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Interaction of Deoxyguanosine Nucleotide Analogs with Human Telomerase[1]

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ABSTRACT

To maintain the telomeres at the ends of the chromosomes, telomerase in human cells adds a repeating sequence of nucleotides (TTAGGG) to the 3′-end of each chromosome using an RNA component of the enzyme as the template for DNA synthesis. Because of the selective expression of this enzyme in cancer cells, we have evaluated the interaction of human telomerase with several deoxyguanosine nucleotides of clinical importance. 2′,3′-dideoxyguanosine 5′-triphosphate, 6-thio-2′-deoxyguanosine 5′-triphosphate (T-dGTP), carbovir 5′-triphosphate, and D-carbocyclic-2′-deoxyguanosine 5′-triphosphate (D-CdG-TP) inhibited telomerase activity by 50% when these analogs were present at only 2 to 9 times the dGTP concentration. The L-enantiomer of CdG-TP was far less inhibitory, thereby demonstrating the stereoselectivity of telomerase for nucleotide substrates. T-dGTP was incorporated into the DNA by telomerase in the absence of dGTP, but unlike dGTP there was little extension of the DNA chain after its incorporation. These results indicate that the metabolites of three clinically useful agents (6-mercaptopurine, 6-thioguanine, and Abacavir) can inhibit human telomerase activity, and it is possible that the effect of these nucleotides on telomerase activity or telomere function could contribute to the mechanism of action of these agents.

Telomerase is the DNA polymerase that is responsible for the maintenance of telomeres at the ends of the chromosomes. This enzyme is functionally a reverse transcriptase, and its active site has recently been shown to be related to that of other reverse transcriptases (Lingner et al., 1997). Because telomerase activity is present in tumor cells but not in most somatic cells, it has been suggested that this enzyme would be a good target for antitumor drug development (Morin, 1995; Parkinson, 1996; Sharma et al., 1997). Furthermore, inhibition of this activity by antiviral nucleoside analogs could result in toxicity to normal cells that express telomerase. It is possible that the metabolites of some clinically useful nucleoside analogs could interfere with telomerase activity and contribute to either their therapeutic activity or toxicity.

Numerous nucleotide analogs (ddGTP, ddATP, ddTTP, 3′-deoxy-2′,3′-didehydrothymidine 5′-triphosphate, 3′-azido-3′-deoxythymidine 5′-triphosphate, 7-deaza-dATP, 7-deaza-dGTP, arabinofuranosyl-guanine 5′-triphosphate, and 2′-fluoro-5-methyl-arabinofuranosyl uracil 5′-triphosphate) have previously been shown to inhibit telomerase activity (Morin, 1989; Strahl and Blackburn, 1994, 1996; Chen et al., 1995; Fletcher et al., 1996; Pai et al., 1998). In this work, we have studied the interaction of five deoxyguanosine nucleotide analogs, 6-thio-2′-deoxyguanosine 5′-triphosphate (T-dGTP), 5′-triphosphate of carbovir (CBV-TP), ddGTP, D-carbocyclic-2′-deoxyguanosine 5′-triphosphate (D-CdG-TP), and L-carbocyclic-2′-deoxyguanosine 5′-triphosphate (L-CdG-TP), with telomerase isolated from human cells to increase our understanding of the substrate requirements of this important enzyme. T-dGTP is the active metabolite of both 6-mercaptopurine and 6-thioguanine, which are two drugs used in the treatment of acute leukemias (Elion, 1989), CBV-TP is the active metabolite of Abacavir, an agent that has recently been approved for the treatment of AIDS (Foster and Faulds, 1998), and D-CdG-TP is the active metabolite of D-CdG, an agent with activity against herpes simplex virus, cytomegalovirus, and hepatitis-B virus (Bennett et al., 1993). Because T-dGTP, D-CdG-TP, and L-CdG-TP have a 3′-hydroxyl, extension of the DNA chain after the incorporation of one of these nucleotide analogs is possible. Therefore, in addition to inhibition studies, the ability of the human telomerase enzyme to incorporate these analogs into DNA in the absence of dGTP was also measured.

Experimental Procedures

Materials. L-CdG-TP, D-CdG-TP, and CBV-TP were obtained from Sierra Bioresearch (Tucson, AR). T-dGTP was obtained from

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ABBREVIATIONS: T-dGTP, 6-thio-2′-deoxyguanosine 5′-triphosphate; CBV-TP, 5′-triphosphate of carbovir; T-dGMP, 6-thio-2′-deoxyguanosine 5′-monophosphate; L-CdG-TP, L-carbocyclic-2′-deoxyguanosine 5′-triphosphate; D-CdG-TP, D-carbocyclic-2′-deoxyguanosine 5′-triphosphate; PMSF, phenylmethylsulfonyl fluoride.

Dr. Jonathan Maybaum at the University of Michigan, Ann Arbor, MI. dGTP, dATP, dTTP, and ddGTP were purchased from Pharmacia Biotech (Piscataway, NJ). Leupeptin was purchased from Calbiochem (La Jolla, CA), pepstatin A from Calbiochem or Boehringer Mannheim (Indianapolis, IN), proteinase K from Boehringer Mannheim, RNase A from Sigma Chemical Co. (St. Louis, MO), and RNasin from Promega (Madison, WI).

Preparation of S100 Cell Extracts. Extracts were prepared from either CEM or HeLa cells. CEM cells obtained from the American Type Culture Collection (Rockville, MD) were grown as described (Parker et al., 1997). The extraction procedure used for CEM cells has been used with modifications for a variety of cell types (Counter et al., 1992; Nilsson et al., 1994). PBS-washed CEM cells (10^8-10^9 cells) were resuspended in 2.5 cell volumes of buffer [10 mM HEPES, 3 mM KCl, 1 mM dithiothreitol, 1 mM $MgCl_2$, 100 μM phenylmethylsulfonyl fluoride (PMSF), 10 μM pepstatin A, 5 μM leupeptin, 10 U/ml RNasin] and homogenized 15 times on ice in a 7-ml Dounce homogenizer with pestle B. The homogenate was incubated on ice for 30 min, and then spun 10 min at 5°C at 13,500g_{av} (12,000 rpm) in a Beckman SW50.1 rotor. NaCl was added to the supernatant to a final concentration of 0.1 M, and it was spun at 5°C for 1 h at 100,000g_{av} (38,000 rpm) in a Beckman Ti70.1 rotor. Glycerol was added to the supernatant (S100) to a final concentration of 20% v/v. Extraction from HeLa cells (obtained from the National Cell Culture Center, Minneapolis, MN) was similar to the procedure used for CEM cells, but followed a procedure designed specifically for HeLa cells (Morin, 1989) with minor modifications. Refrigerated or frozen PBS-washed HeLa cells (1–1.5 × 10^9) were suspended in 5 ml of lysis buffer per 10^9 cells and incubated on ice for 10 min. Our lysis buffer also contained 5 μM pepstatin A, 5 μM leupeptin, and 10 U/ml RNasin. Cells were homogenized and then centrifuged for 20 min at 5°C in a cold Beckman Ti70.1 rotor at 8000g_{av} (11,000 rpm) to pellet the nuclear extract. The addition of high salt buffer and 100,000g_{av} centrifugation were as described (Morin, 1989), except for an increase in centrifugation time to 2 h and the use of a Beckman SW 50.1 rotor. The supernatant, S100 extract, was dialyzed versus two 250-ml portions of dialysis buffer to which we added 0.2 mM EGTA, 1 μM pepstatin A, 1 μM leupeptin, and 1 U/ml RNasin—dialysis first overnight then 2 to 4 h after buffer change. After dialysis, the S100 cell extract was centrifuged for 30 min at 5°C in a Beckman Ti70.1 rotor at 15,000g_{av} (15,000 rpm) and the precipitate was discarded. Pepstatin A, leupeptin, RNasin, and PMSF were added to the supernatant to final concentrations of 10 μM, 5 μM, 10 U/ml, and 100 μM, respectively. The CEM and HeLa cell extracts were aliquoted, frozen on dry ice, stored at −70°C, and were used within 7 months.

Standard Telomerase Assay. Telomerase activity in 20 μl of S100 cell extract was assayed in a final reaction volume of 40 μl. Reaction components were as specified by Counter et al. (1992) except for the addition of 1 mM EGTA and the following concentration changes: 2 mM $MgCl_2$, 1.25 μM [α^{32}P]-dGTP (40 μCi, 800 Ci/mmol) (ICN, Costa Mesa, CA), and 2 μM oligonucleotide primer $(TTAGGG)_3$ (Genosys Biotechnologies, Inc., The Woodlands, TX). Tubes were incubated at 30°C for the desired time, and the reactions were stopped by adding 0.1 μg/μl RNase A and 10.6 mM EDTA (final concentrations). After incubation at 37°C for 15 min, proteinase K (0.4 μg/μl) and SDS (0.2%, w/v) were added to each sample, and the samples were incubated for 15 min at 37°C (45 μl total volume). The unincorporated radioactivity was removed from each sample by centrifugation (MicroSpin G-25 column; Pharmacia Biotech Inc., Piscataway, NJ). The samples were extracted with 25:24:1 phenol/chloroform/isoamyl alcohol (pH 7.9, Tris-saturated). tRNA (50 μg; Sigma Chemical Co.) was added to each sample, and the nucleic acids were precipitated twice with ethanol (67% ethanol and 0.67 M ammonium acetate). The precipitated nucleic acids were washed with 70% ethanol, resuspended in electrophoresis loading buffer (80% v/v formamide, 10% w/v sucrose, 8.9 mM Tris-borate, 1 mM EDTA, 0.02% w/v bromophenol blue), heated to 100°C, cooled on ice, and analyzed by electrophoresis on a 10% (w/v) polyacrylamide gel containing 6.7 M urea (35 cm × 42.5 cm × 0.4 mm; 1.5 h at 80 W constant power). Autoradiographs were 1- to 2-week exposures of Kodak X-OMAT AR film with a DuPont Cronex Lightning Plus intensifying screen at −70°C.

Inhibition by dGTP Analogs. Telomerase extract in assay buffer was incubated with 1.25 μM [^{32}P]dGTP and seven to nine concentrations of one of the nucleotide analogs (ddGTP, T-dGTP, CBV-TP, L-CdG-TP, or D-CdG-TP). The mixtures were incubated for 45 min at 30°C, and the incorporation of [^{32}P]dGMP into DNA was determined as described above. Using the autoradiographs, each sample, which corresponded to one analog concentration, was visually ranked as showing no inhibition, some inhibition, substantial inhibition, or complete inhibition. Allowance was made for low recovery when indicated by an added internal standard [prelabeled $(TTAGGG)_2$] or by the low-molecular-weight nontelomerase product bands. An approximate IC_{50} was determined for each experiment by averaging the log of the lowest analog concentration with inhibition and the log of the highest analog concentration with less than complete inhibition.

Incorporation of dGTP Analogs into DNA. The assays were done as described above except that radiolabeled dATP (2.5 μM [α^{32}P]-dATP, 80 μCi, 800 Ci/mmol) (ICN, Costa Mesa, CA) was used instead of dGTP, and $MgCl_2$ and dTTP concentrations were both reduced to 0.5 mM. Cold dGTP or dGTP analogs (ddGTP, T-dGTP, CBV-TP, L-CdG-TP, or D-CdG-TP) were added at 0.25 mM after an initial measurement showed identical results with 0.1, 0.25, and 0.5 mM. The assay solutions were incubated for 2 h at 30°C. Product purification and imaging was identical with that given above except that films were exposed for at least 2 weeks to compensate for the lower incorporation of radiolabeled dATP. Results were visually assessed.

Results

Characterization of Baseline Telomerase Activity (data not shown). S100 from both CEM and HeLa cell lines showed telomerase activity with the characteristic primer extension banding pattern on the autoradiographs. RNase A and proteinase K pretreatment confirmed both the protein and RNA dependence of the activity. The complement $(CCCTAA)_3$ showed no primer extension above background and $(GGGTTA)_2$ showed a banding pattern shifted three base pairs from that of $(TTAGGG)_2$.

Inhibition by dGTP Analogs. Representative gels from inhibition experiments with T-dGTP and CBV-TP are shown in Figs. 1 and 2, respectively. The 45-min incubation time was chosen for the inhibition experiments because the overall rate of incorporation of label under the experimental conditions in the absence of inhibitor was determined to be increasing over time between 0 and 60 min. The approximate IC_{50} values for the five analogs are shown in Table 1, where they are listed in order of their effectiveness as telomerase inhibitors: ddGTP ≥ CBV-TP ≥ T-dGTP ≥ D-CdG-TP ≫ L-CdG-TP. The inhibitory effects of ddGTP, CBV-TP, T-dGTP, and D-CdG-TP were similar with approximate IC_{50} values ranging from 2 to 9 times the concentration of dGTP. L-CdG-TP was dramatically less inhibitory than its enantiomer with an IC_{50} greater than 64 times the experimental concentration of dGTP, which indicated that telomerase could distinguish between D and L enantiomers of nucleotide substrates. These results support the observation of Pai et al. (1998) with the D and L enantiomers of 2′-fluoro-5-methyl-arabinofuranosyl uracil 5′-triphosphate.

Incorporation of dGTP Analogs into DNA. Figure 3 shows the results from one of our three incorporation exper-

iments. We chose to use radiolabeled dATP rather than dTTP in the analog incorporation experiment, because, as reported previously for extracts from human embryonic kidney 293 cell line (Fletcher et al., 1996), we were unable to detect the characteristic telomerase banding pattern in the presence of limiting radioactive dTTP. If telomerase is accurate in its nucleotide additions, then the smallest visible product when using labeled dATP should be a 21 mer (primer + TTA). A light band was seen in the 21-mer position in the absence of added dGTP and analog (negative control, lane 1) and only in the presence of S100 cell extract. Under these same conditions, a light 22-mer band was also seen. Because the 22nd nucleotide should be dGTP, any products larger than 21 mers in the absence of added dGTP or dGTP analog probably result from synthesis using endogenous dGTP in the crude extract or an alternative nucleotide: TTP or dATP. The samples with dGTP showed the characteristic laddering with bands at six nucleotide intervals above the 26 mer (lanes 8 and 9). As observed by Fletcher et al. (1996), excess dTTP and dGTP and limiting dATP shifted the pause site to the second thymine.

Our results clearly indicated that T-dGTP was a substrate for human telomerase activity (lane 7), although it was used less efficiently than dGTP. More bands were seen with T-dGTP than with any of the other dGTP analogs tested. Bands corresponding to a 21 mer, 22 mer, 23 mer, and 24 mer were clearly visible in the presence of T-dGTP with the 23-mer band being most intense. There was no evidence of any higher molecular weight products, which indicated that the T-dGTP is not an equivalent alternative substrate to dGTP, and that two to three incorporations of 6-thio-2′-deoxyguanosine 5′-monophosphate (T-dGMP) seem to lead to dissociation of telomerase from the DNA primer. This result does not necessarily indicate that telomerase cannot extend primers past the incorporation of three T-dGMPs, but may only indicate that the enzyme dissociates from the DNA primer after the incorporation of the T-dGMP. In cell-free experiments, once the enzyme has dissociated from the primer, the excess of starting primer does not favor reassociation with a partially extended primer. Even though the DNA bands created in the presence of T-dGTP only contained one molecule of radiolabeled dAMP per DNA molecule, they were much more intense than the higher-molecular-weight bands formed in the presence of dGTP, which contained more molecules of radiolabeled dAMP per DNA molecule. Because

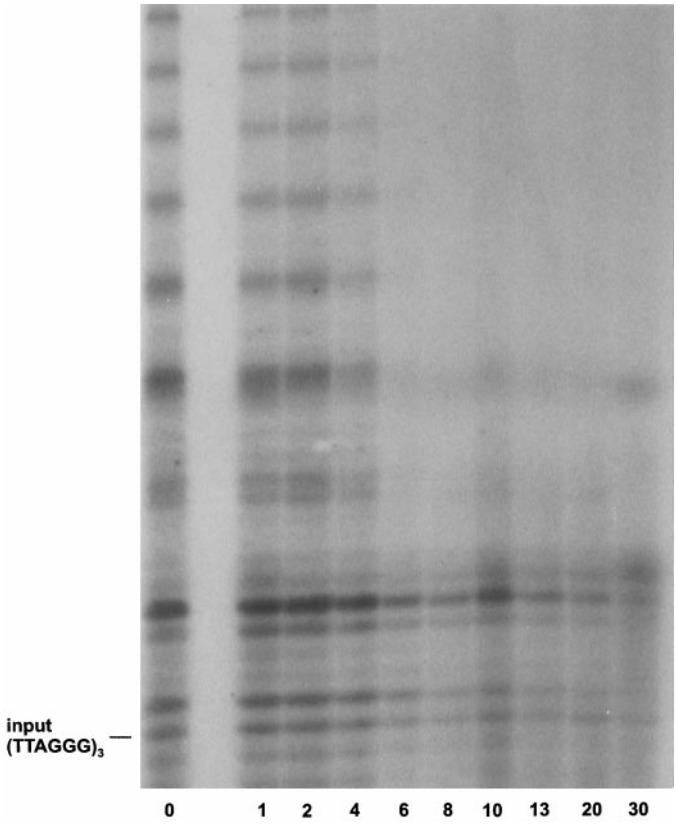

Fig. 1. Inhibition of telomerase activity by T-dGTP. Telomerase activity was measured in HeLa cell extract in the presence of 2 μM $(TTAGGG)_3$, 1.25 μM [α^{32}P]-dGTP, 2 mM dTTP, 2 mM dATP, and increasing amounts of T-dGTP (0, 1, 2, 4, 6, 8, 10, 13, 20, and 30 μM T-dGTP).

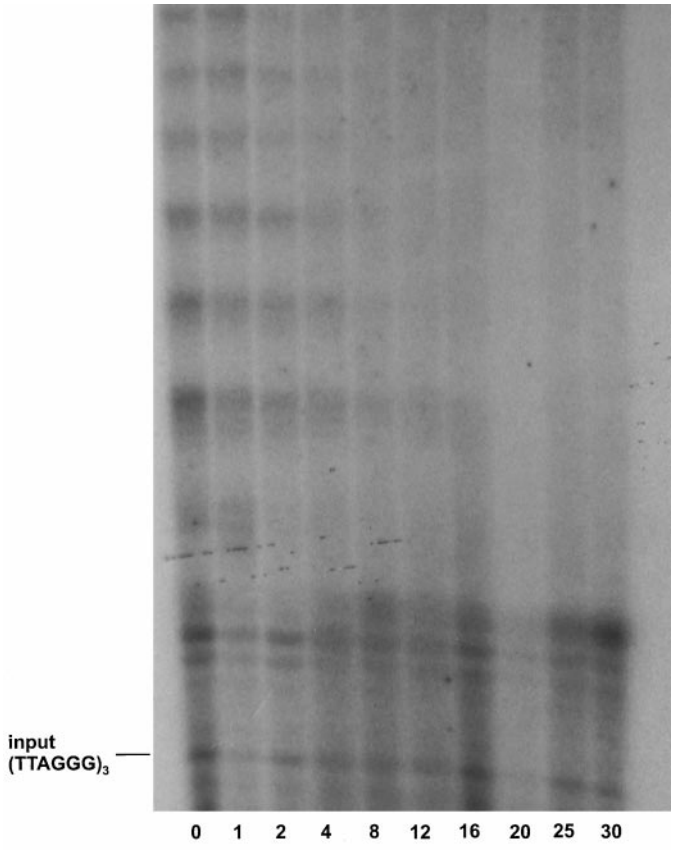

Fig. 2. Inhibition of telomerase activity by CBV-TP. Telomerase activity was measured in HeLa cell extract in the presence of 2 μM $(TTAGGG)_3$, 1.25 μM [α^{32}P]-dGTP, 2 mM dTTP, 2 mM dATP, and increasing amounts of CBV-TP (0, 1, 2, 4, 8, 12, 16, 20, 25, and 30 μM CBV-TP).

TABLE 1
Inhibition of telomerase activity by deoxyguanosine nucleotide analogs

Analog	IC_{50}[a]
	μM
ddGTP	3 ± 2
CBV-TP	6 ± 1.5
T-dGTP	8 ± 2
D-CdG-TP	11 ± 2
L-CdG-TP	80–160[b]

[a] IC_{50} values shown (mean ± S.D.) were averages of three to four determinations in the presence of 1.25 μM dGTP. Results from both cell lines were included in the averages.
[b] One of the three determinations for L-CdG-TP only yielded a lower limit.

of the differences in the specific activities of these products, smaller molecular products displaying similar intensities actually represent many more molecules. Therefore, the intensity of the T-dGTP products suggested that telomerase extended many primers using T-dGTP and that the primary difference between dGTP and T-dGTP was the effect of T-dGTP on the processivity of the telomerase enzyme. In other words, telomerase continues synthesis on the original primer with dGTP, whereas telomerase dissociates from the original primer and initiates DNA synthesis on another primer when the substrate is T-dGTP.

Use of the other nucleotides by telomerase was less clear. With D-CdG-TP, the banding pattern (lane 5) was not different from that in the control lane (lane 1), but we consistently observed that the intensity of the DNA bands that were 21 and 22 nucleotides long (lane 5) was greater than that in the control lane. Because the first addition of a guanine nucleotide to the primer DNA would be at position 22, these results suggested that telomerase was able to incorporate one D-CdG nucleotide, but that it was not able to add another D-CdG nucleotide after the incorporation of the first. The increased intensity of these bands suggests that the telomerase dissociated from the DNA primer and reinitiated on a new primer.

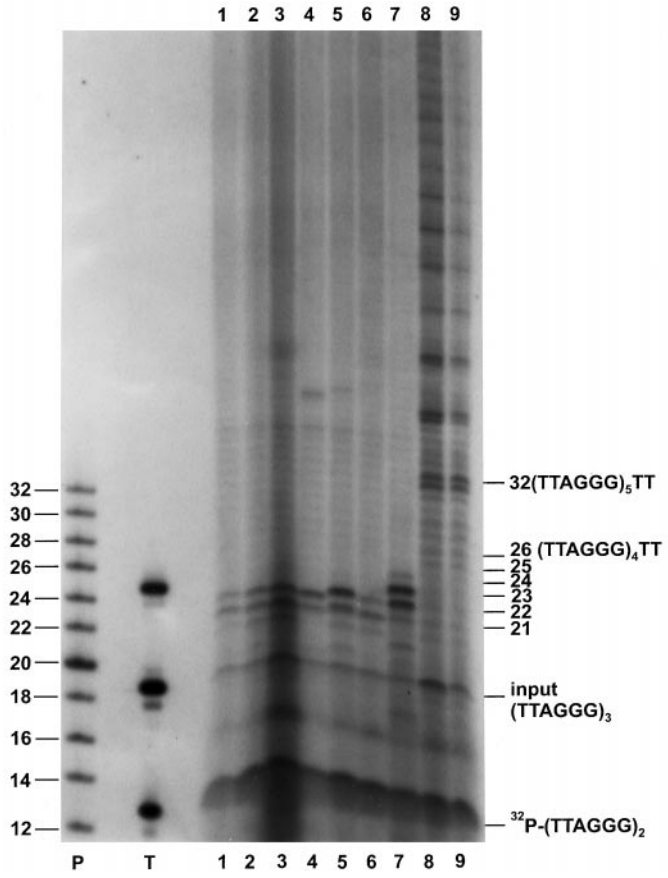

Fig. 3. Use by telomerase of dGTP analogs as substrates. Telomerase activity was measured in the presence of 2.5 μM [α^{32}P]-dATP, 0.5 mM dTTP, 2 μM $(TTAGGG)_3$, and the following: no dGTP or dGTP analog (Lane 1), 18 μM CBV-TP (Lane 2), 250 μM CBV-TP (Lane 3), 250 μM ddGTP (Lane 4), 250 μM D-CdG-TP (Lane 5), 250 μM L-CdG-TP (Lane 6), 250 μM T-dGTP (Lane 7), or 250 μM dGTP (Lanes 8 and 9). ^{32}P-labeled oligonucleotide sizing markers from Pharmacia Biotech (Piscataway, NJ) are in Lane P. Lane T is a ^{32}P-labeled mixture of $(TTAGGG)_2$, $(TTAGGG)_3$, and $(TTAGGG)_4$. This photographed gel was from one of three incorporation experiments.

In contrast there was very little, if any, incorporation of L-CdG-TP into the DNA product by human telomerase (lane 6). With L-CdG-TP, we observed bands in the same position and having similar intensity to the no dGTP/no analog negative control bands.

We consistently saw a slight increase over background in the intensity of the DNA of chain length of 22 nucleotides with CBV-TP (Fig. 3, lanes 2 and 3) and ddGTP (lane 4), but it was very low. Therefore, it is not possible from these results to unequivocally determine whether or not these two compounds were substrates for the human telomerase. Morin (1989) and Strahl and Blackburn (1996) did not detect the incorporation of ddGMP into DNA by human telomerase.

Discussion

Our results confirmed the previously reported inhibitory effect of ddGTP on telomerase activity and indicated that CBV-TP, T-dGTP, and D-CdG-TP were also inhibitors of human telomerase activity. The IC_{50} values for inhibition of telomerase activity by these nucleotides were similar to the concentration of dGTP used in the assay, which indicated that the affinities of these nucleotides were similar to that for the natural substrate, dGTP. Because two of the nucleotides studied in this work are formed from agents that are currently used in the treatment of human diseases, the interaction of human telomerase with these nucleotides could have clinical significance.

6-Mercaptopurine and 6-thioguanine are metabolized to T-dGTP in human cells, and it is believed that the incorporation of T-dGMP into DNA is responsible for the antitumor activity of these agents (Tidd and Paterson, 1974; Nelson et al., 1975; Elion, 1989). T-dGTP is a good substrate for the DNA polymerases involved in DNA replication and once incorporated into the newly synthesized DNA chain, these DNA polymerases are able to add new nucleotides past the incorporation of T-dGMP (Yoshida et al., 1979; Ling et al., 1991). Although T-dGTP competes with dGTP for incorporation into DNA by DNA polymerases, it is not an inhibitor of DNA synthesis. Considerable effort has been extended to understand the consequences of the incorporation of T-dGMP into DNA (Maybaum et al., 1987; Iwaniec et al., 1991; Ling et al., 1992; Swann et al., 1996; Uribe-Luna et al., 1997; Krynetskaia et al., 1999), but the action that results in toxicity is still not clearly defined. Our data indicated that T-dGTP is also a substrate for the human telomerase and suggested that T-dGMP could be incorporated into the telomeres of tumor cells in patients treated with either 6-mercaptopurine or 6-thioguanine.

Telomeric DNA is believed to form G-tetrads (Sundquist and Klug, 1989; Williamson et al., 1989; Williamson, 1994), and the substitution of 2′-deoxyguanosine by 6-thio-2′-deoxyguanosine in G-rich oligodeoxyribonucleotides has been shown to inhibit the formation of G-tetrad structures in DNA (Rao et al., 1995). It is possible that the incorporation of T-dGMP into these structures in a cell could interfere with G-tetrad formation, which could result in disruption of telomere function. Others have shown that the inhibition of telomerase activity in rapidly proliferating cells does not result in the immediate inhibition of cell growth (Strahl and Blackburn, 1996). However, it is possible that the disruption

of telomere function could have a more immediate impact on cell viability than an inhibition of telomere synthesis.

Abacavir is one of the most efficacious of the nucleoside analogs when given as a single agent and has recently been approved for the treatment of AIDS (Foster and Faulds, 1998). The active form of Abacavir is CBV-TP (Daluge et al., 1997; Faletto et al., 1997), which is a substrate for the HIV reverse transcriptase and causes DNA chain termination due to the lack of 3′-OH (Parker et al., 1991). Our results indicate that CBV-TP is an inhibitor of human telomerase activity, which supports the observation of Yegorov et al. (1996) that indicated that treatment of mouse embryonic fibroblasts with carbovir inhibited telomerase activity. Others have shown that the inhibition of telomerase activity in proliferating cells does not result in the immediate inhibition of cell growth, but it does result in shortening of the telomeres that eventually (after about 20 generations) results in cell death (Parkinson, 1996). Because anti-HIV agents must be given over the remaining life span of the patients, it is possible that the continued inhibition of telomerase activity in stem cells by Abacavir, or other anti-HIV nucleoside analogs, could eventually result in a delayed toxicity to the patient.

It is possible that the observed interactions of the metabolites of these agents with human telomerase could contribute to either their efficacy (6-thioguanine or 6-mercaptopurine) or toxicity (Abacavir or D-CdG) of these agents. Additional studies evaluating the effect of these agents in intact cells are needed to clarify the role of this enzyme in the activity of these compounds.

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