochondria of myopathic HIV-infected patients after long-term treatment with AZT (5).

Recently, it has been shown that treatment of chronically hepatitis B virus (HBV)-infected woodchucks with FIAC, which is rapidly metabolized to FIAU, was associated with severe toxicity, and all animals died within 6 months of treatment (22). Similarly, a clinical trial of FIAU for the treatment of HBV infection was recently halted because of severe steatosis and fatal lactic acidosis in trial participants after prolonged exposure to the drug (41). These biochemical and clinical observations have led some to propose that toxicities observed in HIV- or HBV-infected patients treated with antiviral nucleoside analogs may be due to a reduction in mitochondrial DNA synthesis and that this reduction may be due to the inhibition of polymerase γ (9).

To determine whether there is a universal mechanism by which all nucleoside analogs exert their toxic effects on cells, human DNA polymerases α, β, γ and ε were purified and the K_i/K_m values were determined for 16 nucleotide analogs with each enzyme under identical conditions of ionic strength, pH, divalent metal ion concentration, and DNA substrate. Seven of these nucleoside analogs were assessed as inhibitors of mitochondrial DNA synthesis in an in vitro cell culture assay in which the ratio of mitochondrial to cellular DNA was determined after prolonged exposure of the cells to clinically relevant concentrations of drug. The data reported herein suggest that inhibition of purified mitochondrial DNA polymerase γ may not be an accurate predictor of the potential in vitro toxicity of antiviral nucleoside analogs to cells or mitochondria. In addition, inhibition of mitochondrial DNA synthesis does not appear to be an accurate predictor of the potential in vivo toxicity of antiviral nucleoside analogs.

MATERIALS AND METHODS

Compound abbreviations. ACVTP, acyclovir triphosphate; AZT, 3′-azido-3′-deoxythymidine; BuPdGTP, N^2-(butylphenyl)-2′-deoxyguanosine 5′-triphosphate; 935U83, 3′-fluoro-2′,3′-dideoxy-5-chlorothymidine; COMDP, carbonyldiphosphonate; ddC, 2′,3′-dideoxycytidine; ddI, 2′,3′-dideoxyinosine; ddNTP, 2′,3′-dideoxynucleoside 5′-triphosphate; ddTTP, 3′-deoxythmidine; DHPGTP, (−)-ganciclovir triphosphate; d4T, 3′-deoxy-2′,3′-didehydrothymidine; dNTP, deoxynucleoside triphosphate; DTT, dithiothreitol; FIAC, 2′-fluoro-2′-deoxyarabinofuranosyl-5-iodocytidine; FIAU, 2′-fluoro-2′-deoxyarabinofuranosyl-5-iodouridine; FLGTP, 3′-fluoro-2′,3′-dideoxyguanosine 5′-triphosphate; FLT, 3′-fluoro-3′-deoxythymidine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid;

* Corresponding author. Mailing address: Division of Biochemistry, Burroughs Wellcome Co., 3030 Cornwallis Rd., Research Triangle Park, NC 27709. Phone: (919) 315-4599. Fax: (919) 315-0656.

524W91 [(−) FTC], (−)-β-L-2′,3′-dideoxy-5-fluoro-3′-thiacytidine; 523W91 [(+) FTC], (+)-β-D-2′,3′-dideoxy-5-fluoro-3′-thiacytidine; 3TC [(−)SddC], (−)-β-L-2′,3′-dideoxy-3′-thiacytidine; (+)SddC, (+)-β-D-2′,3′-dideoxy-3′-thiacytidine; NEM, *N*-ethylmaleimide; TP, triphosphate.

Materials. [*methyl*-^{3}H]dTTP (84.7 Ci/mmol), [5,5′-^{3}H]dCTP (49.5 Ci/mmol), [8,5′-^{3}H]dGTP (26 Ci/mmol), [8-^{3}H(N)]dATP (18.8 Ci/mmol), and [^{32}P]dCTP (3,000 Ci/mmol) were from Dupont-New England Nuclear, Wilmington, Del. Calf thymus DNA was from Calbiochem, San Diego, Calif. DNase I from bovine pancreas, aphidicolin, NEM, DTT, pepstatin A, leupeptin, *N*-lauroyl sarcosine, and phenylmethylsulfonyl fluoride were from Sigma Chemical Co., St. Louis, Mo. RPMI 1640 medium, penicillin-streptomycin (100×), phosphate-buffered saline, prehybridization and hybridization solution, ultrapure 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), proteinase K, and fetal bovine serum were purchased from Gibco, Grand Island, N.Y. DEAE-cellulose (DE52), phosphocellulose (P11), and DE81 paper were from Whatman, Clifton, N.J. DNase-free RNase from bovine pancreas was purchased from Boehringer Mannheim, Indianapolis, Ind. Restriction enzymes were purchased from New England Biolabs, Beverly, Mass. Ultrapure 2′-deoxynucleoside 5′-triphosphates, 2′,3′-dideoxynucleoside 5′-triphosphates, and DNA-cellulose (native) were from Pharmacia LKB Biotechnology Inc., Piscataway, N.J. Hydroxylapatite (Bio-Gel HT) was from Bio-Rad Laboratories. The clone MTTK-MERRF was purchased from the American Type Culture Collection. The plasmid pSF2124 was provided by Michelle Davis of the Wellcome Research Laboratories. BuPdGTP and COMDP were kindly provided by George Wright of the University of Massachusettes Medical School. FLTTP was kindly provided by Brian O'Hara of Lederle Laboratories. All other nucleoside and nucleoside triphosphate analogs were synthesized at Burroughs Wellcome Co.

Purification of human DNA polymerases. HeLa S3 cells were grown in cell culture as described previously (44). DNA polymerase β was purified as described previously (42). DNA polymerases α, γ, and ε were purified together through the phosphocellulose step as described previously (42). Fractions from the phosphocellulose column that contained polymerases α, γ, and ε, which eluted between 0.2 and 0.4 M KCl, were pooled, dialyzed against 1 liter of 25 mM potassium phosphate (pH 7.5) for 16 h, and loaded, at a flow rate of 0.6 ml/min, onto a hydroxylapatite column (1.0 by 20 cm). The column was washed with 20 ml of dialysis buffer, eluted with a 200-ml linear gradient from 0 to 1.0 M potassium chloride, and then washed with 40 ml of 0.5 M potassium phosphate (pH 7.5). Fractions of 2.5 ml were collected and assayed for polymerase activity. Fractions that contained polymerases γ and ε, which eluted at 0.4 M potassium chloride, were pooled, and the enzyme activities were separated by DNA-cellulose chromatography (42). Fractions from the hydroxylapatite column that contained polymerase α, which eluted in the 0.5 M potassium phosphate wash, were pooled, and the enzyme was further purified by DNA-cellulose chromatography (42). All four polymerases were stored at −20°C in buffer containing 50% glycerol.

Enzyme characterization. In addition to their chromatographic properties, the human polymerases were identified by their K_m values for nucleotide substrates and sensitivities to aphidicolin, NEM, ddTTP, BuPdGTP, and COMDP (50 mM HEPES [pH 7.5] was substituted for 50 mM Tris-HCl). Assays were conducted as described below with activated calf thymus DNA and 5 μM [^{3}H]dTTP (2 μM [^{3}H]dGTP was used as the competing substrate with BuPdGTP).

Preparation of activated calf thymus DNA. Activated calf thymus DNA was prepared as described previously (4) and was stored at −20°C.

Enzyme assays. Reaction mixtures contained 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 10 μg of bovine serum albumin, 10 μg of activated calf thymus DNA, polymerase, nucleotide substrates, and inhibitors in a total volume of 100 μl. Polymerase β reaction mixtures contained 4 mM NEM. The K_m value for each nucleotide substrate was determined with the other three nucleotide substrates present at 50 μM. Similarly, K_i values for nucleotide analogs were determined with the noncompeting nucleotide substrates present at 50 μM. Reactions (37°C) were initiated by the addition of enzyme, and aliquots (15 μl) were removed at five time points and spotted onto DE81 filter paper. The paper was washed five times for 5 min each time with 125 mM Na_2HPO_4, once for 2 min with water, once for 2 min with ethanol, dried, and counted in Ready Safe liquid scintillation fluid. To determine the K_i values, the substrate concentration was held fixed and the inhibitor concentration was varied. Apparent K_i values were determined by nonlinear least-squares fitting of the data. K_i values were calculated from the following relationship: apparent $K_i = K_i\ (1 + [S]/K_m)$. Values are reported as means ± standard errors.

Determination of total cellular and mitochondrial DNA synthesis inhibition. Molt-4 cells were seeded at 2×10^5 cells per ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 U of penicillin G per ml, and 10 μg of streptomycin sulfate per ml. To determine the effects of the compounds on mitochondrial DNA, various concentrations of each compound were added at the time that the cells were split. Cells were counted to monitor cell growth by using a hemacytometer. Cells were split every 2 to 3 days, when the cell density reached approximately 10^6 cells per ml.

Cells were harvested by centrifugation for 10 min at 1,000 × *g*. Cell pellets were washed twice with phosphate-buffered saline, resuspended at 10^8 cells per ml in phosphate-buffered saline, and stored at −70°C. Cells were diluted with 10 volumes of lysing buffer (0.5 M EDTA [pH 8.0], 0.5% Sarkosyl, 100 mg of proteinase K per ml) and were incubated at 50°C for 3 h. The samples were deproteinized by phenol-chloroform extraction. The DNA extract was then treated with DNase-free RNase for 1 h at 37°C. Samples were extracted with phenol-chloroform and precipitated overnight at −20°C with 2 volumes of ethanol. The precipitate was dissolved in 10 mM Tris (pH 8.0)–0.1 mM EDTA. Excess salt was removed by centrifugation through a Centricon centriprep column.

Total cellular DNA was sheared by expulsion through a 26-gauge needle. DNA was denatured as described in the Bio-Rad Bio-Dot SF Micro filtration apparatus instruction manual, and 10 to 20 μg was slot blotted onto a nitrocellulose membrane. The α-^{32}P-labeled probes were hybridized to the blots as described below, and the ratio of mitochondrial to cellular DNA was quantitated with a Molecular Dynamics Phosphor Imager. The 50% inhibitory concentrations were determined by linear regression according to the Hill equation for inhibition (39).

Specificities of the mitochondrial and cellular DNA probes. The mitochondrial and cellular DNA probes were examined to determine whether they were specific for mitochondrial DNA and cellular DNA, respectively. Total cellular DNA (10 to 20 μg) was digested with the restriction enzyme *Bam*HI and was analyzed on an 0.8% acrylamide gel as described previously (32).

DNA was transferred to nitrocellulose membranes by the procedure of Southern (52). Nitrocellulose membranes containing the transferred DNA were prehybridized for 4 h at

42°C in prehybridization-hybridization buffer (6× SSC [pH 7.0]; 5× Denhardt's solution, which is 0.1% [wt/vol] polyvinylpyrrolidone, 0.1% [wt/vol] Ficoll type 400, and 0.1% [wt/vol] bovine serum albumin; 100 mg of sheared, denatured salmon sperm DNA per ml as competitor DNA; and 50% deionized formamide). The prehybridization solution was then discarded, and hybridizations were performed in 6× SSC–5× Denhardt's solution–100 mg of sheared salmon sperm DNA per ml, 50% deionized formamide–random-primed [α-^{32}P]dCTP-labeled DNA probe (18, 19). A 1.5-kb *Pst*I-*Hin*dIII gel-purified restriction fragment specific for human mitochondrial DNA, MTTK-MERRF, was used to detect mitochondrial DNA (1, 48, 58). A 2.5-kb DNA fragment specific for 18S RNA was used for the detection of cellular DNA (15). The blots were hybridized overnight at 42°C.

The mitochondrial DNA probe hybridized exclusively to the 16.5-kb mitochondrial DNA, and the cellular DNA probe hybridized to the 9- and 6-kb cellular DNA fragments (data not shown). Thus, any cross-reactivity of the DNA probes was below the detection limit under the assay conditions described here.

RESULTS AND DISCUSSION

Human DNA polymerases. DNA polymerases α, β, γ, and ε were isolated from HeLa S3 cells and were characterized by their sensitivities to aphidicolin, NEM, ddTTP, BuPdGTP, and COMDP. Each DNA polymerase exhibited a unique pattern of sensitivity to these inhibitors that was similar to those reported by others (24, 27, 49, 56, 57) (data not shown).

Inhibition constants, with mammalian DNA polymerases, for many of the antiviral nucleoside triphosphate analogs included in the present study have been reported by others. However, in many cases, these data have been collected by using different reaction conditions and, more importantly, by using different DNA substrates with the different enzymes. This makes comparison of K_i values difficult. Therefore, in the present study, K_i values were determined for all four enzymes at the same pH, ionic strength, and divalent metal ion concentration and with the same nucleic acid substrate. Because inhibitors competitive with each of the four dNTP substrates were to be examined and because all of the enzymes could use activated calf thymus DNA as a substrate, activated calf thymus DNA was chosen as the DNA substrate. The conditions chosen were those for which all four enzymes readily catalyzed the incorporation of nucleotides into activated calf thymus DNA.

The K_m values for each dNTP substrate with all four polymerases are given in Table 1. These values were similar to the values determined by others (11, 25). In particular, the low K_m values for polymerase γ are diagnostic of this enzyme. Sixteen nucleoside analog triphosphates were compared as inhibitors of the human DNA polymerases (Table 2). In most cases, the K_i values against polymerases α, β, and γ reported herein were similar to the literature values for d4TTP (6), carbovir triphosphate (42), ddNTPs (12, 26, 54), 935U83TP (34), ACVTP (53), DHPGTP (53), (+)SddCTP (25), and 3TCTP [(−)SddCTP] (25).

The data in the literature for AZTTP, of all of the compounds examined in the present study, are the most variable. As discussed above, when comparing K_i values it is important that the data obtained with the same DNA substrate be compared. The reported K_i values for AZTTP inhibition of dTMP incorporation into endonuclease-activated DNA vary for polymerase α between 45 and 650 μM, for polymerase β between 0.67 and 810 μM, and for polymerase γ between 0.23 and 73 μM (11, 12, 25). The reason for this dramatic disparity is not clear. However, in all cases, AZTTP is a less potent inhibitor than the ddNTPs. In particular, for inhibition of polymerase γ, ddCTP is 20- to 2,000-fold more potent than AZTTP. In the present study, ddCTP was approximately 500-fold more potent than AZTTP.

TABLE 1. Human DNA polymerase α, β, γ, and ε K_m values for nucleotide substrates[a]

Enzyme	K_m (μM) for the following nucleotide substrate:			
	dTTP	dGTP	dCTP	dATP
α	2.4 ± 0.2	0.90 ± 0.05	1.4 ± 0.1	1.3 ± 0.1
β	1.6 ± 0.2	1.4 ± 0.1	1.4 ± 0.1	1.2 ± 0.1
γ	0.17 ± 0.01	0.14 ± 0.01	0.17 ± 0.01	0.16 ± 0.02
ε	3.0 ± 0.1	2.5 ± 0.3	2.4 ± 0.3	3.0 ± 0.2

[a] Assays were conducted as described in Materials and Methods.

None of the polymerases were differentially inhibited by the four ddNTPs. In addition, substitution of the 5-methyl group of thymine with a chloro group did not affect the potency of inhibition (935U83TP versus FLTTP and FLGTP). Additionally, substitution of the 5-hydrogen of the cytidine base with a fluorine atom did not result in differential inhibition by 3TCTP [(−)SddCTP] versus that by 524W91TP or in differential inhibition by (+)SddCTP versus that by 523W91TP. Thus, the structure of the sugar moiety and not the nucleobase was the determinant of the potency of inhibition. This finding was not surprising because polymerases must use all four dNTP substrates with similar efficiencies. Finally, the nuclear replicative polymerases α and ε were, in general, considerably less sensitive to inhibition by the antiretroviral nucleotide analogs than were the repair polymerase, polymerase β, and the mitochondrial polymerase, polymerase γ.

Effect of nucleoside analogs on the ratio of mitochondrial to cellular DNA of Molt-4 cells. Seven nucleoside analogs were tested as inhibitors of mitochondrial DNA synthesis in Molt-4 cells (Table 3). Mitochondrial DNA synthesis in Molt-4 cells was particularly sensitive to ddC. The ratio of mitochondrial to cellular DNA was decreased 80% after 5 days of treatment with 0.05 μM ddC; however, there was no apparent increase in the doubling times of the treated cells (data not shown). At 0.5 μM ddC, there was a 92% reduction in the ratio of mitochondrial DNA to cellular DNA. Finally, after 5 days of treatment with 5 μM ddC, there was significant cell death. These results were similar to the results of Chen and Cheng (9) and are consistent with the potent inhibition of mitochondrial DNA polymerase γ by ddCTP described above. In contrast to the results with ddC, 524W91 did not reduce the ratio of mitochondrial to cellular DNA at concentrations up to 100 μM, and no effect of 524W91 on cell growth was observed.

Thymidine analogs were also examined as inhibitors of mitochondrial DNA synthesis. FLT reduced the ratio of mitochondrial to cellular DNA by 59% after 7 days of treatment at 0.05 μM drug and by 86% at 0.5 μM drug. Significant cell death was observed after 5 days of treatment with 5 μM FLT. An IC_{50} of 0.02 μM was calculated after 7 days of treatment with FLT (Table 3). As discussed above, FLTTP was a potent inhibitor of mitochondrial DNA polymerase γ. Exposure of Molt-4 cells to 0.1, 1, 10, and 100 μM d4T for 6 days resulted in dose-dependent reductions in the ratio of mitochondrial to cellular DNA. d4T at 10 μM significantly decreased cell growth and mitochondrial DNA synthesis. Once again, these results are consistent with the potent inhibition of polymerase γ by d4TTP. AZT at concentrations up to 100 μM caused no

TABLE 2. Human DNA polymerase K_i values for nucleotide analogs[a]

Substrate	Inhibitor	Polymerase α		Polymerase β		Polymerase γ		Polymerase ε	
		K_i (μm)	K_i/K_m	K_i (μM)	K_i/K_m	K_i (μM)	K_i/K_m	K_i (μM)	K_i/K_m
dTTP	AZTTP	140 ± 20	58	290 ± 20	180	8.7 ± 0.7	51	400 ± 50	130
	ddTTP	90 ± 10	38	1.3 ± 0.2	0.81	0.030 ± 0.003	0.18	70 ± 10	23
	935U83TP	24 ± 5	10	2.5 ± 0.4	1.6	0.068 ± 0.007	0.40	44 ± 6	15
	d4TTP	120 ± 10	50	1.2 ± 0.3	0.75	0.048 ± 0.005	0.28	59 ± 9	20
	FLTTP	8.8 ± 0.9	3.6	1.7 ± 0.3	1.1	0.036 ± 0.004	0.21	ND[b]	ND
dGTP	ddGTP	27 ± 6	30	1.7 ± 0.3	1.2	0.016 ± 0.002	0.12	67 ± 9	27
	(−)C_{bvr}TP[c]	6.9 ± 0.9	7.7	340 ± 30	240	14 ± 2	100	410 ± 80	160
	FddGTP	4.0 ± 0.6	4.4	1.1 ± 0.2	0.79	0.017 ± 0.003	0.12	44 ± 9	18
	ACVTP	0.96 ± 0.08	1.1	13 ± 2	9.3	5.0 ± 0.5	36	62 ± 9	25
	DHPGTP	13 ± 2	14	53 ± 9	38	30 ± 7	210	80 ± 10	32
dCTP	ddCTP	90 ± 20	64	1.2 ± 0.2	0.86	0.015 ± 0.003	0.09	70 ± 10	29
	523W91TP	110 ± 20	79	0.47 ± 0.09	0.34	0.043 ± 0.007	0.25	140 ± 30	58
	524W91TP	130 ± 20	86	17 ± 2	12	6.0 ± 0.8	35	150 ± 20	63
	(+)SddCTP	70 ± 10	50	0.44 ± 0.08	0.31	0.031 ± 0.005	0.18	140 ± 20	58
	(−)SddCTP	110 ± 20	79	13 ± 3	9.3	4.0 ± 0.6	24	120 ± 30	50
dATP	ddATP	64 ± 8	49	1.1 ± 0.2	0.92	0.018 ± 0.002	0.11	67 ± 9	22

[a] Inhibition constants were determined as described in Materials and Methods.
[b] ND, not determined.
[c] (−) C_{bvr}TP, (−)carbovir triphosphate.

significant decrease in the ratio of mitochondrial to cellular DNA after 7 days of exposure to the cells, and no effect of AZT on cell growth was observed. Consistent with these findings, AZT did not inhibit mitochondrial DNA synthesis in CEM cells (10) or the neuronal PC12 cell line (29), and AZTTP was a much weaker inhibitor of polymerase γ. In contrast, although FIAU caused no reduction in the ratio of mitochondrial to cellular DNA after 7 days of exposure of the cells to 0.1 or 0.5 μM drug, significant cell death was observed after exposure to 5 μM FIAU for 2 days. FIAU was the only nonobligate chain-terminating nucleotide analog included in the present study. Incorporation of FIAU monophosphate or its metabolites at internal positions in DNA may not result in the depletion of DNA but may still cause cytotoxicity (41). Finally, 935U83 caused no reduction in the ratio of mitochondrial to cellular DNA after 7 days of exposure of cells to 0.1, 1, 10, or 100 μM drug, and no effect on cell growth was observed. Similar results have been reported in CEM cells (14). This result was surprising because 935U83TP was a potent inhibitor of polymerase γ.

TABLE 3. Inhibition of DNA polymerase γ, inhibition of mitochondrial DNA synthesis, and dose-limiting clinical toxicities of anti-HIV and anti-HBV nucleoside analogs

Compound	Inhibition of polymerase γ[a]	IC_{50} (μM) for inhibition of mitochondrial DNA synthesis[b]	Dose-limiting clinical toxicity
AZT	+	>100[c]	Hematologic, myopathy
d4T	+++	10	Peripheral neuropathy
FLT	+++	0.02[d]	Hematologic[e]
935U83	+++	>100	MTD[f] not established
FIAU	ND[g]	>5[c,d]	Lactic acidosis, steatosis
524W91	+	>100	MTD not established
3TC	+	>200[h]	MTD not established
ddC	+++	0.002[d]	Peripheral neuropathy
ddI	+++[i]	ND	Peripheral neuropathy, pancreatitis

[a] K_i value of <0.1 μM. (+++), 0.1 to 1.0 μM (++), 1.0 to 10 μM (+) >10 μM (−).
[b] IC_{50}s were determined from day 7 exposure values, with the exception of those of FIAU and ddC, which were determined from the day 5 harvest values, and that of d4T, which was determined from the day 6 harvest values, because of the toxicities that were observed.
[c] Highest concentration tested.
[d] Cell death was observed at 5 μM without selective depletion of mitochondrial DNA.
[e] Data from reference 7.
[f] MTD, maximum tolerated dose.
[g] ND, not determined.
[h] Data from reference 8.
[i] Inhibition by ddATP.

In vitro and in vivo toxicities of antiviral nucleoside analogs. The results presented herein demonstrated no clear quantitative or qualitative correlation between the inhibition of DNA polymerases, particularly mitochondrial DNA polymerase γ, and the inhibition of mitochondrial DNA synthesis in Molt-4 cell culture. Furthermore, these data indicate that inhibition of either isolated DNA polymerases or mitochondrial DNA synthesis in vitro may not be predictive of in vivo toxicity (Table 3) (17).

One limitation of extrapolating the results of studies such as those described here to predict the clinical toxicities of nucleoside analogs is that the cell line chosen may not be a good model for the target tissue. Molt-4 cells were chosen to study the effects of nucleoside analogs on mitochondrial DNA synthesis for several reasons. First, Chen and Cheng (9) reported that ddC is a potent inhibitor of mitochondrial DNA synthesis in the Molt-4 cell line. Second, Molt-4 cells are a T-lymphoid leukemic cell line that grows rapidly in culture. Third, efficient anabolism of many of the nucleoside analogs included in the present study has been demonstrated in Molt-4 cells.

Inhibition of DNA polymerase γ by a nucleoside analog triphosphate indicates that a potential exists for the nucleoside analog to inhibit mitochondrial DNA synthesis in vitro. Whether or not the nucleoside analog will inhibit mitochon-

drial DNA synthesis depends on the efficiency of mitochondrial anabolism of the nucleoside analog to the triphosphate form or on the efficiency of cytoplasmic anabolism of the analog to the triphosphate form and transport of the triphosphate into the mitochondria. Mitochondria contain nucleoside kinases with substrate specificities distinct from those contained in the cytoplasm (16, 40). Furthermore, the mitochondrial membrane contains nucleoside and nucleotide transporters with permeant specificities different from those in the plasma membrane (23, 28). Thus, whole-cell anabolism and transport studies provide little information regarding the levels of nucleoside analog triphosphate in mitochondria. Consequently, there are not sufficient data in the literature on any antiretroviral nucleoside analog to provide a sound mechanistic basis for concluding that inhibition of isolated polymerase γ will be predictive of inhibition of mitochondrial DNA synthesis in vitro or in vivo. The results presented herein with 935U83 serve as a prime example. 935U83TP was a potent inhibitor of polymerase γ. However, 935U83 did not inhibit mitochondrial DNA synthesis at concentrations up to 100 μM. Whether this lack of inhibition of mitochondrial DNA synthesis was due to inefficient mitochondrial anabolism of the drug or to inefficient transport of the triphosphate into mitochondria will require further study. A similar discrepancy has been reported for (+)SddC and (−)SddC (8).

The dose-limiting clinical toxicities of ddI, ddC, and d4T include peripheral neuropathy and, in the case of ddI, pancreatitis. Peripheral neuropathies are pathologies that have in some cases been linked to structural abnormalities of mitochondria causally related to genetic deficiencies of enzymes of energy metabolism (43, 48, 58). Thus, because mitochondrial DNA polymerase γ is potently inhibited by ddCTP, ddATP, and d4TTP and because exposure of CEM cells to ddC, ddI, or d4T results in delayed cytotoxicity that is correlated with a reduction in the mitochondrial DNA contents of these cells, inhibition of mitochondrial DNA synthesis has been proposed to be the mechanism of induction of peripheral neuropathy in HIV-infected individuals treated with ddC, ddI, or d4T (6). Mitochondrial abnormalities were observed in Schwann cells in a rabbit model of ddC-induced severe peripheral neuropathy (3). However, no mitochondrial abnormalities were observed in neurons, and axonal degeneration occurred concurrently with myelin changes (2, 3, 20). Therefore, it is not clear whether or not the clinical distal axonopathy caused by these nucleoside analogs can be explained by this simple mechanism.

Prolonged treatment of HIV-infected individuals with AZT may be associated with a mitochondrial myopathy (13, 37); however, this remains a controversial issue (33, 50). AZTTP has been reported to inhibit DNA synthesis in isolated mitochondria (51); however, AZTTP was found in the present study and by others to be, compared with the other ddNTPs, a relatively weak inhibitor of mitochondrial DNA polymerase γ (25, 42). Furthermore, the data herein and those of others indicate that AZT does not inhibit mitochondrial DNA synthesis in vitro (10). Clearly, there is not at present sufficient biochemical evidence to support the mechanism that has been proposed as an explanation for AZT-induced myopathy. In fact, if AZT does induce a myopathy, it may be due to something other than the direct inhibition of mitochondrial DNA synthesis (38).

Although all of the nucleoside analogs included in the present study are structurally related, they have diverse biological properties. This diversity includes differences in mechanisms of cell permeation, differences in anabolic and catabolic pathways, differences in inhibition of cellular and mitochondrial DNA synthesis, and differences in the effects on intermediary metabolism. Thus, a restricted approach of attempting to understand nucleoside toxicities by evaluating their effects on mitochondrial DNA polymerase γ is unlikely to be informative and is not warranted by the data presented herein.

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