0026-895X/96/010160-06$3.00/0

MOLECULAR PHARMACOLOGY, 50:160–165 (1996).

2′,3′-Didehydro-3′-deoxythymidine: Regulation of its Metabolic Activation by Modulators of Thymidine-5′-triphosphate Biosynthesis

GURPREET S. AHLUWALIA, WEN-YI GAO, HIROAKI MITSUYA, and DAVID G. JOHNS

Laboratory of Medicinal Chemistry, Basic Sciences Division (G.S.A., D.G.J.), and Experimental Retrovirology Section, Medicine Branch, Clinical Sciences Division (W.-Y.G., H.M.), National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892-4255

Received October 27, 1995; Accepted March 8, 1996

SUMMARY

The anti-human immunodeficiency virus (anti-HIV) agent 2′,3′-didehydro-3′-deoxythymidine (D4T), like other 2′,3′-dideoxynucleosides, requires conversion to its 5′-triphosphate to exert its pharmacological effect. Although D4T-triphosphate is unusually potent as an inhibitor of HIV-1 reverse transcriptase, the phosphorylation of the drug at low dose levels is inefficient because of its low affinity as an alternate substrate for the initial phosphorylation enzyme thymidine kinase. Because thymidine kinase is under feedback regulatory control by the physiological deoxynucleoside-5′-triphosphate dTTP, we examined the effect on D4T phosphorylation and thus, potentially, on its antiviral activity, of a variety of agents that lower intracellular dTTP pools. We found that agents that inhibit the *de novo* pyrimidine biosynthetic pathway have the ability to increase D4T phosphorylation, the most effective being two inhibitors of thymidylate formation, methotrexate and 5-fluoro-2′-deoxyuridine, compounds that block the enzymes dihydrofolate reductase and thymidylate synthetase, respectively. Because HIV itself lacks the capacity to synthesize dTTP and the other deoxynucleoside triphosphates essential for viral replication, combinations of D4T with modulatory agents that deplete host-cell dTTP, unlike conventional anti-HIV drug monotherapy directed solely at viral enzymes, have the ability to inhibit replication of mutant HIV strains as well as of wild-type virus.

D4T [stavudine (Zerit)] (Fig. 1), a ddN with significant anti-HIV activity, has been the subject of extensive exploratory study (1–8) and has recently been introduced into general clinical use. The potency of the pharmacologically active 5′-triphosphate form of the drug (D4T-TP) as an inhibitor of HIV-1 reverse transcriptase is almost identical to that of the corresponding 5′-triphosphate of the widely used anti-HIV agent AZT (5, 8, 9). Similarly, in most whole-cell assay systems, D4T, compared with AZT at relatively high dose levels, is equivalent to the latter in anti-HIV activity; at low dose levels, however, D4T has been reported to be significantly less active than AZT, apparently reflecting its less efficient conversion to the 5′-monophosphate and then the 5′-triphosphate level (7, 8, 10). Consequently, there has been considerable interest in methods of increasing the phosphorylation of this agent and thus decreasing the dosage required for clinical activity.

Thymidine kinase (EC 2.7.1.21) is generally (although not universally) regarded as the enzyme responsible for a critical step in the pharmacological activation of the drug [i.e., its initial phosphorylation to D4T-MP (7, 11–14)], although D4T is a relatively inefficient substrate for this enzyme (K_m, 138 versus ~3 μM for AZT) (11, 15). Thymidine kinase is well known to be subject to feedback regulation by dTTP, with elevated levels of the latter resulting in decreased kinase activity and, conversely, subphysiological levels resulting in increased activity (16, 17). In an early study, Baba *et al.* (10) noted that the nucleoside analogue ribavirin, an agent known to increase intracellular dTTP pools, brought about a highly significant decrease in the anti-HIV activity of both D4T and AZT, although the effect of ribavirin on phosphorylation of these two agents was not measured. The purpose of the current study was to quantify the effects on D4T phosphorylation of agents, which, in contrast to ribavirin, are known to have the property of lowering dTTP pools and thus potentially increasing the activity of the drug. A preliminary account of some of the results has appeared (18).

Experimental Procedures

Materials. HU, PHA, and nucleoside and nucleotide standards were purchased from the Sigma Chemical Co. (St. Louis, MO) or from Pharmacia (Piscataway, NJ). D4T, AZT, ribavirin, tiazofurin, mycophenolic acid, MTX, pyrazofurin, 5-fluoro-2′-deoxyuridine, and brequinar were supplied by Dr. Karl Flora (Pharmaceutical Resources Branch, National Cancer Institute, Bethesda, MD). Recombinant

ABBREVIATIONS: D4T, 2′,3′-didehydro-3′-deoxythymidine; HIV, human immunodeficiency virus; ddN, 2′,3′-dideoxynucleoside; D4T-MP, D4T-5′-monophosphate; D4T-TP, D4T-5′-triphosphate; dTTP, thymidine-5′-triphosphate; MTX, methotrexate; FUdR, 5-fluoro-2′-deoxyuridine; dNTP, deoxynucleoside-5′-triphosphate; AZT, 3′-azido-3′-deoxythymidine; HU, hydroxyurea; PHA, phytohemagglutinin; PBM cells, peripheral blood mononuclear cells.

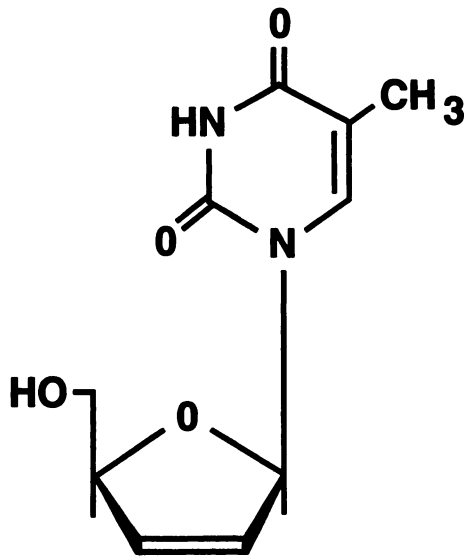

Fig. 1. Structure of D4T.

interleukin-2 was purchased from R & D Systems (Minneapolis, MN). Radioimmunoassay kits for p24 Gag (group-specific antigen) protein were purchased from DuPont (Boston, MA). [*methyl*-^{3}H]D4T (36.6 Ci/mmol) and [*methyl*-^{3}H]AZT (14 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA).

Cells. MOLT-4 cells (American Type Culture Collection, Rockville, MD) were grown at 37° in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% heat-treated (56° for 60 min) fetal bovine serum, 44 μg/ml gentamycin, and 4 mM L-glutamine in a humidified atmosphere of 95% air/5% CO_2. Cells were verified to be in logarithmic growth at time of use. Typically, a cell concentration of ~1 × 10^6/ml was used in metabolism studies. PBM cells were isolated from heparinized venous blood of healthy donors and incubated for 48 hr with PHA (10 μg/ml) in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 15 units/ml recombinant interleukin-2, 4 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. An HIV-1 strain ($ERS104_{pre}$) was isolated, as described previously, from a patient with advanced HIV-1 infection before antiviral therapy (19).

Determination of anti-HIV-1 activity. PHA-stimulated PBM were plated onto 24-well tissue culture plates at a density of 1 × 10^6 cells/well, and drugs (D4T and/or FUdR) were added in 2 ml of supplemented RPMI 1640 medium. After incubation for 2 hr, cells were exposed to 2500 HIV-1 50% tissue culture infective doses/well. At 4 days after infection, half of the culture medium was replaced with fresh culture containing the same concentrations of drugs as were initially added. On day 8, the medium was harvested, and the amount of p24 protein was determined through radioimmunoassay. All assays were conducted in quadruplicate.

Cellular metabolism studies. Ten-milliliter aliquots of MOLT-4 cell suspensions in logarithmic growth (10^6 cells/ml) were preincubated for 2 hr with putative dTTP modulators at the concentrations indicated in individual tables and figures. ^{3}H-Labeled D4T (5 μM, 5 μCi/ml) was then added, and after a 5-hr incubation period, cell suspensions were centrifuged, and cell pellets were washed with 1 ml of cold 0.85% sodium chloride solution and extracted with 0.4 ml of 60% methanol. Extracts were heated for 1 min at 95° and, after centrifugation (2 min at 12,000 × *g*), 200 μl of the supernatant was subjected to chromatography on an ion-exchange Partisil 10-SAX column using an previously described elution sequence (20). One-minute fractions were collected, and radioactivity was determined through scintillation counting.

For combination studies on the effect of AZT on D4T metabolism, MOLT-4 cells were preincubated for 15 min with a range of concentrations of unlabeled AZT (0.5–5.0 μM) and then exposed for 5 hr to [^{3}H]D4T (2.5 μM), or were preincubated for 15 min with varying concentrations of unlabeled D4T (0.5–10.0 μM) and then exposed for 5 hr to [^{3}H]AZT (0.5 μM). Analysis of the respective drug metabolites was carried out by high performance liquid chromatography as described above. All cellular metabolism studies were repeated twice (occasionally three times) without significant variation noted between different MOLT-4 cultures. Results shown in figures and tables represent the average of duplicate analyses from single experiments, with duplicate values differing by <5%.

Determination of intracellular dNTP concentrations. dNTP concentrations in MOLT-4 cell extracts were determined according to a modification of the chromatographic method of Garrett and Santi (21). Cells (7 × 10^7 cells/sample) were exposed to the dTTP modulator or to saline for 5 hr. After incubation, cells were washed and extracted with 60% methanol. The methanol extracts were freeze-dried and redissolved in water, the ribonucleotides present quantitatively were removed through treatment with sodium periodate in the presence of cyclohexylamine-HCl, and the deoxynucleoti-

TABLE 1

Effect of modulators of dTTP on D4T metabolism in MOLT cells

MOLT-4 cells growing in log phase (~10^6 cells/ml) were treated with either the listed dTTP modulators or saline (control) for 2 hr before the addition of radiolabeled D4T (5 μM, 50 μCi/10 ml). Cells were incubated with the radiolabeled drug for an additional 5 hr. After incubation, cells were extracted and analyzed for radiolabeled D4T metabolites as described in Experimental Procedures. Values in parentheses represent percentage of control.

Treatment	D4T	D4T-MP	D4T-DP	D4T-TP
		pmol/10^6 cells		
None (control)	9.2 (100)	0.032 (100)	0.072 (100)	0.236 (100)
2′-Deoxyguanosine				
5 μM	11.3 (122)	0.276 (860)	0.307 (430)	1.169 (500)
10 μM	9.8 (106)	0.155 (480)	0.187 (260)	0.727 (310)
Pyrazofurin				
1 μM	7.9 (85)	0.330 (1030)	0.233 (320)	1.277 (540)
5 μM	11.5 (124)	0.289 (900)	0.228 (320)	0.918 (390)
Brequinar				
5 μM	13.0 (141)	0.377 (1180)	0.259 (360)	1.130 (480)
MTX				
1 μM	10.1 (109)	0.136 (430)	0.342 (480)	1.529 (650)
5 μM	10.8 (117)	0.141 (440)	0.387 (540)	1.513 (640)
FUdR				
0.25 μM	13.4 (144)	0.191 (600)	0.441 (610)	2.424 (1030)
1.00 μM	10.9 (118)	0.167 (520)	0.364 (510)	2.029 (860)
HU				
50 μM	8.4 (91)	0.127 (307)	0.167 (232)	0.831 (352)
100 μM	9.2 (100)	0.112 (350)	0.101 (140)	0.153 (65)

TABLE 2

Effect of inosine monophosphate dehydrogenase inhibitors on D4T metabolism in MOLT-4 cells

MOLT-4 cells growing in log phase (~10^6 cells/ml) were treated with the listed IMPD inhibitors for 2 hr before the addition of radiolabeled D4T (5 μM, 50 μCi/10 ml). Cells were incubated with the radiolabeled drug for an additional 5 hr. After incubation, cells were extracted and analyzed for radiolabeled D4T metabolites as described in Experimental Procedures. Values in parentheses represent percentage of control.

Treatment	D4T	D4T-MP	D4T-DP	D4T-TP
		pmol/10^6 cells		
None (control)	9.2 (100)	0.040 (100)	0.087 (100)	0.382 (100)
Ribavirin (10 μM)	8.4 (91)	0.008 (20)	0.004 (5)	0.020 (5)
Tiazofurin (10 μM)	10.3 (112)	0.009 (23)	0.003 (3)	0.020 (5)
Mycophenolic acid (2 μM)	8.6 (93)	0.008 (20)	0.004 (5)	0.022 (6)

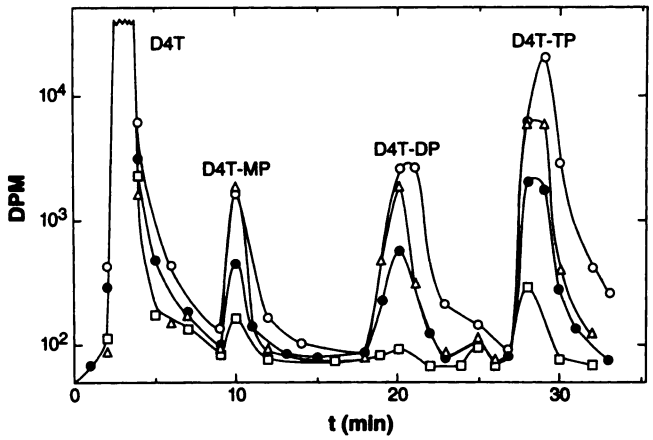

Fig. 2. Chromatographic separation of ^{3}H-labeled metabolites arising from D4T in the presence of modulators of dTTP pool size. MOLT-4 cells (~10^6 cells/ml) were preincubated for 2 hr with 0.25 μM FUdR, 100 μM HU, or 10 μM ribavirin. ^{3}H-Labeled D4T (5 μM, 5 μCi/ml) was then added. After 5 hr of further incubation, cells were harvested and subjected to methanol extraction, and the extracts were chromatographed through ion-exchange high performance liquid chromatography as described in Experimental Procedures. Representative chromatograms from three experiments are shown, with duplicate values varying by <5%. ●, Control; ○, plus FUdR; △, plus HU; □, plus ribavirin. Note logarithmic scale (*ordinate*).

des were stabilized through the addition of glycerine. The samples were analyzed for dATP, dGTP, dCTP, and dTTP on a prestandardized ion-exchange high performance liquid chromatographic system as described previously (21).

Results

Effects on D4T phosphorylation of inhibitors of dTTP formation. The effects on D4T phosphorylation by MOLT-4 cells of six known inhibitors of dTTP biosynthesis are listed in Table 1. Inhibitors acting at a variety of sites on the pyrimidine nucleotide biosynthetic pathway were purposely selected (see Discussion). Inhibitors were incubated with MOLT-4 cells for 2 hr before the addition of radiolabeled D4T (5 μM). The levels of the parent nucleoside and of D4T-MP, D4T-5′-diphosphate, and D4T-TP were determined chromatographically after an additional 5 hr of incubation.

All of the inhibitors except brequinar were examined at two concentrations, with the lower concentration being below the known cytotoxic levels of these agents over short incubation periods. All agents were effective in increasing D4T phosphorylation, but the rank order when measured at the indicated subtoxic concentrations and at the termination of the arbitrarily selected 5-hr incubation period varied depending on whether the monophosphate or triphosphate of D4T was used as the index of effectiveness (Table 1). For D4T-5′-MP, the effectiveness of stimulation was greatest with brequinar (11.8-fold), and the rank order was brequinar > pyrazofurin > 2′-deoxyguanosine > FUdR > MTX > HU. For D4T-5′-TP, the pharmacologically active form of the drug, effectiveness was greatest with FUdR (10.3-fold) and the rank order was FUdR > MTX > pyrazofurin > 2′-deoxyguanosine > brequinar > HU. No consistent effect on parent nucleoside (D4T) accumulation was noted with any of the modulators (Tables 1 and 2).

Effect of increases in dTTP pool size on D4T phosphorylation. The effects of three agents that are known to bring about increases rather than decreases in dTTP pools are shown in Table 2. At the concentrations studied, these compounds were equally effective in reducing D4T-MP and D4T-TP pools to ~20% of control values, although when compared on a molar basis, mycophenolic acid was several-fold more effective than ribavirin or tiazofurin. As indicated above, an inhibitory effect of ribavirin on D4T anti-HIV activity has been described by Baba *et al.* (10), although phosphorylation of D4T was not measured by these investigators. The contrasting effects of a phosphorylation inhibitor (riba-

TABLE 3

Effects of AZT on D4T metabolism and of D4T on AZT metabolism in MOLT-4 cells

MOLT-4 cells growing in log phase were incubated with either radiolabeled D4T (2.5 μM, 50 μCi/10 ml) in the presence or absence of AZT (0.5–5.0 μM) or radiolabeled AZT (0.5 μM, 75 Ci/10 ml) in the presence or absence of varying concentrations of D4T (0.5–10 μM). The nonradiolabeled modulator (i.e., AZT for D4T metabolism and D4T for AZT metabolism) was added to the cell suspensions 15 min before the addition of the radiolabeled drug. Cells were incubated with radiolabeled drug for an additional 5 hr. After incubation, cells were extracted and analyzed for radiolabeled D4T or AZT metabolites as described in Experimental Procedures. Values in parentheses represent percentage of control.

Effect of AZT on D4T metabolism in MOLT-4 cells

AZT	D4T-MP	D4T-DP	D4T-TP
μM		*pmol/10^6 cells*	
0	0.036 (100)	0.095 (100)	0.417 (100)
0.5	0.013 (36)	0.014 (15)	0.056 (13)
1.0	0.010 (28)	0.009 (9)	0.035 (8)
2.5	0.007 (20)	0.004 (4)	0.023 (6)
5.0	0.006 (16)	0.003 (3)	0.019 (5)

Effect of D4T on AZT metabolism in MOLT-4 cells

D4T	AZT-5′-monophosphate	AZT-5′-diphosphate	AZT-5′-triphosphate
μM		*pmol/10^6 cells*	
0	5.999 (100)	1.153 (100)	1.575 (100)
0.5	5.805 (97)	1.082 (94)	1.545 (98)
1.0	5.559 (93)	1.039 (90)	1.461 (93)
5.0	5.271 (88)	1.078 (93)	1.457 (93)
10.0	5.216 (87)	1.020 (88)	1.434 (91)

TABLE 4

Effect of dTTP modulators on dNTP pools in MOLT-4 cells

MOLT-4 cells (7×10^7/cells/sample) were exposed to the dNTP modulator or to saline for 5 hr. Cells were then washed and extracted with 60% methanol. Deoxynucleotides were determined by the method of Garrett and Santi (21). The experiment was repeated twice and the results shown are the average of duplicate analyses, with duplicate values differing by <5%. Values in parentheses represent percentage of control.

Modulator	dTTP	dCTP	dATP	dGTP
		pmol/10^6 cells		
None	112.1 (100)	18.9 (100)	49.9 (100)	24.7 (100)
Ribavirin (10 μM)	263.7 (235)	22.3 (118)	43.3 (87)	17.8 (72)
2'-Deoxyguanosine (10 μM)	54.2 (48)	15.6 (83)	69.1 (138)	152.5 (617)
Pyrazofurin (1 μM)	61.3 (55)	15.2 (80)	59.2 (119)	15.0 (61)
Brequinar (5 μM)	64.9 (58)	14.0 (74)	60.4 (121)	14.7 (60)
MTX (1 μM)	40.8 (36)	18.1 (96)	47.4 (95)	15.1 (61)
FUdR (0.25 μM)	41.8 (37)	16.5 (87)	60.8 (122)	11.3 (46)
HU (50 μM)	80.5 (72)	17.7 (94)	25.9 (52)	15.3 (62)

virin) and two phosphorylation stimulators (FUdR and HU) on formation of D4T-MP, D4T-5'-diphosphate, and D4T-TP are shown graphically in Fig. 2; it is particularly notable that accumulation at the 5'-monophosphate level, a characteristic and possibly undesirable feature of the related anti-HIV thymidine analogue AZT (22), is not seen with D4T in either the control or the drug-treated incubation mixtures.

Effect of AZT on D4T phosphorylation. In confirmation of a previous observation in CEM cells (7), AZT, a known and highly efficient substrate for thymidine kinase, was found to be effective as a competitive substrate inhibitor in blocking D4T phosphorylation in the MOLT-4 cell line; D4T, on the other hand, had very little effect on AZT phosphorylation, even at 20 times the concentration of the latter drug (Table 3).

Effect of modulators on dNTP pools. As indicated above, inhibitors of dTTP formation were selected for their potential abilities, based on available knowledge of their biochemical sites of action, to modulate the feedback regulation of thymidine kinase through effects on dTTP pool size. Studies were therefore carried out to quantify directly the effects of the agents studied on dTTP and other dNTP pools. As shown in Table 4, all agents that increased D4T phosphorylation brought about a decrease in dTTP pools, with the most effective at the concentrations studied being MTX and the least effective being HU. 2'-Deoxyguanosine, as would be anticipated, caused in addition a significant (6-fold) increase in dGTP. HU (50 μM) was more effective in lowering dATP than in lowering dTTP, an effect that is typical of this agent, particularly at low dose levels (23). Conversely, ribavirin (10 μM), a highly effective inhibitor of D4T phosphorylation (Fig. 2 and Table 2), increased dTTP pools by 2.4-fold. Tiazofurin and mycophenolic acid were not examined in the latter context, but the ability of the latter of these two compounds to increase dTTP levels has been reported previously (24).

Effect of higher concentrations of modulators of dTTP synthesis on phosphorylation. As noted above, the lower concentrations of dTTP synthesis modulators examined were selected as being below the level of cytotoxicity for MOLT-4 cells. As shown in Table 1, however, with all modulators except MTX, the higher of the two concentrations used resulted in less enhancement of D4T phosphorylation than was seen with the lower level. Further study of this phenomenon was carried out with HU, a compound whose antiviral and biochemical effects alone and in combination we have studied extensively in the PHA/PBM system (25–27). The effect of a wide range of HU concentrations (25–1000 μM) on D4T-TP and dTTP pools was determined. As shown in Fig. 3, enhancement of D4T-TP formation was maximal in the MOLT-4 system at 50 μM HU and declined to ~25% of control values at 1000 μM. dTTP pools declined to ~60% of control values over the range of 25–100 μM HU and showed little further decrease at higher HU concentrations, effects that are compatible with partial cell-cycle arrest and increased thymidine kinase levels at the lower HU doses and generalized dNTP depletion and consequent cytotoxicity at the higher dose levels. These results with MOLT-4 cells are in marked contrast to HU effects in PHA/PBM cells, a slowly replicating system that is much more resistant to HU cytotoxicity (25, 26).

Effect of dTTP depletion on the anti-HIV-1 effectiveness of D4T. The effect of FUdR, the agent with the greatest stimulatory effect on D4T-TP formation, was examined in the PHA/PBM anti-HIV-1 assay system. FUdR (0.2 μM) brought about an 8-fold decrease (from 0.5 to 0.06 μM) in the D4T concentration required for a 50% reduction in HIV-1 p24 antigen production (IC_{50}). FUdR alone showed only slight anti-HIV activity at this concentration. A detailed comparative study in the PHA/PBM and other anti-HIV assay systems of the effects on D4T activity of FUdR and the other modulatory agents examined will form the subject of a future communication.

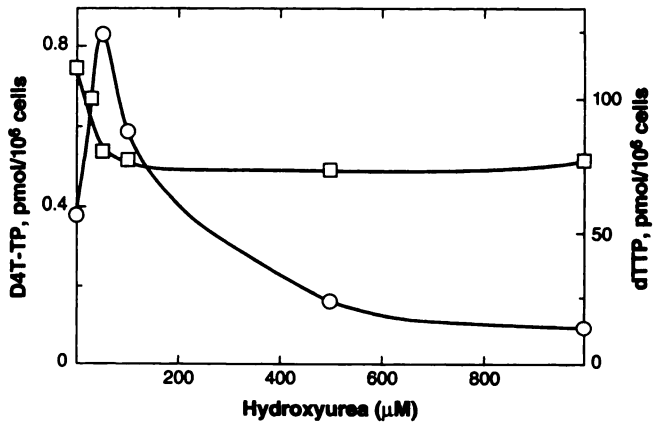

Fig. 3. Effect of HU concentration (25–1000 μM) on D4T-TP pool size and dTTP pool size in MOLT-4 cells. MOLT-4 cells (~10^6 cells/ml) were preincubated for 2 hr with HU at the concentrations indicated. ^{3}H-Labeled D4T (5 μM; 5 μCi/ml) was then added. After a 5-hr further incubation, cells were harvested and subjected to methanol extraction, and D4T-TP and dTTP levels were determined as described in Experimental Procedures. Representative values from two experiments are shown, with duplicate values varying by <5%. ○, D4T-TP (pmol/10^6 cells); □, dTTP (pmol/10^6 cells).

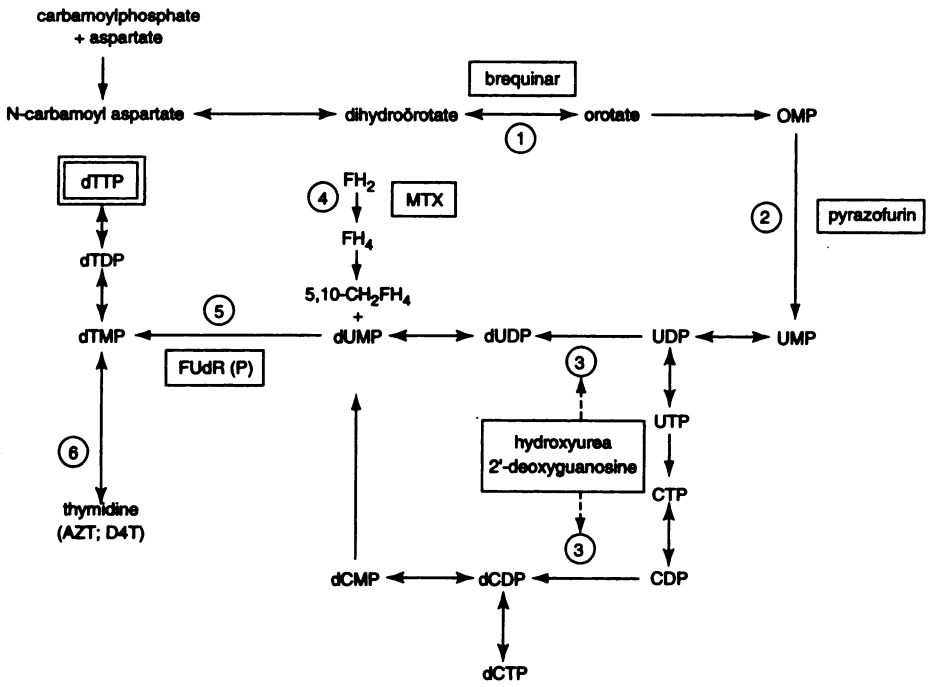

Fig. 4. Schematic representation of sites of action on the pyrimidine nucleotide biosynthetic pathway of selected modulators of dTTP pool size. *1*, Dihydroorotate dehydrogenase. *2*, OMP decarboxylase. *3*, Ribonucleotide reductase. *4*, Dihydrofolate reductase. *5*, Thymidylate synthetase. *6*, Thymidine kinase.

Discussion

The putative sites of action of the inhibitors studied on the pyrimidine nucleotide biosynthetic pathway are depicted graphically in Fig. 4. Because of their ability at higher dose levels to inhibit host cell DNA and/or RNA biosynthesis and thus to act as antitumor agents, most of these agents have been subjected to intensive prior study. Considered in decreasing order of their relative clinical significance, MTX has been established for many years as an antineoplastic agent and, because of its ability to block the formation of tetrahydrofolate cofactors, is an inhibitor of *de novo* purine biosynthesis as well as of the conversion of deoxyuridylate to thymidylate (28). FUdR, although less frequently used clinically than MTX, is an effective antitumor agent; as its 5′-monophosphate, FUdR inhibits thymidylate synthetase and thus, like MTX, blocks the conversion of deoxyuridylate to thymidylate. HU, as an inhibitor of the M2 subunit of ribonucleotide reductase, is particularly effective in lowering dATP pools, but at higher dose levels, it can act as a general inhibitor of deoxynucleotide biosynthesis; in addition to its routine use in chronic myelogenous leukemia, HU has proved to be useful in the treatment of hemoglobinopathies, particularly sickle-cell anemia (29).

Of the remaining agents examined, brequinar (30) and pyrazofurin, although intensively investigated as experimental antitumor agents, have been limited in their clinical usefulness because of toxicity. 2′-Deoxyguanosine is mainly of biochemical interest because of its ability, as its 5′-triphosphate, to act as a regulatory feedback inhibitor of the ribonucleotide reductase-catalyzed reduction of UDP and CDP, precursors of dTTP and dCTP in the *de novo* pathway (31) (see Fig. 4).

In terms of use in combination with D4T, low-dose MTX and FUdR seem to have the greatest potential applicability in view of the extensive familiarity with the pharmacological properties of these two agents in cancer chemotherapy. HU, although least toxic at the clinical level of all of the agents examined, seems to be of lesser interest in this context because of the wide intercellular variability of its effects on dNTP levels: although the formation of dTTP was inhibited in the MOLT-4 system examined here, more typical of HU in mammalian cell systems, particularly at low levels, is an increase in both dTTP and dCTP pools, with only dATP pools reproducibly decreased (23, 27), an apparent consequence of the absence of an effective alternate salvage pathway for the generation of the latter deoxynucleotide.

In terms of anti-HIV-1 activity, of possible equal significance to the increase in D4T-5′-triphosphate levels seen with agents that lower dTTP levels is the absence in the virus of either salvage or *de novo* enzyme systems for the generation of dTTP; synthesis of HIV-1 DNA at both the prereplicational and replicational stages is entirely dependent on the host cell for this and other deoxynucleotides. In resting, HIV-1-infected human PBM cells, the *in vitro* test system that probably most closely resembles the mixture of cell types harboring the virus *in vivo*, we observed the level of dTTP to vary considerably among individual donors but to average ~4.1 ± 0.1 μM (25), slightly below the K_m of dTTP for HIV-1 reverse transcriptase (9, 32). Thus, because the dTTP level is already below half-saturation of the enzyme in untreated PBM cells, further, even modest, modulator-induced decreases will have a nondiscriminatory inhibitory effect on replication of wild-type and mutant virus alike, entirely dependent as both are on host-cell dNTPs. The potentiation of D4T by agents that restrict dTTP pools, like the potentiation of 2′,3′-dideoxyinosine by agents that restrict dATP pools (26), is thus an example of inhibition of HIV-1 replication resulting from appropriate manipulation of host cell as well as viral enzyme activities. Of probable greater importance, because the antiviral activity of D4T, dideoxyinosine, and other ddNs depends not solely on the absolute level of the active 5′-triphosphate generated but rather on the ddNTP/dNTP ratio, agents that have the double action of inhibiting dNTP formation while simultaneously increasing ddNTP levels thus have greater potential utility than do agents affecting only one of these factors (27). The K_m of D4T-TP for HIV-1 reverse

transcriptase is ~0.01 μM (9, 32) (i.e., ~500-fold the affinity of dTTP). Thus, for example, raising the level of D4T-TP by ~5-fold while simultaneously lowering the level of dTTP by 3-fold through the use of modulators would exert an overall 15-fold favorable change in the ddNTP/dNTP ratio and the consequent anti-HIV effect of D4T as a reverse transcriptase inhibitor and viral DNA chain terminator without any change in the administered dosage of the latter compound. In these respects, the FUdR/D4T or HU/dideoxyinosine (26) combinations differ from conventional anti-HIV drug monotherapy directed solely at viral-specific enzymes such as reverse transcriptase or HIV protease, in which inhibition of wild-type virus results in a selective advantage to the otherwise inefficient replication of resistant strains (33–35). Particularly when combined with dideoxynucleosides, whose action is specifically potentiated by depletion of dNTP pools, inhibition of thymidylate synthetase, dihydrofolate reductase, ribonucleotide reductase, and other host-cell enzymes participating in synthesis of the dNTPs essential for HIV DNA synthesis and, therefore, HIV replication offers a useful and possibly superior alternative to existing anti-HIV drug therapy.

Acknowledgments

We thank Sandra Taubenkibel for excellent secretarial assistance.

References

1. Lin, T.-S., R. F. Schinazi, and W. N. Prusoff. Potent and selective *in vitro* activity of 3′-deoxythymidin-2′-ene (2′-deoxy-2′:3′-didehydrothymidine) against human immunodeficiency virus. *Biochem. Pharmacol.* **36**:2713–2718 (1987).
2. Hamamoto, Y., H. Nakashima, T. Matsui, A. Matsuda, T. Ueda, and N. Yamamoto. Inhibitory effect of 2′:3′-didehydro-2′,3′-dideoxynucleosides on infectivity, cytopathic effects, and replication of human immunodeficiency virus. *Antimicrob. Agents Chemother.* **31**:907–910 (1987).
3. Baba, M, R. Pauwels, P. Herdewijn, E. De Clercq, J. Desmyter, and M. Vandeputte. Both 2′:3′-dideoxythymidine and its 2′,3′-unsaturated derivative (2′,3′-dideoxythymidinene) are potent and selective inhibitors of human immunodeficiency virus replication *in vitro*. *Biochem. Biophys. Res. Commun.* **142**:128–134 (1987).
4. Balzarini, J., G.-J. Kang, M. Dalal, P. Herdewijn, E. De Clercq, S. Broder, and D. G. Johns. The anti-HTLV (anti-HIV) and cytotoxic activity of 2′:3′-didehydro-2′,3′-dideoxyribonucleosides: a comparison with their parental 2′,3′-dideoxyribonucleosides. *Mol. Pharmacol.* **32**:162–167 (1987).
5. Matthes, E., C. Lehmann, D. Scholz, M. von Janta-Lipinski, K. Gaertner, H. A. Rosenthal, and P. Langen. Inhibition of HIV-associated reverse transcriptase by sugar-modified derivatives of thymidine 5′-triphosphate in comparison to cellular DNA polymerases α and β. *Biochem. Biophys. Res. Commun.* **148**:78–85 (1987).
6. August, E. M., M. E. Marongiu, T.-S. Lin, and W. N. Prusoff. Initial studies on the cellular pharmacology of 3′-deoxythymidine-2′-ene(d4T): a potent and selective inhibitor of human immunodeficiency virus. *Biochem. Pharmacol.* **37**:4419–4422 (1988).
7. Ho, H.-T., and M. J. M. Hitchcock. Cellular pharmacology of 2′,3′-dideoxy-2′,3′-didehydrothymidine, a nucleoside analog active against human immunodeficency virus. *Antimicrob. Agents Chemother.* **33**:844–849 (1989).
8. Balzarini, J., P. Herdewijn, and E. De Clercq. Differential patterns of intracellular metabolism of 2′:3′-didehydro-2′,3′-dideoxythymidine and 3′azido-2′,3′-dideoxythymidine, two potent anti-human immunodeficiency virus compounds. *J. Biol. Chem.* **264**:6127–6133 (1989).
9. Mansuri, M. M., M. J. M. Hitchcock, R. A. Buroker, C. L. Bregman, I. Ghazzouli, J. V. Desiderio, J. E. Starrett, R. Z. Sterzycki, and J. C. Martin. Comparison of biological activities *in vitro* and toxicity *in vivo* of three thymidine analogues (D4T, FddT and AZT) active against HIV. *Antimicrob. Agents Chemother.* **34**:637–641 (1990).
10. Baba, M., R. Pauwels, J. Balzarini, P. Herdewijn, E. De Clercq, and J. Desmyter. Ribavirin antagonizes inhibitory effects of pyrimidine 2′:3′-dideoxynucleosides but enhances inhibitory effects of purine 2′,3′-dideoxynucleosides on replication of human immunodeficiency virus *in vitro*. *Antimicrob. Agents Chemother.* **31**:1613–1617 (1987).
11. Marongiu, M. E., E. M. August, and W. H. Prusoff. Effect of 3′-deoxythymidin-2′-ene(d4T) on nucleoside metabolism in H9 cells. *Biochem. Pharmacol.* **39**:1523–1528 (1990).
12. Hitchcock, M. J. M.: 2′,3′-Didehydro-2′,3′-dideoxythymidine (D4T), an anti-HIV agent. *Antiviral Chem. Chemother.* **2**:125–132 (1991).
13. Eriksson, S., B. Kierdaszuk, B. Munch-Petersen, B. Öberg, and N. G. Johansson. Comparison of the substrate specificities of human thymidine kinase 1 and 2 and deoxycytidine kinase toward antiviral and cytostatic nucleoside analogs. *Biochem. Biophys. Res. Commun.* **176**:586–592 (1991).
14. Munch-Petersen, B., L. Cloos, G. Tyrsted, and S. Eriksson. Diverging substrate specificity of pure human thymidine kinases 1 and 2 against antiviral dideoxynucleosides. *J. Biol. Chem.* **266**:9032–9038 (1991).
15. Furman, P. A., J. A. Fyfe, M. H. St. Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **83**:8333–8337 (1986).
16. Bresnick, E., and R. J. Karjala. End-product inhibition of thymidine kinase activity in normal and leukemic human leukocytes. *Cancer Res.* **24**:841–846 (1964).
17. Black, M. E., and D. E. Hruby. A single amino acid substitution abolishes feedback inhibition of vaccinia virus thymidine kinase. *J. Biol. Chem.* **267**:9743–9748 (1992).
18. Gao, W.-Y., D. G. Johns, G. Ahluwalia, and H. Mitsuya. Enhancement of the anti-HIV-1 activity of 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T) by the thymidylate synthetase inhibitor FUdR: an anti-HIV strategy with a host-cell target. *AIDS Res. Human Retroviruses* **11**:S161 (1995).
19. Shirasaka, T., R. Yarchoan, M. C. O'Brien, R. N. Husson, B. D. Anderson, E. Kojima, T. Shimada, S. Broder, and H. Mitsuya. Changes in drug sensitivity of human immunodeficiency virus type 1 during therapy with azidothymidine, dideoxycytidine, and dideoxyinosine: an in vitro comparative study. *Proc. Natl. Acad. Sci. USA* **90**:562–566 (1993).
20. Masood, R., G. S. Ahluwalia, D. A. Cooney, A. Fridland, V. E. Marquez, J. S. Driscoll, Z. Hao, H. Mitsuya, C.-F. Perno, S. Broder, and D. G. Johns. 2′-Fluoro-2′:3′-dideoxyarabinosyladenine, a metabolically stable analogue of the antiretroviral agent 2′,3′-dideoxyadenosine. *Mol. Pharmacol.* **37**: 590–596 (1990).
21. Garrett, C., and D. V. Santi. A rapid and sensitive high pressure liquid chromatography assay for deoxyribonucleoside triphosphates in cell extracts. *Anal. Biochem.* **99**:268–273 (1979).
22. Törnevik, Y., B. Ullman, J. Balzarini, B. Wahren, and S. Eriksson. Cytotoxicity of 3′-azido-3′-deoxythymidine correlates with 3′-azidothymidine-5′-monophosphate (AZTMP) levels, whereas anti-human immunodeficiency virus (HIV) activity correlates with 3′azidothymidine-5′-triphosphate (AZTTP) levels in cultured CEM T-lymphoblastoid cells. *Biochem. Pharmacol.* **49**:829–837 (1995).
23. Bianchi, V., E. Pontis, and P. Reichard. Changes of deoxyribonucleoside triphosphate pools induced by hydroxyurea and their relation to DNA synthesis. *J. Biol. Chem.* **261**:16037–16042 (1986).
24. Lowe, J. K., L. Brox, and J. F. Henderson. Consequences of inhibition of guanine nucleotide synthesis by mycophenolic acid and virazole. *Cancer Res.* **37**:736–743 (1977).
25. Gao, W.-Y., T. Shirasaka, D. G. Johns, S. Broder, and H. Mitsuya. Differential phosphorylation of azidothymidine, dideoxycytidine, and dideoxyinosine in resting and activated peripheral blood mononuclear cells. *J. Clin. Invest.* **91**:2326–2333 (1993).
26. Gao, W.-Y., D. G. Johns, and H. Mitsuya. Anti-human immunodeficiency virus type 1 activity of hydroxyurea in combination with 2′,3′-dideoxynucleosides. *Mol. Pharmacol.* **46**:767–772 (1994).
27. Gao, W.-Y., D. G. Johns, S. Chokekijchai, and H. Mitsuya. Disparate actions of hydroxyurea in potentiation of purine and pyrimidine 2′:3′-dideoxynucleoside activities against replication of human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **92**:8333–8337 (1995).
28. Allegra, C. J., K. Hoang, G. C. Yeh, J. C. Drake, and J. Baram. Evidence for direct inhibition of *de novo* purine synthesis in human MCF-7 breast cells as a principal mode of metabolic inhibition by methotrexate *J. Biol. Chem.* **262**:13520–13526 (1987).
29. Donehower, R. C. Hydroxyurea, in *Cancer Chemotherapy: Principles and Practice* (B. A. Chabner and J. M. Collins, eds.). J.B. Lippincott, Philadelphia, 225–233 (1990).
30. Maroun, J., J. Ruckdeschel, R. Natale, R. Morgan, B. Dallaire, R. Sisk, and J. Gyves. Multicenter phase II study of brequinar sodium in patients with advanced lung cancer. *Cancer Chemother. Pharmacol.* **32**:64–66 (1993).
31. Lowe, J. K., and G. B. Grindey. Inhibition of growth rate and deoxynucleoside triphosphate concentrations in cultured leukemia L1210 cells. *Mol. Pharmacol.* **12**:177–184 (1976).
32. North, T. W., R. C. Cronn, K. M. Remington, and R. T. Tandberg. Direct comparisons of inhibitor sensitivities of reverse transcriptases from feline and human immunodeficiency viruses. *Antimicrob. Agents Chemother.* **34**:1505–1507 (1990).
33. Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, M. S. Saag, and G. M. Shaw. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature (Lond.)* **373**:117–122 (1995).
34. Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature (Lond.)* **373**:123–126 (1995).
35. Coffin, J. M. HIV population dynamics *in vivo*: implications for genetic variation, pathogenesis and therapy. *Science (Washington D. C.)* **267**:483–489 (1995).

Send reprint requests to: Dr. David G. Johns, Laboratory of Medicinal Chemistry, Basic Sciences Division, Building 37, Room 5B22, NCI, NIH, Bethesda, MD 20892-4255.