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EFFECTOR STUDIES OF 3′-AZIDOTHYMIDINE NUCLEOTIDES WITH HUMAN RIBONUCLEOTIDE REDUCTASE

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Abstract—The 5′-mono-, di- and triphosphate derivatives (N_3dTMP, N_3dTDP and N_3dTTP respectively) of 3′-azidothymidine (N_3dThd), a new drug for the treatment of the acquired immune deficiency syndrome (AIDS), were synthesized. The abilities of these analog nucleotides to mimic the effector properties of the corresponding thymidine nucleotides with human ribonucleotide reductase were studied. Surprisingly, the mode of inhibition of CDP reduction by dTTP and dTDP was found to be competitive versus CDP. The K_i values were 22 and 78 μM respectively. Inhibition by N_3dTTP and N_3dTDP was considerably weaker, with K_i values of 1200 and 550 μM. Neither dTMP nor N_3dTMP produced significant inhibition at concentrations up to 500 μM. dTTP was an essential activator for GDP reduction. In the presence of the accessory activator, ATP, the activation constant for dTTP was 7.8 μM. N_3dTTP was neither an activator of GDP reduction nor an inhibitor of the activation by dTTP. In view of the intracellular concentrations of these analog nucleotides reached after incubations with N_3dThd [Furman *et al.*, *Proc. natn. Acad. Sci. U.S.A.* **83**, 8333 (1986)] and the weakness of their interactions with ribonucleotide reductase, it is unlikely that the antiviral or toxic effects of N_3dThd can be attributed to direct effects on this enzyme. The possible indirect effects caused by alterations in the pools of the natural effectors are discussed.

Azidothymidine (BW A509U, Retrovir®), 3′-azido-2′,3′-dideoxythymidine (N_3dThd†), is currently being developed for treatment of the acquired immune deficiency syndrome (AIDS) [1]. Its clinical efficacy is probably due to its antiviral properties. Studies *in vitro* [2] have demonstrated that submicromolar concentrations of N_3dThd inhibit the replication of the causative agent, human immunodeficiency virus (HIV). The chemotherapeutic target appears to be the HIV reverse transcriptase [3]. N_3dThd is metabolically converted by cellular enzymes to mono-, di- and triphosphate azido-analogs of the respective thymidine nucleotides [3]. N_3dTTP effectively competes with dTTP for binding to reverse transcriptase with a K_i of 0.04 μM compared to the K_m of 2.8 μM for dTTP [3]. Moreover, incorporation of N_3dTMP into DNA causes chain termination of DNA synthesis.‡ Host cells are relatively resistant to N_3dThd because the affinity of N_3dTTP for cellular DNA polymerase α is weaker by at least two orders of magnitude [3].

Although the mechanism of action of N_3dThd is likely to be related to the interaction of N_3dTTP with the viral reverse transcriptase, it is important to know whether the azidonucleotide analogs affect other key enzymes involved in DNA synthesis. In this respect, ribonucleotide reductase is an obvious candidate since it catalyzes the rate-limiting production of the deoxynucleotide precursors of DNA. Furthermore, ribonucleotide reductase is highly regulated by dTTP, which is a feedback inhibitor of pyrimidine ribonucleotide reduction and an essential activator of guanine ribonucleotide reduction (reviewed in Refs. 4–7). The interactions of partially purified human ribonucleotide reductase with dTMP, dTDP, dTTP and the respective azido-analogs are presented herein.

MATERIALS AND METHODS

Reagents. ATP and ultrapure deoxynucleoside triphosphates were obtained from Pharmacia Biochemicals, [U-^{14}C]CDP (450–547 Ci/mol) from New England Nuclear, and [8-^{14}C]GDP (60 Ci/mol) from Amersham. Both radiolabeled nucleotides were purified by ion exchange chromatography as previously described [8]. Alkaline phosphatase from *Escherichia coli* and tributylammonium pyrophosphate were purchased from Sigma. Other reagents used in the synthesis of the nucleotides of N_3dThd were the highest grade available from Aldrich. The sources for other materials are listed elsewhere [8].

N_3dTMP, N_3dTDP and N_3dTTP were synthesized according to the method previously described for preparing deoxynucleoside triphosphate analogs [9, 10]. Several modifications were incorporated. N_3dThd (1.5 mmol) was dissolved in 4 ml of trimethylphosphate and 1 ml of triethylamine with stirring at −10° (ice/methanol). Phosphorus oxychloride (3.1 mmol) was added to the mixture, which was stirred for 15 min at −10°, incubated for 18 hr at −20°, and then neutralized with 15 ml of 0.5 M

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† Abbreviations: N_3, 3′-azido-3′-deoxy-; HIV, human immunodeficiency virus; DTT, dithiothreitol; and Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

‡ M. H. St. Clair, C. A. Richards, T. Spector, K. J. Weinhold, W. H. Miller, A. L. Langlois and P. A. Furman, manuscript submitted for publication.

triethylamine in water. The resulting N_3dTMP was purified as described below with a 73% yield.

N_3dTMP was converted to the triethylammonium salt from the ammonium salt by dissolving the powder in 0.5 M triethylammonium bicarbonate and evaporating the solution to dryness two times. This product (0.84 mmol) was further dried by evaporating twice from acetonitrile. It was then dissolved in 5 ml hexamethylphosphoramide and mixed with 4.2 mmol of 1,1-carbonyldiimidazole and stirred for 18 hr at room temperature. Methanol (0.6 ml) was added and, after 1 hr of stirring, 4.2 mmol of a 0.5 M solution of tributylammonium pyrophosphate (dissolved in hexamethylphosphoramide) were added and stirred for an additional 2 hr. The mixture was neutralized with 50 ml of 50 mM ammonium bicarbonate, and N_3dTDP and N_3dTTP were purified from this mixture as described below. The yields (relative to starting N_3dThd) were 1% and 42% respectively.

The 5'-mono-, di- and triphosphate derivatives of N_3dThd were purified by chromatography on DEAE Sephadex A-25 with a gradient of 50 to 500 mM ammonium bicarbonate. Excess ammonium bicarbonate was removed by evaporation *in vacuo* at 35°. Further desalting and final purification were accomplished by preparative reverse phase HPLC chromatography (Alltech RSIL C18). All three compounds, isolated as the ammonium salt, were >97% pure as assessed by anion-exchange HPLC, base/phosphate ratios, ultraviolet absorption spectra and cleavage by alkaline phosphatase to N_3dThd.

Ribonucleotide reductase. Human ribonucleotide reductase was purified from Detroit 98 cells (human sternal marrow) by the method previously described [11]. Product formation was linear with respect to time for at least 20 min at 37° (with and without inhibitors). The enzyme preparation had a ratio of ribonucleotide reductase to nucleoside diphosphate kinase >4:1.

Ribonucleotide reductase assays. The assays for CDP reduction were performed as previously described [12] with some minor modifications. The 0.05 ml reaction mixture contained the indicated concentrations of [^{14}C]CDP (130 Ci/mol) and inhibitor, 5 mM ATP, 6 mM $MgCl_2$, 5 mM DTT, 100 mM sodium Hepes, pH 7.4, and enzyme. A new assay was developed for GDP reduction. The reaction mixtures (0.02 ml) contained 100 mM sodium Hepes, pH 7.4, 50 μM [^{14}C]GDP (60 Ci/mol), 5 mM DTT, 4 mM $MgCl_2$, 2 mM ATP, enzyme and the indicated amounts of dTTP or N_3dTTP. These reaction mixtures were incubated for 20 min at 37° and then terminated by the addition of 5 μl of 100 mM hydroxyurea and 60 mM EDTA. A reaction with the enzyme added after the stopping reagent served as the blank. The samples were heated at 100° for 4 min. The nucleosides were then converted to nucleosides by incubating the mixture at 37° for 30 min with 5 μl (0.5 I.U.) of alkaline phosphatase. Denatured protein was removed by centrifugation, and guanosine and deoxyguanosine carriers (0.5 μmol) were added to 10 μl of the supernatant fraction. The mixture was then spotted on polyethyleneimine cellulose u.v.-impregnated sheets that had been converted to the borate form [13]. The plates were developed in 0.1 M borate for 1 hr. The guanosine and deoxyguanosine spots (R_f values of 0.07 and 0.5 respectively) were located by u.v. illumination, cut out, and quantitated by liquid scintillation counting.

Blank values (about 0.03 and 0.5% of the total radioactivity for CDP and GDP reduction respectively) were subtracted from reaction rates. Between 0.5 and 10% of the substrates were converted to products during the course of the reactions. All velocities are expressed as arbitrary numbers.

Miscellaneous. Kinetic constants were calculated from initial velocities using the computer programs described by Cleland [14]. Data from reactions containing inhibitors were tested for conformity to the common models of enzyme inhibition as previously described [15]. The concentrations of ATP and Mg^{2+} in the free and complexed forms were calculated by the method of Storer and Cornish-Bowden [16].

RESULTS

Inhibition of CDP reduction. The data of Fig. 1A and 1B, demonstrate that, compared to dTTP and dTDP, N_3dTTP and N_3dTDP were considerably weaker inhibitors of human ribonucleotide reductase. These data are also presented in the form of Dixon plots [17] in Fig. 2, A and B. However, in order to quantitate the inhibition constants, the mechanism of inhibition must be determined. Therefore, detailed analyses of the inhibition by dTTP and dTDP with respect to CDP as the variable substrate were performed. Statistical analysis [15] of these data (Fig. 3, A and B) showed that the inhibition was strictly competitive for both inhibitors. These experi-

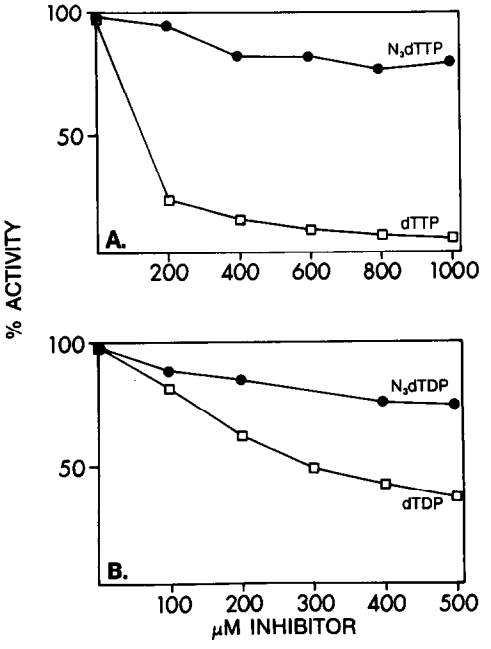

Fig. 1. Inhibition of human ribonucleotide reductase by Thd and N_3dThd nucleotides. Velocities were measured in the presence of the indicated concentrations of dThd and N_3dThd nucleoside triphosphates (A) and diphosphates (B). The standard reaction conditions were used with 5 μM [^{14}C]CDP.

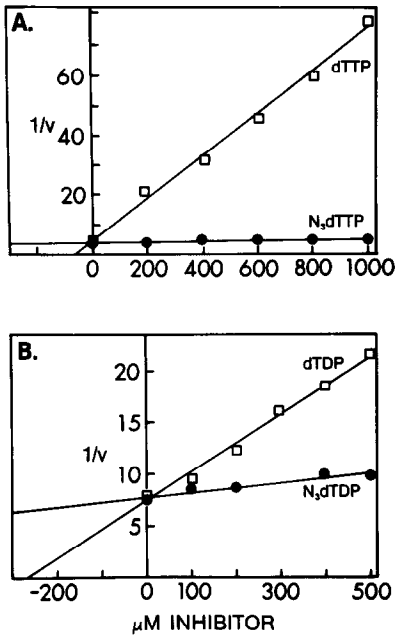

Fig. 2. Dixon replots of the inhibition of human ribonucleotide reductase by dThd and N_3dThd nucleotides. The data of Fig. 1 are presented for dThd and N_3dThd nucleoside triphosphate (A) and diphosphates (B).

ments were repeated with ribonucleotide reductase purified from different batches of Detroit 98 cells and with newly purchased dTTP and dTDP, which were >99 and 94% homogeneous (respectively), as assessed by HPLC. The competitive K_i values for replicate determinations were 20 ± 3.0, 28 ± 4.6, and $20 \pm 4.4\ \mu M$ for dTTP, and 75 ± 12 and $74 \pm 13\ \mu M$ for dTDP. The average K_m for CDP reduction (five determinations) was 2.4 ± 0.17 (S.D.) μM with 8.4 to 20% SE on individual determinations. Thus, K_i values were also calculated from the x-axis intercepts of the Dixon plots according to the competitive inhibition model [17]. Values of $21 \pm 4.1\ \mu M$ for dTTP, $84 \pm 6.9\ \mu M$ for dTDP, $1200 \pm 340\ \mu M$ for N_3dTTP, and $550 \pm 190\ \mu M$ for N_3dTDP were obtained.

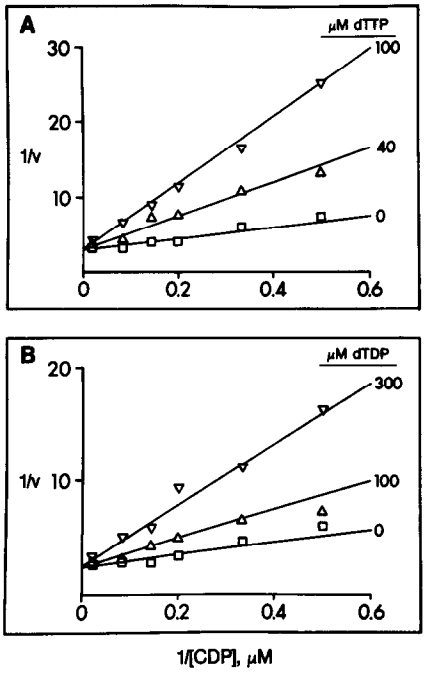

Fig. 3. Competitive inhibition of human ribonucleotide reductase by dTTP (A) and dTDP (B). Data are fit to the competitive inhibition model. Constants are reported in the text.

The inhibition of CDP reduction by dTTP was also assessed with the concentration of CDP fixed at $50\ \mu M$ and that of ATP-Mg^{2+} varied. This proved to be a very complicated experiment because both free ATP and free Mg^{2+} (that not in the ATP–Mg^{2+} complex) competed with the complex and inhibited the reaction. Because the inhibition by free ATP was stronger than that by free Mg^{2+}, it was necessary to hold the concentration of free Mg^{2+} to a constant minimum level (0.5 mM) in excess over ATP and dTTP. This maintained the level of free ATP between 0.01 and 0.05 mM and minimized the inhibition by free Mg^{2+} and free ATP. The resulting pattern of inhibition was noncompetitive (data not shown), with K_{is} and K_{ii} values of 160 ± 75 and $360 \pm 110\ \mu M$ respectively. The activation constant (concentration of ATP producing half-maximum activation of CDP reduction) for ATP was 0.48 ± 0.14 (SD) mM for three determinations. The values of these constants are similar to those previously determined by others (reviewed in Ref. 6).

Neither dTMP nor N_3dTMP produced significant inhibition when tested at concentrations up to $500\ \mu M$ with the initial concentration of CDP at $5\ \mu M$.

Activation of GDP reduction. The abilities of dTTP and N_3dTTP to activate ribonucleotide reductase to reduce GDP were studied. In agreement with other investigations [18–20], dTTP was found to be an essential activator and ATP to be an accessory activator. No GDP reduction was detected in the absence of dTTP. Furthermore, ATP, which did not support GDP reduction alone, increased the maximum velocity in the presence of dTTP by about 3-fold. The data of Fig. 4 show the effect of increasing concentrations of dTTP and N_3dTTP on the rate of GDP reduction in the presence of 2 mM ATP. The activation constant (concentration of activator producing half-maximum activation) for dTTP was $7.8 \pm 0.51\ \mu M$. N_3dTTP did not support GDP reduction significantly.

N_3dTTP was also tested as an inhibitor of GDP reduction with the concentrations of GDP, ATP and $MgCl_2$ identical to those described in Fig. 4. Less than 15% inhibition was observed with the concentration of dTTP fixed at $10\ \mu M$ and that of N_3dTTP varied from 50 to $500\ \mu M$.

DISCUSSION

The abilities of the nucleotide metabolites of N_3dThd to mimic the effector properties of thymidine nucleotides with human ribonucleotide reductase were studied. In order to predict their

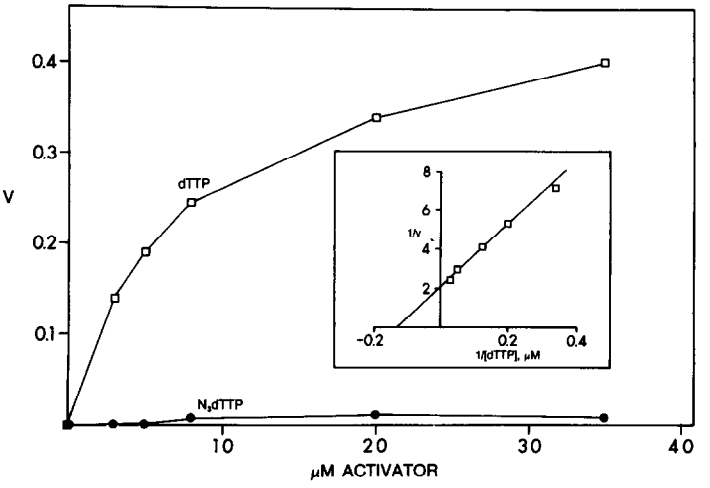

Fig. 4. Activation of GDP reduction by dTTP and N_3dTTP. The concentrations of [^{14}C]GDP, ATP and Mg^{2+} were 50 μM, 2 mM and 3.5 mM respectively. The concentrations of the activators are indicated in the figure. Insert: double-reciprocal replot of the activation vs the concentration of dTTP. The apparent activation constant for dTTP was 7.8 ± 0.51 μM.

potential to produce effects *in vivo*, it is important to compare the ratio of the intracellular concentrations of the analogs to their kinetic constants. A recent study demonstrated that human T-lymphocytes metabolically convert N_3dThd to approximately 1 mM N_3dTMP and 2–8 μM N_3dTDP and N_3dTTP [3]. Therefore, concerning the inhibition of CDP reduction, these concentrations are considerably lower than the inhibition constants of the analog nucleotides. Moreover, N_3dTTP neither activated nor inhibited GDP reduction. Therefore, it is highly unlikely that any of the antiviral or toxic effects of N_3dThd are related to direct interactions of its nucleotide metabolites with ribonucleotide reductase. However, because N_3dThd causes profound perturbations in the pools of the natural effectors [3], it is important to consider the indirect effects.

Furman *et al.* [3] found that the incubation of either HIV-infected or uninfected cells with 50 μM N_3dThd caused the dTTP pool to decrease from 45 to 2.5 μM. In addition, the dCTP and dGTP pools decreased by approximately 95 and 50%, respectively, whereas the dATP pool increased by 300%. While dATP is a general feedback inhibitor of ribonucleotide reduction, dTTP is a specific inhibitor of pyrimidine ribonucleotide reduction (reviewed in Refs. 4–7). Our finding that the inhibition by dTTP was competitive with respect to CDP is surprising in light of the common dogma that the inhibition is allosteric. Although it is possible that the competitive inhibition patterns result from mutually exclusive binding of dTTP and CDP at allosteric sites, this is not an expected mechanism for an allosteric regulator because it allows for the reversal of inhibition by the substrate. A search of the literature did not reveal other inhibition studies with CDP as the varied ligand. However, the claim of Eriksson *et al.* [19] that inhibition by dTTP is not evident at high concentrations of CDP is consistent with our data. The present finding of noncompetitive inhibition versus the activator, ATP, is in agreement with other studies [20] and is also compatible with competitive inhibition versus CDP. Due to the technical complexity of the studies, where the concentration of ATP is varied (see Results), and the likelihood that saturating CDP would increase the apparent K_i values [21], the value obtained versus CDP (ATP saturating) is probably a more representative indicator of the inhibitory potency of dTTP. This value of 22 μM is also close to the intracellular concentration of dTTP [3, 22]. Thus, regulation of CDP reduction by dTTP should be sensitive to small fluctuations in the concentration of dTTP. However, because the pool of dCTP in N_3dThd-treated cells did not increase when that of dTTP decreased, it appears that the dCTP pool was modulated by other means. It is possible that the reduction of CDP was inhibited by the increased pool of dATP or that the salvage pathway was inhibited. Another less likely explanation is that the pool of dTDP had increased to an inhibitory level. Although dTDP also inhibits CDP reduction (this study and Cory *et al.* [23]), it is weaker than dTTP and is unlikely to accumulate [3].

Finally, the N_3dThd-related decrease in the dGTP pool could have been caused by the depletion of dTTP to levels that were significantly below the concentration of its activation constant of 7.8 μM. Other investigators have found similar values for this constant (reviewed in Ref. 6).

Note added in proof: A recent unpublished reinvestigation by L. W. Frick, D. J. Nelson and P. A. Furman discovered that a technical problem had resulted in erroneous determinations of the dCTP pool sizes reported earlier [3]. It is now clear that N_3dThd caused the dCTP pool to increase rather than decrease. This increase is consistent with the above predicted effect of a lowered dTTP pool on human ribonucleotide reductase.

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