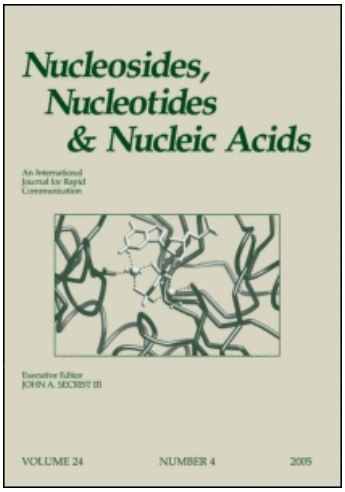


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Synthetic Nucleosides and Nucleotides. 43. Inhibition of Vertebrate Telomerases by Carbocyclic Oxetanocin G (C.OXT-G) Triphosphate Analogues and Influence of C.OXT-G Treatment on Telomere Length in Human HL60 Cells

Toyofumi Yamaguchi[a]; Mineo Saneyoshi[a]; Hazuki Takahashi[b]; Sachiko Hirokawa[b]; Rie Amano[b]; Xiaohong Liu[c]; Motoko Inomata[c]; Tokumi Maruyama[d]
[a] Department of Biosciences and Biotechnology Research Center, Teikyo University of Science and Technology, Uenohara, Yamanashi, Japan [b] Department of Biosciences, Teikyo University of Science and Technology, Uenohara, Yamanashi, Japan [c] Biotechnology Research Center, Teikyo University of Science and Technology, Uenohara, Yamanashi, Japan [d] Faculty of Pharmaceutical Sciences at Kagawa Campus, Tokushima Bunri University, Shido, Sanuki, Kagawa, Japan

To cite this Article Yamaguchi, Toyofumi , Saneyoshi, Mineo , Takahashi, Hazuki , Hirokawa, Sachiko , Amano, Rie , Liu, Xiaohong , Inomata, Motoko and Maruyama, Tokumi(2006) 'Synthetic Nucleosides and Nucleotides. 43. Inhibition of Vertebrate Telomerases by Carbocyclic Oxetanocin G (C.OXT-G) Triphosphate Analogues and Influence of C.OXT-G Treatment on Telomere Length in Human HL60 Cells', Nucleosides, Nucleotides and Nucleic Acids, 25: 4, 539 — 551
To link to this Article: DOI: 10.1080/15257770600684217
URL: http://dx.doi.org/10.1080/15257770600684217

Nucleosides, Nucleotides, and Nucleic Acids, 25:539–551, 2006

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770600684217

Taylor & Francis
Taylor & Francis Group

SYNTHETIC NUCLEOSIDES AND NUCLEOTIDES. 43. INHIBITION OF VERTEBRATE TELOMERASES BY CARBOCYCLIC OXETANOCIN G (C.OXT-G) TRIPHOSPHATE ANALOGUES AND INFLUENCE OF C.OXT-G TREATMENT ON TELOMERE LENGTH IN HUMAN HL60 CELLS

Toyofumi Yamaguchi and Mineo Saneyoshi □ *Department of Biosciences and Biotechnology Research Center, Teikyo University of Science and Technology, Uenohara, Yamanashi, Japan*

Hazuki Takahashi, Sachiko Hirokawa, and Rie Amano □ *Department of Biosciences, Teikyo University of Science and Technology, Uenohara, Yamanashi, Japan*

Xiaohong Liu and Motoko Inomata □ *Biotechnology Research Center, Teikyo University of Science and Technology, Uenohara, Yamanashi, Japan*

Tokumi Maruyama □ *Faculty of Pharmaceutical Sciences at Kagawa Campus, Tokushima Bunri University, Shido, Sanuki, Kagawa, Japan*

□ *Telomerase, responsible for telomere synthesis, is expressed in ∼90% of human tumor cells but seldom in normal somatic cells. In this study, inhibition by carbocyclic oxetanocin G triphosphate (C.OXT-GTP) and its analogues was investigated in order to clarify the susceptibility of telomerase to various nucleotide analogues. C.OXT-GTP competitively inhibited telomerase activity with respect to dGTP. However, C.OXT-GTP had a potent inhibitory effect on DNA polymerase α. It was examined whether the nucleoside (C.OXT-G) was able to alter telomere length in cultured human HL60 cells. Contrary to expectation, long-term treatment with 10 μM C.OXT-G was found to cause telomere lengthening.*

Keywords Carbocyclic oxetanocin; DNA polymerase; Telomerase; Telomere lengthening; Telomere shortening

Received 31 December 2005; accepted 1 February 2006.

We thank Dr. Teruo Azuma, Nikkou Branch Institute, National Research Institute of Fisheries Science, Fisheries Research Agency, for kindly providing immature male cherry salmon. This work was supported in part by the High-Technology Research Center Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

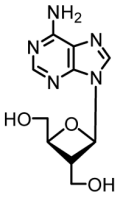
This article is dedicated to Professor Eiko Ohtsuka on the occasion of her 70th birthday.

Part 42 of this series: Yamaguchi, T., Yamada, R., Tomikawa, A., Shudo, K., Saito, M., Ishikawa, F., and Saneyoshi, M. 2000. *Biochem. Biophys. Res. Commun.* 279, 475–481.

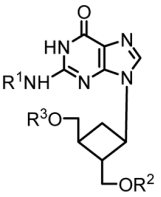
Address correspondence to Toyofumi Yamaguchi, Department of Biosciences and Biotechnology Research Center, Teikyo University of Science and Technology, 2525 Yatsusawa, Uenohara, Yamanashi 409-0193, Japan. E-mail: toyofumi@ntu.ac.jp

INTRODUCTION

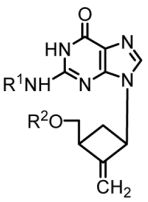
Vertebrate telomerase is a cellular endogenous reverse transcriptase in which the internal RNA serves as a template for directing the telomere DNA sequence, $(TTAGGG)_n$, thus contributing to the maintenance of telomere length.[1–3] In order to obtain selective inhibitors that can be employed as useful tools for studying telomerase, the susceptibility of the enzyme to nucleotide analogues has been investigated. The results have shown that human telomerase is inhibited more potently by dGTP analogues than by dTTP analogues bearing the same modified sugar.[4,5] Thus, sugar-modified dGTP analogues may be promising telomerase inhibitors. As shown in Scheme 1, carbocyclic oxetanocin G (C.OXT-G) (**1**) has a unique sugar structure and is reported to have excellent activity against herpes simplex virus.[6,7] C.OXT-G was originally designed as a carbocyclic analogue of oxetanocin A, which is a naturally occurring antibiotic.[8] The present article reports the inhibitory effects of C.OXT-GTP (**4**), and its analogues **8**, **11**, and **14**, on telomerases. The effects of C.OXT-G (**1**) on telomere length and growth properties of HL60 cells derived from human leukemia cells are also described.



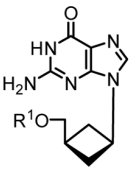
oxetanocin A



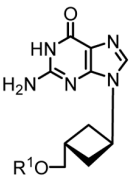
1 (C.OXT-G): R^1=H, R^2=H, R^3=H
2: R^1=isobutyryl, R^2=trityl, R^3=H
3: R^1=isobutyryl, R^2=H, R^3=monophosphate
4 (C.OXT-GTP): R^1=H, R^2=H, R^3=triphosphate



5 (m-C.OXT-G): R^1=H, R^2=H
6: R^1=isobutyryl, R^2=H
7: R^1=isobutyryl, R^2=monophosphate
8 (m-C.OXT-GTP): R^1=H, R^2=triphosphate



9: R^1=H
10: R^1=monophosphate
11: R^1=triphosphate



12: R^1=H
13: R^1=monophosphate
14: R^1=triphosphate

SCHEME 1

In this study, a large amount of telomerase activity was needed for direct detection of the DNA products that were synthesized by telomerase. However, it is difficult to prepare a large amount of human telomerase even from telomerase-positive cells. As it has been reported that high telomerase activity is detectable in several normal organs of the rainbow trout,[9,10] we undertook

purification of telomerase from cherry salmon testis. The partially purified enzyme was used for the primer extension experiments. Cherry salmon DNA polymerases α and δ[11] were also used for similar reasons.

MATERIALS AND METHODS

MicroSpin G-25 and a Sephadex NAP-5 column were purchased from Amersham Biosciences. A digoxigenin-labeled dUTP/dNTP mixture, antidigoxigenin antibody–alkaline phosphatase conjugate and blocking reagent were purchased from Roche Diagnostics. DEAE-cellulose (DE52) and DE-81 filter discs were purchased from Whatman. Detection of fluorescent signals of gels, membranes, or imaging plates (Fuji Photo Film) was performed using a fluorescence image analyzer FLA 3000 (Fuji Photo Film).

Preparation of Nucleoside 5′-Monophosphates (3, 7, 10 and 13)

General procedure: A solution of nucleosides **6**, **9**, **12**, and **2**[6,7] (0.05 mmol) in triethyl phosphate (1 ml) was mixed with phosphorus oxychloride[12] (0.019 ml, 0.2 mmol) at −10°C and the mixture was stored in a refrigerator for one day. After the mixture had been neutralized by addition of saturated aqueous $NaHCO_3$ (2 ml), water (10 ml), and chloroform (10 ml) were added to the mixture. The aqueous phase was separated, diluted with water to 80 ml, and applied to a DEAE-cellulose column (2.5 × 10 cm) preequilibrated with water. Elution was then done with a linear gradient of 0 (500 ml) to 0.3 M (500 ml) triethylammonium bicarbonate (TEAB) (pH 7.8). Appropriate fractions were collected and concentrated under reduced pressure, and water was added to and evaporated from the resulting residue to remove residual TEAB. The products [**7** (66%), **10** (17%), and **13** (50%)] were isolated. For the synthesis of N^2-butyryl-C.OXT-GMP (**3**), the reaction mixture from **2** was poured into ice-cold water (20 ml), stirred at room temperature for 20 min, and then concentrated to ~2 ml under reduced pressure. Acetic acid (8 ml) was added to the solution and the mixture was heated at 100°C for 10 min. The solvent was removed under reduced pressure and the residue was dissolved in saturated aqueous $NaHCO_3$ (10 ml) and chloroform (10 ml). The aqueous phase was treated and chromatographed on a DEAE-cellulose column in a manner similar to that described above. The desired compound **3** was isolated.

Preparation of Nucleoside 5′-Triphosphates (4, 8, 11, and 14)

General procedure: A mixture of **3**, **7**, **10**, or **13** (0.02 mmol) and 1,1′-carbonyldiimidazole (17 mg, 0.1 mmol) was dissolved in *N*,*N*-dimethylformamide (1 ml) (DMF).[13] The reaction mixture was stirred for 3 h at room temperature and methanol (3.2 μl) was then added. After stirring

for 30 min, a solution of tri-*n*-butylammonium pyrophosphate (0.6 M, 0.33 ml) in DMF was added and the mixture was vigorously stirred for 1 day at room temperature. The solvent was removed under reduced pressure, and the residue was dissolved in water (50 ml). This solution was treated with active carbon (0.2 g) and stirred for 15 min, filtered, and then washed well with water. The triphosphate was then eluted from the active carbon with 50 ml of 50% aqueous ethanol containing 3% ammonium hydroxide. The eluate was evaporated and the residue obtained was dissolved in 7 M ammonium hydroxide (1 ml). The mixture was stirred for one day at room temperature and then evaporated. The residue was dissolved in water (50 ml) and the solution was applied to a DEAE-cellulose column (2.5 × 10 cm). Elution was done with a linear gradient of 0 (400 ml) to 0.5 M (400 ml) triethylammonium bicarbonate (TEAB) (pH 7.8). Appropriate fractions were collected and concentrated under reduced pressure. Water was added to the residue and the solvent was evaporated to remove the residual TEAB. The products [**4** (13%) and **8** (8.4%)] were isolated. For the synthesis of **11** (60%) and **14** (61%), treatment with 7 M ammonium hydroxide was omitted. When analyzed by HPLC using TSK-gel DEAE-2SW (Tosoh) and 0.225 M potassium phosphate buffer (pH 6.9) containing 20% CH_3CN as the mobile phase (0.8 ml/min) at room temperature, the retention times of **4**, **8**, **11**, and **14** were 10.4, 11.9, 11.4, and 11.1 min, respectively, and the purities of these compounds assessed by measuring the UV absorption at 250 nm were confirmed to be greater than 95%.

Cells, Tissues, and Enzymes

HeLa cells and HL60 cells were obtained from the Riken RBC Cell Bank (Tsukuba, Japan). Immature cherry salmon were killed and their testes were removed, frozen promptly with solid carbon dioxide, and kept at −80°C. DNA polymerases α and δ were purified from a crude extract of the immature testis as described previously.[11] DNA polymerase, classified previously as ε-polymerase by us, was finally identified as δ-polymerase, because analysis of the amino acid sequence of the tryptic peptides prepared from the major polypeptide of Mr 110,000 showed ∼70% homology with human δ-polymerase (data not shown).

Quantitative Telomerase Assay Based on the "Stretch PCR" Method

Standard telomerase assay[14,15]: A reaction mixture (20 μl) for telomere DNA extension was composed of 50 mM Tris-OAc/50 mM KOAc (pH 8.5), 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 μM denatured TAG-U primer (5′-GTA AAA CGA CGG CCA GTT TGG GGT TGG GGT TGG GGT TG-3′), 10-200 μM dGTP, 10–200 μM dTTP, 10–200 μM dATP, inhibitors, and cell

extract containing telomerase. Incubation was performed for 10 min at 30°C followed by addition of 13 mM EDTA (pH 8.0) (40 μl). The following procedures, isolation of product DNA, stretch PCR using CTA-R reverse primer (5′-CAG GAA ACA GCT ATG ACC CCT AAC CCT AAC CCT AAC CCT-3′), separation by polyacrylamide gel electrophoresis, and estimation of products were carried out as described previously for human HeLa cell extract.[15]

Partial Purification of Telomerase from Immature Cherry Salmon Testis

A crude extract (S-100) (20 ml), which had been prepared from cherry salmon testes (5 g) as described by Tatematsu et al.,[14] was loaded onto a column of DEAE-cellulose (16 ml) preequilibrated with buffer A [20 mM HEPES-KOH (pH 8.0), 2 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.2 mM PMSF, 5 μM leupeptin, 0.5 μM pepstatin A, 10% glycerol]. The column was washed with buffer A (50 ml), followed by elution of the bound fraction with a 0–0.625 M KCl linear gradient in buffer A (150 ml). The fractions containing telomerase activity were collected and the proteins were fractionated with ammonium sulfate. Telomerase was recovered as a precipitate by 70% saturated ammonium sulfate. The precipitate was dissolved in a small amount of buffer B [20 mM HEPES-KOH (pH 8.0), 2 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.2 mM PMSF, 5 μM leupeptin, 0.5 μM pepstatin A, 50 mM KCl, 20% glycerol], dialyzed against buffer B and then stored at −80°C.

Assay of DNA Polymerases α and δ

The reaction mixture (25 μl) was composed of 50 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/ml activated DNA, 20 μM dGTP, 50 μM each dATP, dCTP and [α-^{32}P]dTTP (Amersham Biosciences) (5 kBq), 200 μg/ml bovine serum albumin, 10% glycerol, and enzyme preparation. Incubation was carried out at 37°C for 15 min. The radioactive DNA product was collected on a paper disc (DE 81) and the radioactivity was then measured.[16]

Primer Extension Assay

The assay was performed essentially as described by Tendian et al.[17] The reaction mixture (40 μl) was composed of 50 mM Tris-HOAc (pH 8.5); 50 mM KOAc; 1 mM $MgCl_2$; 1 mM dithiothreitol; 2 μM $(TTAGGG)_3$; 5 μM [α-^{32}P]dTTP; 5 μM dATP; 5 or 0 μM dGTP; 0, 5, or 20 μM C.OXT-GTP (**4**), and 20 μl of partially purified cherry salmon telomerase. Incubation was performed for 1 h at 25°C. The reaction was terminated by adding 0.5 M EDTA (1 μl) and 2 μg/μl RNase A (2 μl) and incubation at 37°C for

15 min. The solution was further treated by adding 10% SDS (1 μl) and 20 μg/μl proteinase K (1 μl) and incubated at 37°C for 15 min. The unincorporated radioactivity was removed from each sample by gel filtration (MicroSpin G-25). The samples were extracted with phenol/chloroform. The reaction products in the aqueous layer (50 μl) were precipitated twice with ethanol (67% ethanol and 0.67 M NH_4OAc) after addition of carrier tRNA (50 μg). The precipitated nucleic acids were analyzed by electrophoresis on a 15% (w/v) polyacrylamide-7 M urea sequencing gel. Autoradiographs were prepared by exposure of imaging plates for one day. Signals were detected using a fluorescence image analyzer.

Preparation of Digoxigenin-Labeled Telomere DNA Probe

S100 crude extract (~2 μg) prepared from HeLa cells[15] was employed according to the standard telomerase assay method described above, except that the CTA-Rh primer (5′-CAG GAA ACA GCT ATG GCC CCT AAC CCT AAC CCT AAC CCT-3′) was used in place of CTA-R for the stretch PCR. DNA products were extracted with phenol/chloroform and precipitated with 0.67 M ammonium acetate and 80% (v/v) ethanol (final concentrations). After washing the precipitates with cold 80% (v/v) ethanol, they were dried and suspended in extension buffer (20 μl) composed of 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% NP40, and 20 μM each oligodeoxyribonucleotide (5′-AGGGTT-3′ and 5′-CCTAAC-3′). The mixture was heated at 95°C for 5 min and promptly chilled in an ice-water bath. It was then mixed with 2 μl of a digoxigenin-labeled dUTP/dNTP mixture and 1 μl (5 units) of a solution of Taq DNA polymerase. After incubation for 1 day at 37°C, two preparations of the reaction mixture were combined. The digoxigenin-labeled DNA was purified by gel filtration using a Sephadex NAP-5 column.

Cell Culture

HL60 cells were cultured in RPMI-1640 medium (Nissui), supplemented with 0.3 mg/L L-glutamine, 70 μg/L kanamycin, and 10% heat-inactivated fetal bovine serum at 37°C under 5% carbon dioxide. The cultures were grown in 25-cm^2 flasks, 6 ml per flask in duplicate, with C.OXT-G (**1**) added to the medium to a final concentration of 10 or 20 μM before passaging. Cells were counted with a hemacytometer and transferred every four days (three to four mean population doublings), 3×10^5 cells per flask being seeded into fresh medium containing analogue or control medium. Remaining cells were collected by centrifugation ($500 \times g$) and suspended in PBS(–). After a further centrifugation, the pelleted cells were stored at −80°C. Simultaneously, cell viability was checked before harvesting by 0.1% trypan blue staining during counting.

DNA Extraction, Restriction Digestion, and Southern Analysis

Cell pellets ($\sim 2 \times 10^6$ cells) were suspended in 50 μl of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA], mixed with 0.5 ml of lysis buffer [10 mM Tris-HCl (pH 8.0), 125 mM EDTA, 10% sodium dodecyl sulfate (SDS), 20 μg/ml RNase A], incubated at 37°C for 1 h, and then mixed with 20 mg/ml proteinase K (5 μl). Incubation was then continued at 50°C overnight, followed by phenol extraction and ethanol precipitation. Genomic DNA ($\sim$10 μg) was resuspended in 45 μl of the mixture [10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT and 30 units of *Rsa* I] and incubated at 37°C for 2 h. Further digestion was performed by addition of 10 μl of the mixture [0.4 M Tris-HCl (pH 7.5), 1 M NaCl and 40 units *Hin*f I] and incubation at 37°C for 2 h. The reaction mixture was further treated by adding 10% SDS (1 μl) and 20 μg/μl proteinase K (1 μl) and incubation was performed at 37°C for 15 min. The resulting digested DNA was treated by phenol extraction and ethanol precipitation.

Approximately 1 μg of digested DNA per lane was electrophoresed on 1% agarose gel. The DNA was transferred to a nylon support membrane by capillary action and then UV-cross-linked to the membrane. Prehybridization of the membranes was performed in a hybridization mixture (6 × SSC, 5 × Denhardt's solution, 0.5% SDS) at 60°C for 1 h, and then hybridization was done with the digoxigenin-labeled telomere probe in hybridization mixture at 60°C overnight in a sealed bag. The membranes were washed in a solution of 0.1 × SSC and 0.1% SDS at 60°C for 30 min and briefly in MBS [0.1 M maleic acid-NaOH (pH 7.5), 0.15 M NaCl] containing 0.3% Tween 20. The membranes were treated with 1 × blocking solution (MBS containing 1% blocking reagent) at room temperature for 40 min and then treated with alkaline phosphatase-conjugated antidigoxigenin antibody. After washes in MBS containing 0.3% Tween 20 for 0.5 h and detection buffer [0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl] for 3 min, the membranes were immersed in staining solution [1 mM 2′-(2-benzothiazoyl)-6′-hydroxybenzothiazole phosphate (Promega), 2.4 M diethanolamine (pH 10), 0.057 mM $MgCl_2$, 0.005% NaN_3] at 37°C for 2–24 h. Signals were detected using a fluorescence image analyzer.

RESULTS

Inhibitory Effects of C.OXT-GTP (4) and Its Analogues 8, 11, and 14 on HeLa Cell Telomerase and DNA Polymerase α and δ

Quantitative telomerase assay based on the "stretch PCR" method[14,15] was performed to investigate the inhibitory effects of some triphosphate derivatives on HeLa cell telomerase activity. As shown in Figure 1A, amplified telomerase products were detected as a ladder consisting of bands spaced six bases apart. The ladders were abolished by incubation (30°C, 3 min)

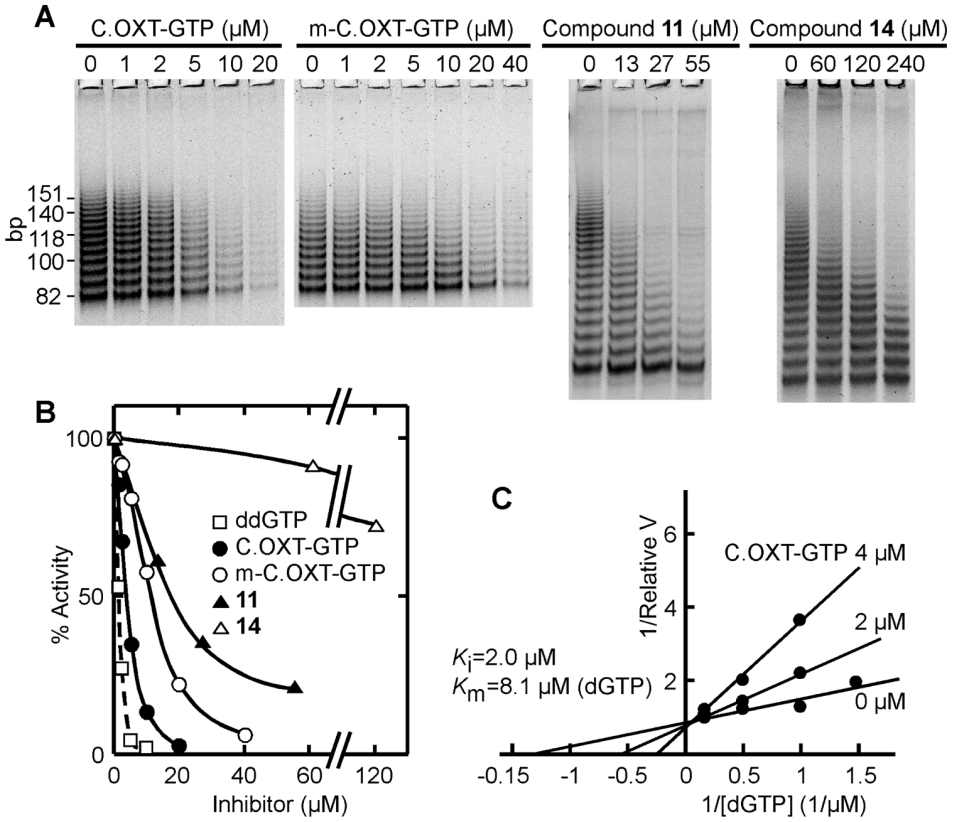


FIGURE 2 Inhibition of telomerase activity by C.OXT-GTP (**4**), m-C.OXT-GTP (**8**), compound **11**, and its *trans*-isomer **14**. (A) Inhibition of telomere DNA ladder formation with various concentrations of inhibitors in the presence of 10 μM dGTP, 200 μM dATP, and 200 μM dTTP. (B) Remaining activities in the presence of 2′,3′-dideoxyguanosine 5′-triphosphate (ddGTP) (clear squares), C.OXT-GTP. (**4**) (solid circles), m-C.OXT-GTP (**8**) (clear circles), and compounds **11** (solid triangles) and **14** (clear triangles) are shown. Activity without inhibitor was taken as 100%. The amount of PCR product comprising a DNA ladder in each lane, as shown in panel A, was quantified using a fluorescence image analyzer and the percentage of activity was estimated. (C) Lineweaver-Burk plot analyses of the inhibitory effects of C.OXT-GTP (**4**). Telomerase activity was measured in the presence of various concentrations of C.OXT-GTP (**4**) and dGTP. Relative reaction velocity (relative V) was calculated by reference to the activities without inhibitor in the presence of 6 μM dGTP, which was taken as 1.

with 1 μg of RNase A or heating (90°C, 5 min) (data not shown). C.OXT-GTP (**4**), m-C.OXT-GTP (**8**), and **11** showed potent inhibitory effects, that of C.OXT-GTP (**4**) being the most marked. However, *trans*-compound **14** of **11** showed only slight inhibition. Lineweaver-Burk plot analysis of C.OXT-GTP (**4**) showed that the mode of inhibition was competitive with respect to dGTP (Figure 1C). The mode of inhibition by m-C.OXT-GTP (**8**) was also competitive with respect to dGTP (data not shown). The K_i values of C.OXT-GTP (**4**) and m-C.OXT-GTP (**8**) were estimated to be 2.0 and 4.9 μM, respectively. Additionally, C.OXT-GTP (**4**) showed potent and moderate inhibitory effects on DNA polymerases α and δ, respectively (Figure 2). On

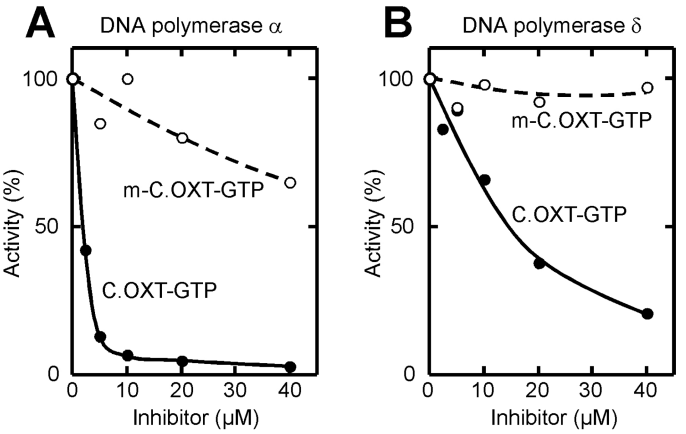


FIGURE 3 Inhibition of DNA polymerases α and δ by C.OXT-GTP (**4**) and m-C.OXT-GTP (**8**). Activities of cherry salmon DNA polymerases were measured in the presence of the indicated concentrations of C.OXT-GTP (**4**) (solid circles) and m-C.OXT-GTP (**8**) (clear circles), 20 μM dGTP, and dATP, dCTP, and [α-^{32}P]dTTP each at 50 μM. Remaining activities of DNA polymerases α (panel A) and δ (panel B) are shown as percentages. Activity without inhibitor was taken as 100%.

the other hand, m-C.OXT-GTP (**8**) showed only very weak or no inhibition against DNA polymerases α and δ.

Incorporation of dGTP Analogues into DNA by Cherry Salmon Telomerase

Partially purified telomerase was obtained from cherry salmon testes. As the properties of fish telomerase were similar to those of the human enzyme (data not shown), it was anticipated that the behavior of the human enzyme would be predictable from the results obtained with the fish enzyme.

The primer extension products that were synthesized by cherry salmon telomerase in the presence of C.OXT-GTP (4) instead of dGTP were analyzed (Figure 3). The experiment was performed using an 18-mer primer, $(5'\text{-TTAGGG-}3')_3$ capable of incorporating two ^{32}P-labeled dTMP molecules at the initial two positions, dAMP at the third position, and dGMP analogues at the fourth position. The primer extension experiment in the presence of [α-^{32}P]dTTP, dATP and dGTP (complete reaction, lane 1 in Figure 3) gave long products of various lengths, as expected. In the absence of dGTP, 19-, 20-, and 21-mer bands were shown (lane 3 in Figure 3). Contrary to expectation, a faint band corresponding to a 22-mer was also detected reproducibly. This has not yet been investigated in detail. The reaction in the presence of C.OXT-GTP (**4**) instead of dGTP gave a distinct 22-mer product (lanes 4 and 5). These results suggest that C.OXT-GTP (**4**) is utilized by telomerase as a substrate and causes chain termination after incorporation at the 3′-terminus of the primer.

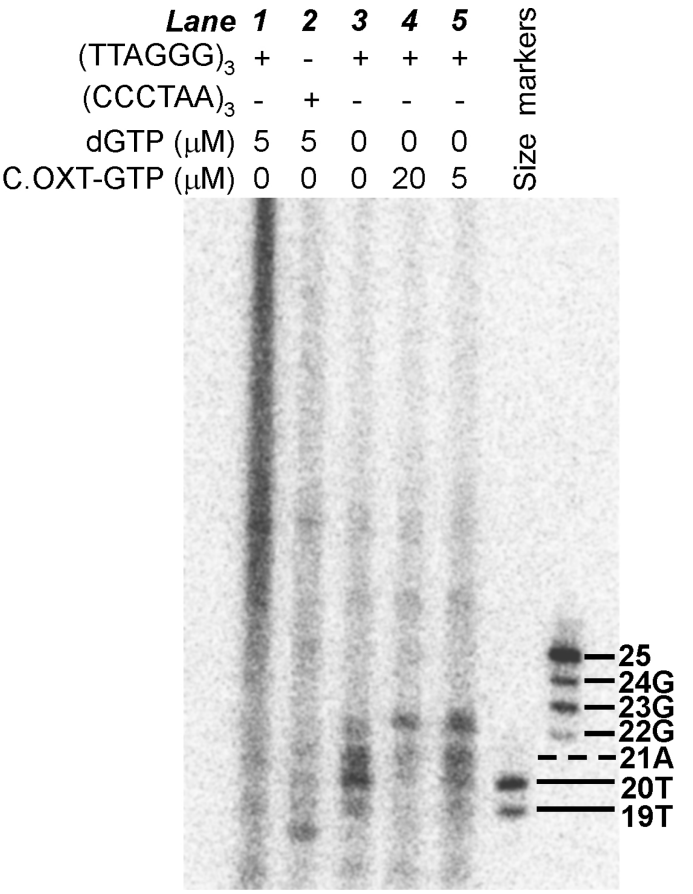


FIGURE 4 Utilization of C.OXT-GTP (**4**) as a substrate by cherry salmon telomerase. Telomerase activity was analyzed in the presence of 5 μM [α-^{32}P]dTTP, 200 μM dATP, 2 μM $(TTAGGG)_3$; 0 or 5 μM dGTP; and 0, 5, or 20 μM C.OXT-GTP (**4**).

Growth Rates and Telomere Length of HL60 Cells Grown in the Presence of C.OXT-GTP (4)

Cultures of HL60 cells were tested with 10 and 20 μM C.OXT-G (**1**) (Figure 4A). For each culture, cells were counted every four days to determine cell growth rates and were seeded quantitatively to maintain logarithmic growth conditions. As shown in Figure 4A, long-term treatment of HL60 cells with C.OXT-G (**1**) inhibited cell growth slightly. Genomic DNAs from HL60 cells cultured with C.OXT-G (**1**) for 0, 16, 32, 48, and 64 days were restriction-digested with *Rsa* I and *Hin*f I, and the telomere lengths were determined by Southern hybridization analysis with a digoxigenin-labeled telomere DNA probe. As shown in Figure 4B, in control experiments (lanes 1, 2, 5, 8, and 11), telomere length showed a broad distribution ranging from ~2 to ~4 kbp and hardly changed during 16 passages (64 days). In contrast,

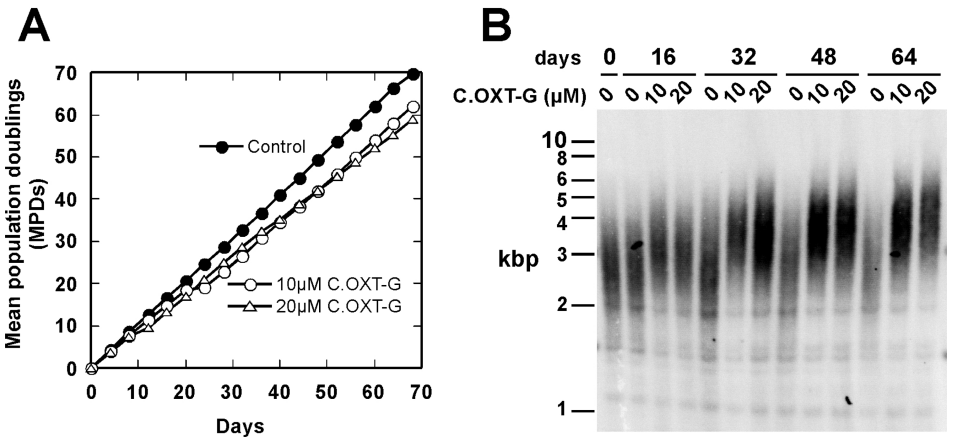


FIGURE 5 Growth rates and telomere lengths of HL60 cells grown in the absence or presence of C.OXT-G (**1**). (A) Mean population doublings (MPDs) of cultures treated with 0 (control culture, solid circles), 10 (clear circles), and 20 μM (clear triangles) C.OXT-G are plotted against days in culture. (B) DNAs from serial passages of HL60 cells grown in the presence of 0, 10, or 20 μM C.OXT-G were digested with *Rsa* I and *Hinf* I, run on a 1% agarose gel, and Southern blotted with a digoxigenin-labeled telomere DNA probe.

progressive telomere lengthening was observed after treatment with 10 and 20 μM C.OXT-G (**1**) (lanes 3, 4, 6, 7, 9, 10, 12, and 13).

DISCUSSION

C.OXT-GTP (**4**) and m-C.OXT-GTP (**8**) inhibited telomerase potently and moderately, respectively, even though each of compounds **4** and **8** was a racemate mixture (Figure 1). Compound **4** showed particularly potent inhibition. The cyclobutane moiety of **4** seems to have a very similar stereostructure to the sugar moiety of naturally occurring oxetanocin A. The K_i value of **4** was estimated to be 2.0 μM, which is somewhat larger than those of 2′,3′-dideoxyguanosine 5′-triphosphate (ddGTP) (0.65 μM) and arabinofuranosylguanine 5′-triphosphate (1.2 μM),[15] and ~4 times smaller than the K_m of dGTP (8 μM). It was noteworthy that human telomerase seems to prefer C.OXT-GTP (**4**) to its natural substrate dGTP. On the other hand, **4** was found to have different inhibitory effects on DNA polymerases α and δ. It should be noted that DNA polymerase α was inhibited potently by 5 μM C.OXT-GTP (**4**), whereas DNA polymerase δ was inhibited only weakly. This result suggests that C.OXT-GTP (**4**) is a useful reagent for discrimination between DNA polymerases α and δ.

As shown in Figure 3, 22-mer bands that incorporated a guanine residue at the 3′-terminus were clearly detectable in the primer extension experiment. This suggests that C.OXT-GTP (**4**) was incorporated into the

3′-terminus of telomere DNA, preventing the next phosphordiester bond formation with the incoming nucleotide.

Since C.OXT-GTP (**4**) showed potent telomerase-inhibitory activity as described above, we investigated whether treatment of HL60 cells with C.OXT-G (**1**) would cause telomere DNA shortening. Although treatment with 10 and 20 μM C.OXT-G (**1**) caused perturbation of telomere maintenance, significant telomere lengthening occurred, contrary to our expectation (Figure 4B). When cultured in the presence of a higher concentration of OXT-G (**1**), growth of HL60 cells was immediately inhibited. From studies using the ciliate *Euplotes*, Fan and Price reported that synthesis of G- and C-rich strands was coordinated, and that treatment with aphidicolin caused a general lengthening of G-rich strands in the telomere.[18] As shown in Figure 2A, C.OXT-GTP (**4**) inhibited DNA polymerase α. Therefore, C.OXT-GTP (**4**) might have an action similar to that of aphidicolin. In contrast, it is well known that 2′,3′-dideoxyguanosine 5′-triphosphate, ddGTP, potently inhibits DNA polymerases β and γ, but does not inhibit DNA polymerase α.[19,20] Strahl and Blackburn demonstrated that ddGTP potently inhibited human telomerase, and that treatment of two immortalized human cell lines with 2′,3′-dideoxyguanosine (ddG) caused telomere shortening.[4] These results suggest that inhibition selectivity between telomerase and DNA polymerase α is critical for telomere shortening.

Further screening of telomerase inhibitors is now under way in our laboratory.

REFERENCES

1. Blackburn, E.H. Structure and function of telomeres. Nature **1991**, 350, 569–573.
2. Harley, C.B.; Futcher, A.B.; Greider, C.W. Telomeres shorten during ageing of human fibroblasts. Nature **1990**, 345, 458–460.
3. Morin, G.B. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell **1989**, 59, 521–529.
4. Strahl, C.; Blackburn, E.H. Effects of reverse transcriptase inhibitors on telomere length and telomerase activity in two immortalized human cell lines. Molecular and Cellular Biology **1996**, 16, 53–65.
5. Yamaguchi, T.; Takahashi, H.; Jinmei, H.; Takayama, Y.; Saneyoshi, M. Inhibition of vertebrate telomerases by the triphosphate derivatives of some biologically active nucleosides. Nucleosides Nucleotides & Nucleic Acids **2003**, 22, 1575–1577.
6. Maruyama, T.; Sato, Y.; Horii, T.; Shiota, H.; Nitta, K.; Shirasaka, T.; Mitsuya, H.; Honjo, M. Synthesis and antiviral activities of carbocyclic oxetanocin analogues. Chemical & Pharmaceutical Bulletin **1990**, 38, 2719–2725.
7. Maruyama, T.; Hanai, Y.; Sato, Y.; Snoeck, R.; Andrei, G.; Hosoya, M.; Balzarini, J.; De Clercq, E. Synthesis and antiviral activity of carbocyclic oxetanocin analogues (C-OXT-A, C-OXT-G) and related compounds. II. Chemical & Pharmaceutical Bulletin **1993**, 41, 516–521.
8. Shimada, N.; Hasegawa, S.; Harada, T.; Tomisawa, T.; Fujii, A.; Takita, T. Oxetanocin, a novel nucleoside from bacteria. Journal of Antibiotics **1986**, 39, 1623–1625.
9. Klapper, W.; Heidorn, K.; Kuhne, K.; Parwaresch, R.; Krupp, G. Telomerase activity in "immortal" fish. FEBS Letters **1998**, 434, 409–412.
10. Yoda, M.; Takahashi, K.G.; Mori, K. 2002. Telomerase activity detected in eyed embryos of rainbow trout *Oncorhynchus mykiss*. Fisheries Science **2002**, 68, 132–137.

11. Tomikawa, A.; Seno, M.; Sato-Kiyotaki, K.; Ohtsuki, C.; Hirai, T.; Yamaguchi, T.; Kawaguchi, T.; Yoshida, S.; Saneyoshi, M. Synthetic nucleosides and nucleotides. 40. Selective inhibition of eukaryotic DNA polymerase α by (β-D-arabinofuranosyl)-2-(*p*-*n*-butylanilino)adenine 5′-triphosphate (BuAaraATP) and Its 2′-up azido analog: synthesis and enzymatic evaluations. Nucleosides & Nucleotides **1998**, 17, 487–501.
12. Yoshikawa, M.; Kato, T.; Takenishi, T. Studies of phosphorylation. III. Selective phosphorylation of unprotected nucleosides. Bulletin of the Chemical Society of Japan **1969**, 42, 3505–3508.
13. Maeda, M.; Patel, A.D.; Hampton, A. Formation of ribonucleotide 2′, 3′-cyclic carbonates during conversion of ribonucleoside 5′-phosphates to diphosphates and triphosphates by the phosphorimidazolidate procedure. Nucleic Acids Research **1977**, 4, 2843–2853.
14. Tatematsu, K.; Nakayama, J.; Danbara, M.; Shionoya, S.; Sato, H.; Omine, M.; Ishikawa, F. A novel quantitative "stretch PCR assay," that detects a dramatic increase in telomerase activity during the progression of myeloid leukemias. Oncogene **1996**, 13, 2265–2274.
15. Yamaguchi, T.; Yamada, R.; Tomikawa, A.; Shudo, K.; Saito, M.; Ishikawa, F.; Saneyoshi, M. Recognition of 2′-deoxy-L-ribonucleoside 5′-triphosphates by human telomerase. Biochemical and Biophysical Research Communications **2000**, 279, 475–481.
16. Yamaguchi, M.; Matsukage, A.; Takahashi, T. Chick embryo DNA polymerase γ. Purification and structural analysis of nearly homogeneous enzyme. Journal of Biological Chemistry **1980**, 255, 7002–7009.
17. Tendian, S.W.; Parker, W.B. Interaction of deoxyguanosine nucleotide analogs with human telomerase. Molecular Pharmacology **2000**, 57, 695–699.
18. Fan, X.; Price, C.M. Coordinate regulation of G- and C strand length during new telomere synthesis. Molecular Biology of the Cell **1997**, 8, 2145–2155.
19. Van der Vliet, P.C.; Kwant, M.M. Role of DNA polymerase γ in adenovirus DNA replication. Mechanism of inhibition by 2′,3′-dideoxynucleoside 5′-Triphosphates. Biochemistry **1981**, 20, 2628–2632.
20. Reardon, J. E. Herpes Simplex Virus Type 1 and Human DNA Polymerase Interactions with 2′-Deoxyguanosine 5′-Triphosphate Analogues. Kinetics of Incorporation into DNA and Induction of Inhibition. Journal of Biological Chemistry **1989**, 264, 19039–19044.