Bioorganic Chemistry **29,** 36–55 (2001)
doi:10.1006/bioo.2000.1194, available online at http://www.idealibrary.com on IDEAL®

Inhibition of Human Telomerase by 7-Deaza-2′-deoxyguanosine Nucleoside Triphosphate Analogs: Potent Inhibition by 6-Thio-7-deaza-2′-deoxyguanosine 5′-Triphosphate

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Received September 1, 2000

We have examined analogs of the previously reported 7-deaza-2′-deoxypurine nucleoside triphosphate series of human telomerase inhibitors. Two new telomerase-inhibiting nucleotides are reported: 6-methoxy-7-deaza-2′-deoxyguanosine 5′-triphosphate (OMDG-TP) and 6-thio-7-deaza-2′-deoxyguanosine 5′-triphosphate (TDG-TP). In particular, TDG-TP is a very potent inhibitor of human telomerase with an IC_{50} of 60 nM. TDG-TP can substitute for dGTP as a substrate for telomerase, but only at relatively high concentrations. Under conditions in which TDG-TP is the only available guanosine substrate, telomerase becomes nonprocessive, synthesizing short products that appear to contain only one to three TDG residues. Similarly, the less potent telomerase inhibitor OMDG-TP gives rise to short telomerase products, but less efficiently than TDG-TP. We show here that TDG-TP, and to a lesser extent OMDG-TP, can serve as substrates for both templated (Klenow exo) and nontemplated (terminal transferase) DNA polymerases. For either polymerase, the products arising from TDG-TP are relatively short, and give rise to bands of unusual mobility under PAGE conditions. These anomalous bands revert, under treatment with DTT, to normal mobility bands, indicating that these products may contain thiol-labile disulfide linkages involving the incorporated TDG residues. This observation of potential TDG-crosslinks may have bearing on the mechanism of telomerase inhibition by this nucleotide analog.

Telomeres are structures located at the ends of chromosomes consisting of long tandem repeats of a DNA sequence and associated proteins (*1*,*2*). These “caps” function to protect the ends of the chromosomes from unwanted degradation or from end-to-end fusion events (*3*). During replication, DNA polymerase fails to replicate the termini of one strand of DNA. This is the so-called “end replication” problem of

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0045-2068/01 $35.00

telomeres (*4,5*), and it has been hypothesized that cellular replication-driven telomere shortening is a mitotic clock controlling cellular senescence (*6,7*).

In humans, the telomeric DNA sequence consists of hundreds to a few thousand repeats of the sequence d(TTAGGG). The telomeric DNA is primarily double-stranded; however, there is a conserved single-stranded 3′-G-rich overhang, which in humans averages more than 150 bases in length (*8,9*). The G-rich, single-stranded telomeric DNA has been hypothesized to form interesting secondary structures. It has been demonstrated *in vitro* that under physiological ionic conditions, G-rich DNA sequences can form G-quadruplex structures. G-quadruplexes are built up of G-tetrads, which are cyclic planar arrays of four guanine residues stabilized through hydrogen bonding involving the Hoogsteen purine face (*10–14*). A wide variety of inter- and intrastrand G-quadruplex structures are known, and it has been hypothesized that these G-quadruplex structures may be important in the function of telomeres (*15*). Many proteins bind preferentially to G-quadruplex structures (*16*), including a yeast nuclease that recognizes and cleaves only G-quartets. When this enzymatic activity is deleted, telomere shortening and cellular senescence occur in yeast (*17–19*).

Telomere shortening associated with cellular replication must be overcome for cells to become immortal. While alternative mechanisms appear to exist (*20,21*), 85–90% of cancer cells depend on the enzyme telomerase to overcome this critical shortening of the telomere. Telomerase is a eukaryotic-specific reverse transcriptase (*22*) that carries its own RNA template (*23*). This ribonucleoprotein elongates the G-rich telomeric DNA on the ends of the chromosomes (*1*). Blackburn and coworkers have proposed a model of telomerase function that includes (a) telomerase binding to the 3′ overhang of the telomere through the association of its complementary RNA sequence; (b) addition of nucleotides to the 3′ DNA end by telomerase utilizing its endogenous RNA as a template; and (c) dissociation of the RNA-DNA hybrid and repositioning of the enzyme for successive rounds of DNA elongation. This last step is known as translocation. It has been postulated that G-quadruplex formation may facilitate unwinding of the DNA–RNA duplex and allow the translocation step to occur (*24–27*). While telomerase has been detected in normal somatic cells as well as immortalized cancer cells, it appears to be tightly controlled in normal cells (*28–30*). Recent results demonstrate that forced expression of telomerase in human cells extends the life of these cell lines far beyond control cells, perhaps conferring immortality (*31*). Inhibition of telomerase via peptide nucleic acids and 2′-O-MeRNA oligonucleotides in immortalized tissue cell culture results in telomere shortening and eventual cell death (*32*). It is apparent that telomerase plays an important role in cell immortality and perhaps tumorigenesis, making telomerase a potential target for the design of selective cancer therapeutics.

One method for inhibiting telomerase is through small molecule interaction with the G-quadruplex telomere structures. Stabilization of the G-quadruplex may hinder telomerase binding to the telomere primer or affect the formation of G-quadruplex structures, which may hinder enzyme translocation (*26,27,33,34*). Porphyrins (*35–37*), disubstituted amidoanthracene-9,10-dione compounds (*38–40*), substituted acridine compounds (*41*), perylene compounds (*42*), and even high concentrations of K^+ ions (*33*) have all been shown to inhibit telomerase activity to some degree through the presumed stabilization of the G-quadruplex.

A variety of other small molecules have been shown to inhibit telomerase activity including berberine (*43*), rhodacyanines (*43*), homoharringtonine (*44*), alterperylenol (*45*), and catechins (*46*). Using a combinatorial screening method, Hayakawa and coworkers identified isothiazolone derivatives as telomerase inhibitors. They hypothesize that the inhibition may be due to covalent modification of the telomerase active site (*47*).

Classical methods of affecting enzymatic reverse transcription have also proven useful in identifying telomerase inhibitors. Numerous nucleotide analogs, including ara-GTP, ddGTP, AZT-TP, ddITP, and ddTTP have been shown to inhibit *Tetrahymena* telomerase *in vitro* (*48*). Inhibition of human telomerase by ddGTP, ddATP, ddTTP, AZT-TP, 2′-deoxy-2′,3′-didehydrothymidine 5′-triphosphate, arabinofuranosyl uracil 5′-triphosphate, 2′-fluoro-5-methyl-arabinofuranosyl uracil 5′-triphosphate, carbovir 5′-triphosphate, D-carbocyclic-2′-deoxyguanosine 5′-triphosphate has been demonstrated *in vitro* (*49–52*). In cell culture, ddG, AZT, AZT-TP, and ddI cause progressive telomere shortening in various human cancer cell lines (*49,53–56*).

Our efforts to inhibit telomerase have focused on the potential role of G-quadruplex structures in telomerase and telomere function. Certain nucleotide analogs, when incorporated into telomeric DNA, might disrupt the formation of G-quadruplex structures, thereby affecting telomerase translocation or telomere function. Oligonucleotides incorporating the 7-deazaguanosine nucleotides are unable to form G-quadruplex structures (*34,57,58*). Fletcher and coworkers demonstrated the inhibitory effects of 7-deaza-2′-deoxyguanosine 5′-triphosphate (7-deaza-dGTP) and 7-deaza-2′-deoxyadenosine 5′-triphosphate (7-deaza-dATP) on human telomerase (*59*). Although they were poor substrates of telomerase, these compounds are known to be effective substrates for several other DNA polymerases (*57–61*). Using the TRAP assay, the inhibition of telomerase by 7-deaza-dGTP was also noted by Pandit and Bhattacharyya (*62*). Another nucleoside, 6-thio-2′-deoxyguanosine, when incorporated into oligonucleotides, inhibits the formation of G-quadruplex structures (*63*). Recently, 6-thio-2′-deoxyguanosine 5′-triphosphate has been shown to inhibit human telomerase (*52*). In this paper we report our studies of 2′-deoxyguanosine nucleotide analogs incorporating modification at both the 6 and 7 positions. The synthesis of these compounds and studies of their ability to inhibit and serve as substrates for human telomerase are reported. We find that one analog in particular, 6-thio-7-deaza-2′-deoxyguanosine 5′-triphosphate (TDG-TP), is a potent inhibitor of human telomerase. TDG-TP is also a weak substrate for telomerase, as well as other DNA polymerases. The observation of unusual products arising from the incorporation of TDG-TP by these polymerases may have bearing on the mechanism of telomerase inhibition by this nucleotide analog.

MATERIALS AND METHODS

Materials. Oligonucleotide primer (Genosys), dNTP's (Pharmacia), [α-^{32}P]dGTP, and [α-^{32}P]dATP (DuPont NEN), PMSF, pepstatin A, and leupeptin (Sigma) were used as purchased without purification. Telomeric primers were synthesized using the Beckman Oligo 1000 automated DNA synthesizer. Deprotected oligonucleotides were purified by either polyacrylamide gel electrophoresis or cartridge (Poly-pack cartridge, Glen Reseach). Phosphoramidites and solid supports were purchased from

Beckman. Telomerase from HeLa cells (National Cell Culture Center) was isolated using the CHAPS extraction method (*34*,*64*).

Telomerase inhibition assay. The telomerase assay (*39*) was a modification of the standard nonamplification procedure. The assay mixture (total of 20 μl) was composed of 50 mM Tris-acetate, pH 8.5, 5 mM β-mercaptoethanol, 1 mM spermidine, 1 mM $MgCl_2$, 1 mM dATP, 1 mM dTTP, 1.56 μM [α-^{32}P]dGTP (800 Ci/mmol), 1 μM oligonucleotide primer, d(TTAGGG)$_3$, and 5 μl of Chaps cell extract (15 mg/ml total protein). The reaction mixture was incubated for 1 h at 30°C and stopped by incubation for 1 min at 90°C. The [α-^{32}P]dGTP labeled telomerase products were ethanol precipitated and separated by electrophoresis on an 8% polyacrylamide sequencing gel and detected by X-ray film (Biomax MS, Kodak) autoradiography and quantification using a laser densitometer and ImageQuant software (Molecular Dynamics). The inhibition studies with the nucleotide analogues were carried out by adding varying concentrations of the analogues into the assay mixture. IC_{50} values were obtained by the method of Fletcher and coworkers (*59*). The autoradiographs were subjected to densitometric analysis and the telomerase activity in each lane was normalized to an internal standard [(TTAGGG)$_3$]. The IC_{50} values were obtained by a computer program (EZ-ED_{50} solfware, Perrella Scientific Inc.), which fits the data to a four-parameter logistic equation.

Telomerase substrate assay. The studies to determine if the nucleotide analogues are substrates for telomerase were performed by a modification of the above telomerase inhibition assay. The [α-^{32}P]dGTP was replaced with unlabeled nucleotide triphosphate analogs (e.g., TDG-TP or OMDG-TP, 0.1–2 mM) and the unlabeled dATP was replaced with 3.12 μM [α-^{32}P]dATP. A 5′-biotinylated d(TTAGGG)$_3$ primer was employed. After the telomerase reaction, the reaction mixture was incubated with 20–30 μl of streptavidin-coated beads (Dynabeads). The reaction products were immobilized on the Dynabeads according to the manufacturer's procedure. The immobilized products were washed at least five times with washing buffer [1 M NaCl and 10 mM Tris–HCl (pH 7.50)], and the reaction products were dissociated from the Dynabeads by incubating the bead reaction complex in 200 μl of 5.0 M guanidine–HCl solution at 90°C for 20 min. Following ethanol precipitation, the telomerase products were analyzed by electrophoresis on an 8% polyacrylamide denaturing gel and autoradiography, as above.

DNA polymerase assay. Reaction mixtures (20 μl) containing 200 μM each of dATP, dCTP, and dTTP, 50 mM Tris-acetate, pH 8.5, 5 mM β-mercaptoethanol, 1 mM spermidine, 1 μl (0.62 μM) [α-^{32}P]dGTP (800 Ci/mmol), 2.5 μl of the same Chaps extract used in the telomerase reactions, and the indicated amount of inhibitor. Reactions were incubated at 30°C for 1 h. Triplicate 1-μl aliquots of each reaction mixture were placed on 3 separate DE81 circular ion exchange filters (Millipore) and washed extensively with 3 M Na_2PO_4, pH 7.5. The filters were air dried and the amount of [α-^{32}P]dGTP-labeled DNA was obtained using a Beckman scintillation counter.

Terminal deoxynucleotidyl transferase (TdT) reactions. In a 30-μl reaction volume containing 0.1 M potassium cacodylate (pH 7.2) and 1 mM $MgCl_2$ was incubated 35 units TdT, 2.4 μM 5′-[^{32}P]-d(TTAGGGTTAGGGTTA) (approx. 30,000 cpm), and 80 μM dGTP, 74 μM TDG-TP, or 86 μM OMDG-TP. The solutions were incubated for the times indicated in the figures. The reactions were quenched by submersion

in boiling water for 2 min. The solutions were cooled on ice before being passed through a G-25 spin column (Pharmacia Biotech) for desalting. Samples were then dried under vacuum and dissolved in 5 μl loading dye (75% formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol). To test the effects of dithiothreitol (DTT), loading dye was prepared as above but containing 10 mM DTT. The samples were then placed in a boiling water bath for 3 min followed by cooling in an ice bath for 4 min before being loaded onto a 12% denaturing (8 M urea) polyacrylamide gel (29:1 acryl:bis). Following electrophoresis the gel was dried and placed in a phosphorimager cassette for 15 h. The image was developed in a Molecular Dynamics 445 SI phosphorimager.

DNA polymerase I large (Klenow) fragment exonuclease minus (Klenow (exo$^-$)) reactions. In a 30-μl reaction volume containing 3 μl 10x Klenow Buffer (Promega) was incubated 10 units Klenow (exo$^-$) (Promega), 2.4 μM 5′-[^{32}P]d(TTAGGGTTAGGGTTA) (approx. 30,000 cpm), 2.7 μM 5′-d[$(C)_{32}$TAACCCTAACCCTAA], and either 80 μM dGTP, 74 μM TDG-TP, or 86 μM OMDG-TP. Primer and template were allowed 30 min to anneal under assay conditions before the addition of enzyme or dNTPs. The samples were then treated as above.

Preparation of 7-deaza-2′-deoxyguanosine nucleoside analogues. 7-deaza-dGTP was purchased from Pharmacia. The noncommercially available nucleotide analogs were synthesized from the common intermediate, diTol-6-chloro-DG (2-amino-6-chloro-9-[2′-deoxy-3′,5′-di-O-(*p*-toluoyl)-β-D-erythropentofuranosyl]-7H-pyrrolo-[2,3-*d*]pyrimidine), which was prepared from 7-deazaguanine (2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-*d*]pyrimidine) as previously reported (*65*,*66*). We employed an improved, one-step synthesis of 7-deazaguanine starting material: Into a stirred solution of DMF (240 ml) and water (40 ml) at room temperature was added 2,4-diamino-6-hydroxypyrimidine (12.6 g, 0.1 mol) and NaOAc (8.2 g, 0.1 mol). After the mixture became homogeneous, 50% aqueous α-chloroacetaldehyde (15.7 g, 0.1 mol) was added all at once, and the reaction mixture was stirred further for 2 days. After evaporation of solvent under vacuum, the residue was triturated with 10 ml of water, cooled to 4°C for 16 h, and filtered. The resulting solid was purified over silica gel column chromatography (20% MeOH/$CHCl_3$) to afford 10.8 g (72% yield) 7-deazaguanine as a buff colored solid, identical to authentic material in all respects (*67*).

OMDG (2-amino-9-(2′-deoxy-β-D-erythro-pentofuranosyl)-6-methoxy-7H-pyrrolo-[2,3-*d*]pyrimidine, 6-methoxy-7-deaza-2′-deoxyguanosine) was prepared from diTol-6-chloro-DG as reported (*66*,*68*,*69*).

NMDG (2-amino-9-2′-deoxy-β-D-erythro-pentofuranosyl)-1-methyl-7H-pyrrolo-[2,3-*d*]pyrimidin-6-one, 1-*N*-methyl-7-deaza-2′-deoxyguanosine). DiTol-6-chloro-DG (332 mg, 0.637 mmol) was refluxed in 2 M NaOH (2.0 ml, 4 mmol) for 4 h. The solution was cooled to room temperature, neutralized with acetic acid, and evaporated *in vacuo*. The residue was applied to an Amberlite XAD-4 column and eluted with H_2O/*i*PrOH (9:1) (*66*). The solvent was removed to give a white solid, which was used directly for the next reaction. The solid was dissolved in 6.4 ml of 0.1 M NaOH and dimethyl sulfate (401.9 mg, 301.5 μl, 3.186 mmol) was added and the reaction was stirred for 2 h. The solvent was evaporated *in vacuo* and the residue was purified by silica gel column chromatography eluting with $CHCl_3$/MeOH (90:10) to give NMDG as a white solid (107 mg, 60% yield). ^{1}H NMR (DMSO-d_6): δ =

6.90 (d, 1H, J = 4 Hz, H-8), 6.42 (dd, 1H, J = 4,8 Hz, H-1′), 6.27 (d, 1H J = 4 Hz, H-7), 4.62 (m, 1H, H-3′), 4.08 (m, 1H, H-4′), 4.03 (m, 2H, H-5′), 3.31 (s, 3H, NMe), 2.55 (m, 1H, H-2′$_a$), 2.26 (m, 1H, H-2′$_b$); ^{13}C NMR (DMSO-d_6): δ = 158.08 (C-6), 153.26 (C-2), 148.81 (C-4), 116.89 (C-8), 102.43 (C-7), 99.10 (C-5), 86.90 (C-1′), 81.90 (C-4′), 71.04 (C-3′), 62.07 (C-5′), 39.52 (C-2′), 27.76(NMe); UV (H_2O) $\varepsilon_{284\ nm}$ = 9700.

TDG (2-amino-9-(2′-deoxy-β-D-erythro-pentofuranosyl)-7H-pyrrolo[2,3-*d*]pyrimidin-6-thione, 6-thio-7-deaza-2′-deoxyguanosine) was prepared from diTol-6-chloro-DG via diTol-TDG (2-amino-9-(2′-deoxy-3′,5′-di-O-(*p*-toluoyl)-β-D-erythro-pentofuranosyl)-7H-pyrrolo[2,3-*d*]pyrimidin-6-thione) by reported procedures (*65*,*68*,*70*).

TMDG (2-amino-9-(2′-deoxy-β-D-erythro-pentofuranosyl)-6-thiomethyl-7H-pyrrolo[2,3-*d*]pyrimidine, 6-thiomethyl-7-deaza-2′-deoxyguanosine). To a cooled solution (0°C) of diTol-TDG (*65*) (300 mg, 0.578 mmol) in 1 ml DMF was added NaH (60% in oil, 25.5 mg, 0.636 mmol). After stirring for 1 h at 0°C, dimethyl sulfate (73.0 mg, 54.7 μl, 0.578 mmol) was added, and the reaction mixture was allowed to stir 1 h. The reaction was quenched with sat. NH_4Cl (aq) and the beige solid filtered. The solid was purified by flash silica gel chromatography (0 to 5% MeOH/$CHCl_3$) to give 2-amino-9-[2′-deoxy-3′,5′-di-O-(*p*-toluoyl)-β-D-erythropentofuranosyl]-6-thiomethyl-7H-pyrrolo[2,3-*d*]pyrimidine as a white solid (221 mg, 72% yield) R_f = 0.27 ($CHCl_3$/MeOH, 95:5). A solution of 2-amino-9-[2′-deoxy-3′,5′-di-O-(*p*-toluoyl)-β-D-erythropentofuranosyl]-6-thiomethyl-7H-pyrrolo[2,3-*d*]pyrimidine (180 mg, 0.338 mmole) in 13 ml of 0.1 N sodium methoxide in methanol was stirred at room temperature for 3 h. The reaction was neutralized with 6 M HCl and evaporated to dryness. The residue was purified by flash silica gel chromatography (0 to 10% MeOH/$CHCl_3$) to give TMDG as an amorphous solid (72 mg, 72% yield), R_f = 0.27 ($CHCl_3$/MeOH, 90:10). ^{1}H NMR (DMSO-d_6): δ = 7.22 (d, 1H J = 6 Hz, H-8), 6.40 (d, 1H J = 4 Hz, H-7), 6.32 (dd, 1H J = 4,8 Hz, H-1′), 4.30 (m, 1H, H-3′), 3.78 (m, 1H, H-4′), 3.49 (m, 2H, H-5′), 2.60 (s, 3H, SMe), 2.35 (m, 1H, H-2′$_a$), 2.12 (m, 1H, H-2′$_b$); ^{13}C NMR (DMSO-d_6): δ = 165.52 (C-6), 158.04 (C-2), 153.44 (C-4), 116.59 (C-8), 102.98 (C-7), 99.37 (C-5), 82.39 (C-1′), 80.40 (C-4′), 72.35 (C-3′), 64.37 (C-5′), 35.93 (SMe), 39.37 (C-2′); UV (H_2O) $\varepsilon_{286\ nm}$ = 7100; HRMS (CI) Calcd for $C_{12}H_{17}N_4O_3S$ 297.102137; found: 297.103044.

Procedure for the preparation of nucleoside triphosphates. The preparation of triphosphates of the 7-deaza-2′-deoxyguanosine nucleoside analogues was carried out using modifications of reported procedures for converting the unprotected nucleosides directly to the triphosphate (*71*). The unprotected nucleosides (150–250 μmol) were treated, at 0°C, with $POCl_3$ (2 eq.) and water (1 eq.) in $PO(OMe)_3$ (1–3 ml). The reaction mixtures were stirred at 0°C for 1 to 4 h and then quenched by the addition of 1 M aqueous triethylammonium bicarbonate. After evaporation of the solvent *in vacuo*, the residue was purified by ion-exchange chromatography (DEAE-Sephadex A-25, 0.0 to 0.5 M triethylammonium bicarbonate). The residual triethylammonium bicarbonate was removed by evaporation with EtOH several times. The yields ranged from 78–140 μmol or roughly 52 to 73%.

The nucleoside monophosphate (70–130 μmol) was dissolved in water and applied to a cation-exchange column (Merck, pyridinium form). The column was washed

with water and tributylamine (1 eq.) was added to the eluate. The solvent was removed and residual water was removed by repeated evaporations with DMF. The residue was then dissolved in anhydrous DMF (1–2 ml) and 1,1′-carbonyldiimidazole (CDI, 5 eq.) in DMF (1 ml) was added. After stirring overnight, the excess CDI was quenched with MeOH (8 eq.) and the solution treated with bis-triethylammonium pyrophosphate (5 eq.) dissolved in DMF. The reaction mixture was allowed to stir 24 h at room temperature. The solvent was evaporated and the residue purified by ion-exchange chromatography as above. The yields ranged from 43–87 μmol or roughly 58 to 67%.

OMDG-TP (2-amino-9-(2′-deoxy-β-D-erythro-pentofuranosyl)-6-methoxy-7H-pyrrolo[2,3-*d*]pyrimidin-5′-triphosphate) was prepared from 187 μmol of unprotected nucleoside afford 72 μmol (40% overall yield) of OMDG-TP. ^{31}P NMR (D_2O): δ = −8.18 (d, $J_{P\text{-}P}$ = 6 Hz, αP), −10.61 (t, $J_{P\text{-}P}$ = 6 Hz, βP), −22.24 (d, $J_{P\text{-}P}$ = 6 Hz, γP); ^{1}H NMR (D_2O): δ = 6.98 (d, 1H J = 4 Hz, H-8), 6.25 (dd, 1H J = 4,8 Hz, H-1′), 6.19 (d, 1H J = 4 Hz, H-7), 4.47 (m, 1H, H-3′), 4.01 (m, 1H, H-4′), 3.77 (m, 2H, H-5′), 3.77 (s, 3H, Ome), 2.50 (m, 1H, H-2′$_a$), 2.33 (m, 1H, H-2′$_b$); ^{13}C NMR (D_2O): δ = 164.49 (C-6), 159.88 (C-2), 154.11 (C-4), 120.80 (C-8), 101.23 (C-7), 99.28 (C-5), 85.87 (C-4′, $J_{C\text{-}P}$ = 8.1 Hz), 83.51 (C-1′), 72.50 (C-3′), 65.44 (C-5′, $J_{C\text{-}P}$ = 4.5 Hz), 54.78 (Ome), 39.11 (C-2′); UV (H_2O) $\varepsilon_{286\ nm}$ = 14700.

NMDG-TP (2-amino-9-(2′-deoxy-β-D-erythro-pentofuranosyl)-1-methyl-7H-pyrrolo[2,3-*d*]pyrimidin-6-one-5′-triphosphate) was prepared from 210 μmol of unprotected nucleoside to afford 92 μmol (44% overall yield) of NMDG-TP. ^{31}P NMR (D_2O): δ = −9.36 (αP, d, $J_{P\text{-}P}$ = 6 Hz), −10.48 (βP, t, $J_{P\text{-}P}$ = 6 Hz), −22.19 (γP, d, $J_{P\text{-}P}$ = 6 Hz); ^{1}H NMR (D_20): δ = 6.91 (d, 1H J = 4 Hz, H-8), 6.27 (dd, 1H J = 5,8 Hz, H-1′), 6.26 (d, 1H J = 4 Hz, H-7), 4.26 (m, 1H, H-3′), 3.75 (m, 1H, H-4′), 3.53 (m, 2H, H-5′), 3.27 (s, 3H, NMe), 2.33 (m, 1H, H-2′$_a$), 2.06 (m, 1H, H-2′$_b$); ^{13}C NMR (D_2O): δ = 161.28 (C-6), 154.11 (C-2), 149.93 (C-4), 119.43 (C-8), 103.58 (C-7), 101.26 (C-5), 84.06 (C-4′, $J_{C\text{-}P}$ = 8.9 Hz), 83.62 (C-1′), 80.52 (C-3′), 65.36 (C-5′), 37.52 (C-2′), 29.11(NMe); UV (H_2O) $\varepsilon_{284\ nm}$ = 9700.

TDG-TP (2-amino-9-(2′-deoxy-β-D-erythro-pentofuranosyl)-7H-pyrrolo[2,3-*d*]-pyrimidin-6-thione-5′-triphosphate) was prepared from 172 μmol of unprotected nucleoside to afford 78 μmol (45% overall yield) of TDG-TP. ^{31}P NMR (D_2O): δ = −8.59 (αP, d, $J_{P\text{-}P}$ = 6 Hz), − 10.62 (βP, t, $J_{P\text{-}P}$ = 6 Hz), −22.31 (γP, d, $J_{P\text{-}P}$ = 6 Hz); ^{1}H NMR (D_2O): δ 7.09 (d, 1H J = 3.5 Hz, H-8), 6.41 (d, 1H J = 3.5 Hz, H-7), 6.24 (dd, 1H J = 4,8 Hz, H-1′), 4.48 (m, 1H, H-3′), 4.01 (m, 1H, H-4′), 3.83 (m, 2H, H-5′), 2.49 (m, 1H, H-2′), 2.26 (m, 1H, H-2′$_b$), ^{13}C NMR (D_2O): δ = 173.62 (C-6), 153.01 (C-2), 148.52 (C-4), 122.87 (C-8), 114.84 (C-7), 105.44 (C-5), 86.06 (C-4′, $J_{C\text{-}P}$ = 8.0 Hz), 86.00 (C-1′), 72.43 (C-3′), 65.40 (C-5′), $J_{C\text{-}P}$ = 4.4 Hz), 39.16 (C-2′); UV (H_2O) $\varepsilon_{344\ nm}$ = 18900.

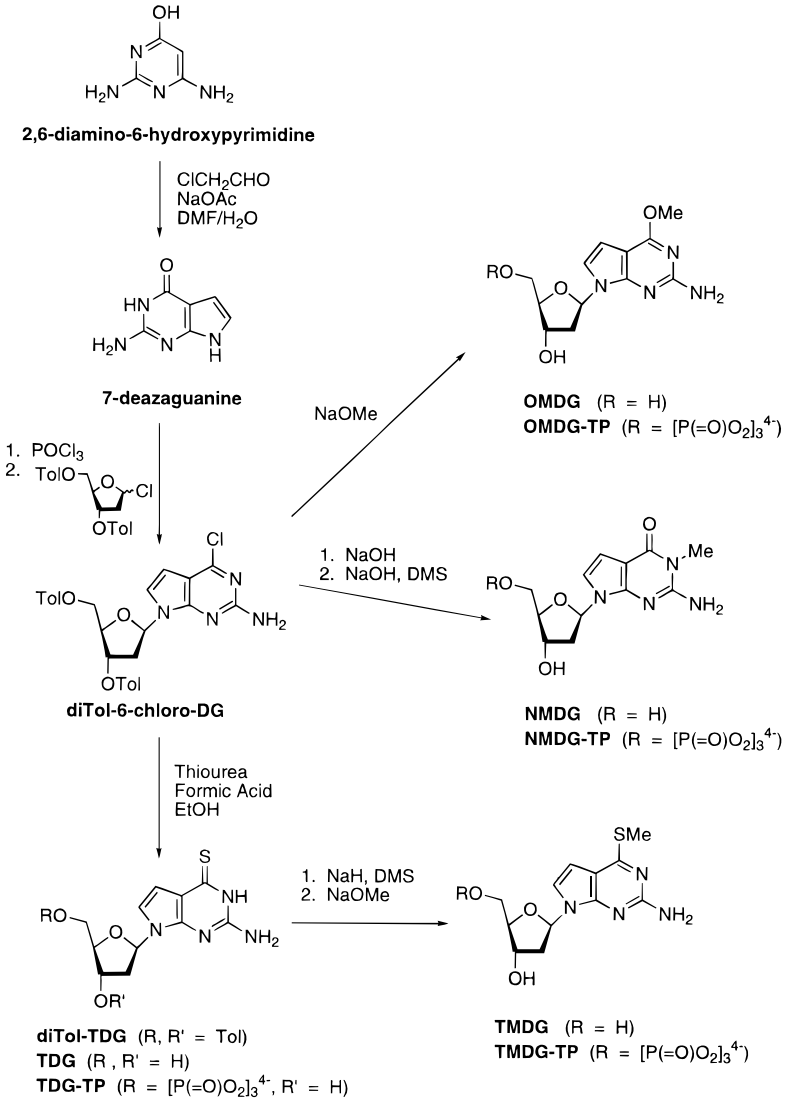
TMDG-TP (2-amino-9-(2′-deoxy-β-D-erythro-pentofuranosyl)-6-thiomethyl-7H-pyrrolo[2,3-*d*]pyrimidin-5′-triphosphate) was prepared from 204 μmol of unprotected nucleoside to afford 105 μmol (51% overall yield) of TMDG-TP. ^{31}P NMR (D_2O): δ = −9.85 (αP, d, $J_{P\text{-}P}$ = 6 Hz), −10.65 (βP, t, $J_{P\text{-}P}$ = 6 Hz), −22.54 (γP, d, $J_{P\text{-}P}$ = 6 Hz); ^{1}H NMR (D_2O): δ = 7.24 (d, 1H J = 4 Hz, H-8), 6.46 (dd, 1H J = 4,8 Hz, H-1′), 6.42 (d, 1H J = 4 Hz, H-7), 4.70 (m, 1H, H-3′), 4.16 (m, 1H, H-4′), 4.10 (m, 2H, H-5′), 2.66 (m, 1H, H-2′$_a$), 2.54 (s, 3H, SMe), 2.40 (m, 1H, H-2′$_b$); ^{13}C

NMR (D_2O): δ = 164.59 (C-6), 160.02 (C-2), 154.33 (C-4), 120.98 (C-8), 101.30 (C-7), 98.53 (C-5), 85.82 (C-4′, $J_{C\text{-}P}$ = 8.1 Hz), 83.34 (C-1′), 72.47 (C-3′), 65.58 (C-5′, $J_{C\text{-}P}$ = 4.5 Hz), 42.89 (SMe), 39.05 (C-2′); UV (H_2O) $\varepsilon_{286\ nm}$ = 7100.

RESULTS

Preparation of nucleotide analogs. In designing nucleotide analogs as potential telomerase inhibitors, we focused on guanosine analogs bearing modifications in the base positions involved in G-quadruplex formation. Fletcher and coworkers had already identified modification in the 7-position, in the form of 7-deazapurine nucleotides, as beneficial for telomerase inhibition (*59*). Our design coupled this modification with further changes at the 1- and 6-positions that we predicted would have an even more profound effect on G-quadruplex formation. We prepared a number of 7-deaza-dG analogs (Scheme 1) with further modifications at the 6-position (TDG (*65,68*)), the 1-position (NMDG), and both the 6- and 1-positions (OMDG (*66,68*) and TMDG). Using chemical synthesis, these nucleosides were converted to the corresponding 5′-triphophates (TDG-TP, NMDG-TP, OMDG-TP, and TMDG-TP) for the telomerase inhibition studies (Scheme 1).

Nucleotide analogues as telomerase inhibitors. Under the reaction conditions employed [d$(TTAGGG)_3$ primer, 1 mM each dTTP and dATP, limiting [α-^{32}P]dGTP], telomerase activity from HeLa cell Chaps extracts affords a ladder of products spaced six bases apart, corresponding to a pause site at the first guanine in the telomere repeat dTTAGGG (*59,72*). These product ladders are clearly visible in the control lanes of gels from the 7-deaza-2′-deoxyguanosine triphosphate analog inhibition studies (Fig. 1). Since telomerase contains an RNA component, RNase A treatment of the reaction mixtures results in a loss of this product ladder (data not shown). Representative gels for the telomerase inhibition by OMDG-TP and TDG-TP are shown in Figs. 1A and 1B. The intensity of these telomerase product ladders decreases with increasing concentration of the nucleotide analog inhibitors. The inhibition of telomerase was determined by quantifying the reduction of the ladder band intensity with increasing concentrations of the nucleotide analogues. The intensity of each lane was normalized with an internal standard of 5′-^{32}P-labeled $(TTAGGG)_3$ to compensate for differences in processing and gel loading (*59*), and the normalized total intensity of telomerase products in each lane was determined as a percent of that in the control lane in order to eliminate variability from film exposure and enzyme preparations. In this manner, the concentration of each nucleotide analog required to produce a 50% reduction of telomerase activity (IC_{50}) relative to the control was determined. The IC_{50} values for telomerase inhibition by the nucleotide analogs are shown in Table 1. As reported previously, the parent nucleotide analog 7-deaza-dGTP is a good inhibitor of human telomerase, with an IC_{50} value of 11 μM. We have confirmed the human telomerase inhibitory property of 7-deaza-dGTP under the exact conditions employed here (HeLa cell Chaps extract telomerase preparation) and have found that its IC_{50} is similar to the IC_{50} of cold dGTP (5 μM) in this assay (data not shown). Two of the 7-deza-dGTP analogs examined here, TMDG-TP and NMDG-TP, are much worse inhibitors of human telomerase, with IC_{50} values of 100 μM. One analog, 6-methoxy-7-deaza-2′-deoxyguanosine-5′-triphosphate (OMDG-TP) inhibits



SCHEME 1.

telomerase with an IC_{50} of 5 μM, similar to that for 7-deza-dGTP. In contrast, 6-thio-7-deaza-2′-deoxyguanosine-5′-triphosphate (TDG-TP) is an extremely potent inhibitor of telomerase, with an IC_{50} value of 0.06 μM.

Nucleotide analogues as telomerase substrates. In order to test for the ability of the two more potent telomerase inhibitors, TDG-TP and OMDG-TP, to serve as substrates for telomerase, a modified telomerase assay was employed. In this telomerase substrate experiment, a limiting concentration (3.12 μM) of [α-^{32}P]dATP

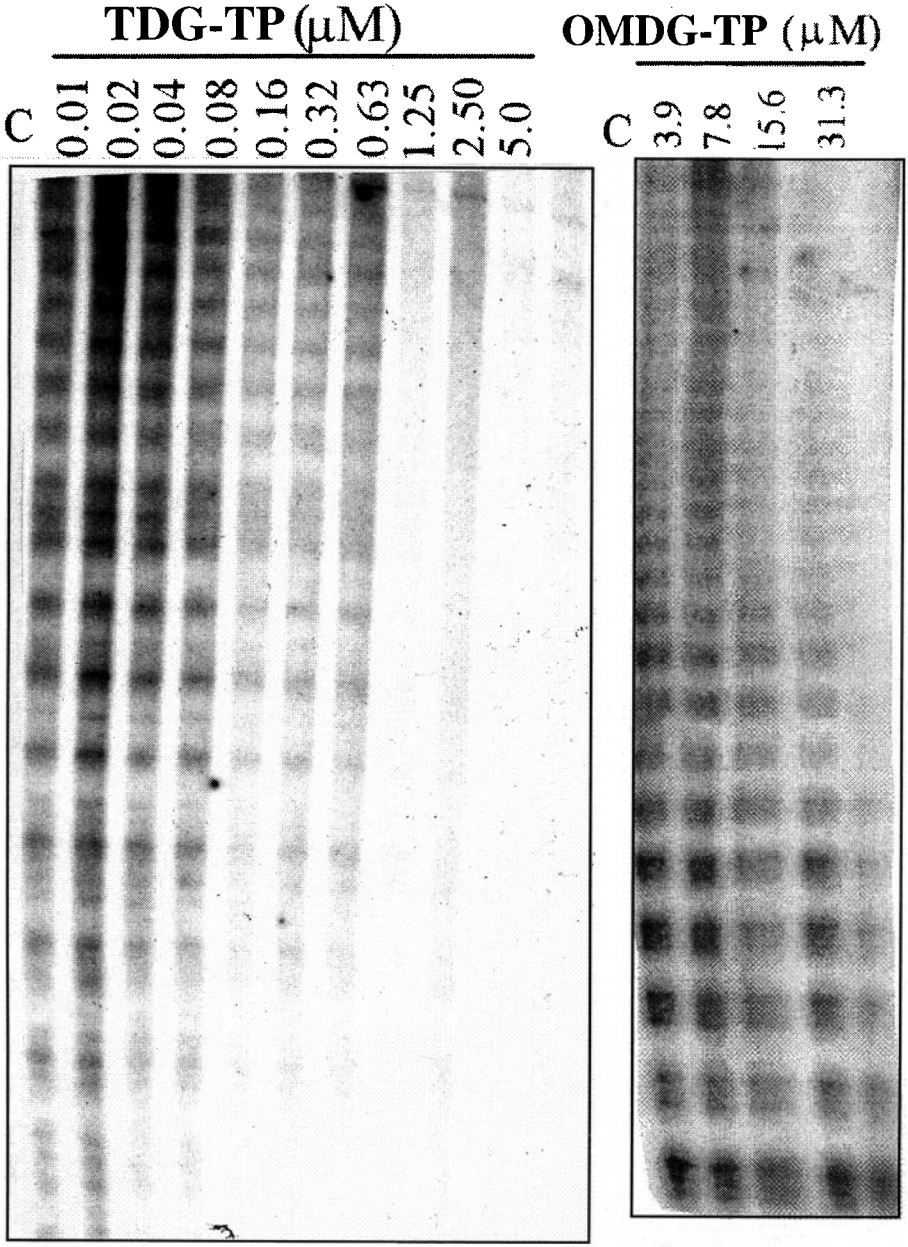


FIG. 1. Inhibition of telomerase ladder with 6-thio-7-deaza-2′-deozyguanosine 5′-triphosphate (TDG-TP) or 6-methoxy-7-deaza-2′-deoxyguanosine 5′-triphosphate (OMDG-TP) in the presence of 1.56 μM [α-^{32}P]dGTP containing 1 mM dTTP, 1 mM dATP, 1 μM d(TTAGGG)$_3$, 5μl of Chaps extract and the indicated concentrations of nucleotide analogs, in μM. The telomerase reactions were run for 60 min, and the products isolated by ethanol precipitation and analyzed by PAGE.