0026-895X/03/6305-1117–1124$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 63:1117–1124, 2003

Vol. 63, No. 5
2269/1059533
Printed in U.S.A.

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Potent Inhibition of Human Telomerase by Nitrostyrene Derivatives

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Received November 6, 2002; accepted February 3, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Telomerase activity is expressed in most types of cancer cells but not in normal somatic cells, suggesting that telomerase may be an important target for cancer chemotherapy. Inhibition of telomerase results in telomere erosion, leading to the subsequent growth arrest of cancer cells followed by senescence or cell death. In this study, we screened a chemical library for the inhibition of human telomerase, identifying three inhibitors. All compounds contained a common nitrostyrene moiety conjugated to different side chains. One of these compounds, 3-(3,5-dichlorophenoxy)-nitrostyrene (DPNS), showed the most potent inhibitory effect, with 50% inhibition at ~0.4 μM and did not inhibit DNA and RNA polymerases, including retroviral reverse transcriptase. A series of enzyme kinetic experiments suggests that DPNS is a mixed-type noncompetitive inhibitor, with an inhibitor-binding site distinct from the binding sites for the telomeric substrate primer and the deoxynucleoside-5′-triphosphates. Extensive propagation of cancer cell line in the presence of DPNS resulted in progressive telomere erosion followed by the induction of senescence phenotype. The results presented here demonstrate that DPNS is a highly selective, small-molecule telomerase inhibitor in vitro and could be useful as a lead molecule for the further development of inhibitors with an improved potential for efficacy in vivo.

Telomeres are the essential and functional components of eukaryotic chromosome ends that protect chromosomes from exonucleolytic degradation or end-to-end fusion and allow the complete replication of the ends (Blackburn, 1991; Greider, 1996). All dividing cells show a progressive loss of telomeric DNA during successive rounds of replication because conventional DNA polymerases cannot synthesize the end sequences of the lagging strand of DNA (Harley et al., 1990, 1994; Hastie et al., 1990; Lingner et al., 1995). Thus, telomere shortening has been proposed as a regulatory mechanism that controls the replicative capacity of primary cells before undergoing cellular senescence, thereby acting as a mitotic clock (Harley, 1991). Based on these situations, cells with extended replicative life spans should have a mechanism to counteract or prevent the cumulative loss of telomeric DNA. In immortal cells, telomere shortening can be arrested by the reactivation of telomerase (Counter et al., 1992; Harley et al., 1994). Telomerase is a ribonucleoprotein enzyme composed of at least two components: the catalytic subunit, hTERT, and the human telomerase RNA (Blackburn, 1992). This enzyme adds short, repetitive telomeric sequences to the chromosome ends by reverse transcriptase activity, thus stabilizing the telomere length.

Whereas telomerase activity has been demonstrated in most immortalized cell lines and in many types of human cancer tissues, it has not been detected in most normal human somatic tissues (Harley et al., 1990, 1994; Kim et al., 1994; Hiyama et al., 1996; Shay and Bacchetti, 1997). The introduction of the telomerase catalytic subunit gene into normal somatic cells prevents telomere erosion and senescence and extends the life spans of the cells (Bodnar et al., 1998; Kiyono et al., 1998; Vaziri and Benchimol, 1998). These findings suggest that telomerase activity is necessary for the proliferation of cancer cells, and the activation of telomerase may be an important step in human carcinogenesis. Taking into account this hypothesis, the inhibition of the telomerase enzyme would result in telomere shortening and subsequent

This work was supported in part by a grant from the Ministry of Health & Welfare through the Molecular Aging Research Center, grant R02-2001-000-00034-0 from the Korea Science and Engineering Foundation (KOSEF), and a grant from KOSEF through the Protein Network Research Center.

Joo Hee Kim and Jun Hyun Kim contributed equally to this work.

ABBREVIATIONS: hTERT, human telomerase reverse transcriptase; TRAP, telomeric repeat amplification protocol; TRF, terminal restriction fragment; DPNS, 3-(3,5-dichlorophenoxy)-nitrostyrene; DNS, 2,3-dichloro-nitrostyrene; NVN, 2-(2-nitrovinyl)-naphthalene; SA-β-gal, senescence-associated β-galactosidase; PD, population doubling; TS, telomeric substrate; dNTP, deoxynucleoside-5′-triphosphate; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PCR, polymerase chain reaction; TRF, terminal restriction fragment; kb, kilobase; BRACO19, 3,6,9-trisubstituted acridine 9-[4-(*N,N*-dimethylamino)phenylamino]-3,6-bis(3-pyrrolodinopropionamido) acridine; BIBR1532, 2-[(*E*)-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid.

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growth arrest of cancer cells because of the effects of sustained telomere erosion followed by senescence or cell death. In contrast to traditional anticancer agents that kill cells within days after administration, telomerase inhibitors require the long lag period (weeks to months) before their effects become apparent, because cellular growth arrest requires a series of DNA replication cycles. Delayed senescence phenotype has been demonstrated in knockout mice of the telomerase RNA component (Blasco et al., 1997) and cell lines that express a dominant-negative form of hTERT (Hahn et al., 1999; Zhang et al., 1999) and by the use of antisense oligonucleotides and small-molecule inhibitors (Herbert et al., 1999; Naasani et al., 1999; Damm et al., 2001; Corey, 2002).

In considering the hypothesis that telomerase may represent a suitable target for specific anticancer therapies, several strategies for the inhibition of telomerase have been designed and evaluated. Among the compounds tested are various types of antisense oligonucleotides designed to hybridize with the template domain of telomerase RNA (Pitts and Corey, 1998; Kondo et al., 2000), reverse transcriptase inhibitors including nucleoside triphosphate analogs (Strahl and Blackburn, 1996), dominant-negative hTERT-derived proteins (Hahn et al., 1999; Zhang et al., 1999), and compounds capable of stabilizing DNA G-quadruplex structures (Perry et al., 1998). A rapidly emerging area for discovering potent telomerase inhibitors is through large-scale screening of chemical small-molecule libraries using a telomerase assay (Hayakawa et al., 1999; Naasani et al., 1999; Damm et al., 2001). In this study, we identified a novel structural class of chemical compounds as inhibitors of human telomerase through the rapid screening of small molecules. The results indicate that these compounds are highly potent and selective telomerase inhibitors in vitro with good potential for further development as promising anticancer agents.

Materials and Methods

Chemical Compounds. The chemical libraries screened for telomerase inhibition in this study were obtained from Lead Genex, Inc. (Taejeon, Korea) and from ChemDiv, Inc. (San Diego, CA), including 15,000 compounds with molecular weights of approximately 400 Da. The synthesis of 3-(3,5-dichlorophenoxy)-nitrostyrene (DPNS) occurred as follows: 3-(3,5-dichlorophenoxy)-benzaldehyde (2.67 g) and nitromethane (0.82 ml) were dissolved in methanol (2 ml). To the reaction mixture was added 5 M aqueous NaOH (2 ml) followed by stirring for 10 min. To this mixture was added methanol (1 ml), and it was stirred for 5 min. The solution was then diluted with ice water (3 ml), and concentrated HCl (3 ml) was added followed by stirring for 10 min. The solution was then extracted with ethyl acetate. The organic layer was washed with concentrated Na_2CO_3. Acetic anhydride (1.02 ml) and a catalytic amount of dimethylaminopyridine was added to the resulting residue, which was then dissolved in dichloromethane (3 ml), followed by stirring for 3 h. The reaction mixture was extracted with ethyl acetate. The solvent was then removed in a rotary evaporator, and the crude product was purified by the use of silica gel column chromatography. The structure of DPNS was confirmed by proton NMR spectroscopy, and the molecular weight was determined to be 311 Da by mass spectrometry.

Cell Lines. The human cervical cancer cell line HeLa was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°C. The human osteosarcoma cell line Saos-2 was maintained in McCoy's 5A medium containing 15% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. For long-term exposure, the cells were grown in 100-mm plastic dishes and exposed to DPNS at a concentration of 1 μM dissolved in 0.1% dimethyl sulfoxide. Every 3 to 4 days, the cells were trypsinized, counted using a hematocytometer, and reseeded at a density of 5×10^5 cells/dish. Control cells were treated with corresponding dimethyl sulfoxide concentrations. Cell growth inhibition was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (Kang and Chung, 2002).

Preparation of Telomerase-Enriched Extracts. Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and then were lysed for 30 min on ice in a CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol, and 0.5 M NaCl). The lysate was then centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was loaded onto a 10 to 40% glycerol gradient (in buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, and 5 mM β-mercaptoethanol) and centrifuged at 25,000 rpm for 24 h at 4°C in a rotor (SW-28; Beckman Coulter, Inc., Fullerton, CA). The fractions were collected from the bottom of the gradient and assayed for telomerase activity in the telomeric repeat amplification protocol (TRAP) assay. The protein concentration was measured with use of the Bradford protein assay kit (Bio-Rad, Hercules, CA).

Telomerase Assay. The TRAP was used for the analysis of telomerase inhibition as described previously (Kim et al., 1994), with minor modifications. Unless indicated, telomerase extension reactions were carried out in TRAP buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween 20, and 1 mM EGTA) containing 200 nM TS primer and 100 μM concentrations of each dNTP. Telomerase-containing fraction (60 ng of protein) was added to the reaction mixture and incubated for 8 min at 37°C in the presence or absence of inhibitors as indicated. The reactions were stopped by heating at 94°C for 90 s and kept on ice. Before proceeding with the PCR reaction, the variable reactant was adjusted to a final concentration of 200 nM for the TS primer and 100 μM for each dNTP. PCR was performed using the forward TS primer and reverse ACX primer for 30 cycles (denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s). As an internal telomerase assay standard, NT primer and TSNT primer were added to the PCR mixture as described previously (Kim and Wu, 1997). Telomerase products were resolved by 10% nondenaturing polyacrylamide gel and visualized by staining with SYBR Green (Molecular Probes, Eugene, OR). The signal intensity was quantified with an image analyzer (LAS-1000 Plus; Fuji Photo Film, Tokyo, Japan). Inhibitors used in this experiment have no inhibitory effect when added after the telomerase reaction.

Determination of Kinetic Constants. For velocity curves, the telomerase activity was plotted as a function of the variable substrates. Michaelis-Menten constants for the substrates and the binding constants of the inhibitor were calculated from Lineweaver-Burk plots. K_i and αK_i were determined as the x-intercept of the linear replots of slope = f(I) and y-intercept = f(I), respectively.

Assays of DNA and RNA Polymerases. Effects of telomerase inhibitors on DNA and RNA polymerases were measured. *Taq* polymerase activity was assayed as described by the manufacturer (Takara, Kyoto, Japan). The amplified products were stained with SYBR Green and quantified by densitometric analysis using the LAS-1000 Plus Image Analyzer (Fujifilm Medical Systems, Stamford, CT). DNA polymerase and reverse transcriptase assays were performed as described by the manufacturer with commercially available Klenow fragment and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The reactions were performed in the presence of digoxigenin-11-dUTP (Roche Diagnostics, Indianapolis, IN), and the products were transferred onto nylon membranes by slot blotting. Using Western blot with anti-digoxigenin-AP, the reaction products were detected and quantified by den-

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sitometric analysis using the LAS-1000 Plus Image Analyzer. RNA polymerase activity was measured under the condition of T7 RNA polymerase assay, as described by the manufacturer (Promega). The reaction products were treated with RQ1 RNase-Free DNase (Promega) stained with RiboGreen RNA quantification reagent (Molecular Probes) and were quantified by using a fluorometer (Tecan Systems Inc., San Jose, CA).

Terminal Restriction Fragment Length Analysis. To measure the telomere length, genomic DNA was digested with *Rsa*I and *Hinf*I and separated onto 0.7% agarose gel. DNA samples were transferred to a nylon membrane (Hybond N^+; Amersham Biosciences Inc., Piscataway, NJ) and hybridized with a probe $(TTAGGG)_{20}$ labeled with digoxigenin-11-dUTP (Roche) using a random priming method. Detection relied on antidigoxigenin antibody conjugated with alkaline phosphotase (Roche). The signal intensity was detected and quantified by use of the LAS-1000 Plus Image Analyzer.

SA-β-Galactosidase Assay. Cells treated with telomerase inhibitors were washed twice in PBS, fixed in 2% formaldehyde/0.2% glutaraldehyde for 5 min at room temperature, washed again in PBS, and incubated for 16 h with β-galactosidase stain solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 40 mM citric acid/sodium phosphate, pH 6, 5 mM potassium ferrocynide, 5 mM ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$. Cells were viewed with use of a Nikon TMS light microscope (Nikon Instruments, Melville, NY) and photographed.

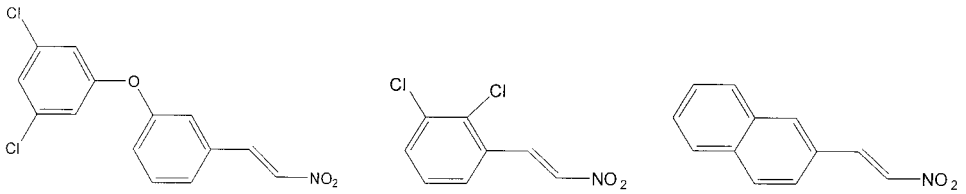
Results

Nitrostyrene Derivatives Selectively Inhibit Telomerase Activity In Vitro. To identify telomerase inhibitors, screening of a chemical small-molecule library was performed by the modified TRAP method using partially purified human telomerase as described under *Materials and Methods*. Three chemicals were selected as potential telomerase inhibitors on the basis of a half-maximal inhibitory concentration (IC_{50}) lower than 3 μM. These chemicals, DPNS, 2,3-dichloro-nitrostyrene (DNS), and 2-(2-nitrovinyl)-naphthalene (NVN), inhibit the in vitro process of telomerase in a dose-dependent manner with IC_{50} values of 0.4, 2.7, and 2.57 μM, respectively. All compounds contained a common nitrostyrene moiety conjugated to different side chains (Fig. 1). As shown in Fig. 2A, at a concentration of 10 μM, DPNS completely inhibited telomerase activity. It was noted that the synthesis of longer extension products was preferentially inhibited to the synthesis of shorter products at varying concentrations of DPNS. A comparison of the band intensities of individual extension products revealed that the IC_{50} values for the two short products (bands 1 and 5) are 0.42 and 0.39 μM, respectively. The formation of the two longer products (bands 10 and 13) is inhibited with IC_{50} values of 0.33 and 0.3 μM, respectively (Fig. 2B).

Effects of DPNS on DNA and RNA Polymerases. To test the specificity of DPNS, we examined the inhibitory effects on activities of DNA and RNA polymerases including reverse transcriptase under the assay conditions described under *Materials and Methods*. DPNS inhibited the telomerase activity to minimum levels at concentrations greater than 1 μM. However, *Taq* polymerase, DNA polymerase, RNA polymerase, and Moloney murine leukemia virus reverse transcriptase were not inhibited at concentrations exceeding the IC_{50} value for telomerase (Fig. 3), suggesting that inhibition by DPNS was highly selective for telomerase. Two other chemicals (DNS and NVN) showed similar selectivity (data not shown).

Mode of Telomerase Inhibition by DPNS. To characterize the mode of inhibition by DPNS, we performed a series of enzyme kinetic experiments. Inhibition was measured as a function of the concentrations of TS primer and dNTPs required for telomerase activity in vitro. The conditions for the linear phase of primer extension by telomerase were determined in the presence of saturating substrate concentrations (200 nM TS primer and 100 μM concentrations of each of dNTP) and different amounts of partially purified telomerase. A linear correlation between reaction time, enzyme concentration, and telomerase production was observed for incubations of less than 10 min (Fig. 4A). Under these conditions, velocity curves were measured for variable TS primer concentrations in the presence or absence of DPNS, and telomerase activity was plotted as a function of the TS primer concentration (Fig. 4B). In the absence of the inhibitor, the maximum enzymatic reaction (V_{max}) was reached with primer concentrations of greater than 4 nM. V_{max} was clearly reduced by the addition of increasing amounts of DPNS. Lineweaver-Burk plots of these data showed inhibition by DPNS to be noncompetitive of mixed type with the TS primer (Fig. 4C). The Michaelis-Menten constants of the TS primer in the absence (K_m) and presence (αK_m) of DPNS (1 μM) were estimated as ~0.5 and ~1.4 nM, respectively. This suggests that the TS primer has a higher affinity to the free enzyme than to the telomerase-DPNS complex (Fig. 4D). The binding constants of DPNS were calculated in the absence (K_i) and presence (αK_i) of the TS primer. The αK_i value (~650 nM) was significantly higher than the K_i value (~180 nM), indicating that DPNS shows a higher affinity to the free enzyme than to the telomerase-TS primer complex (Fig. 4E).

The velocity curve and the resulting Lineweaver-Burk plot were also measured as a function of the concentration of the dNTPs in the presence or absence of DPNS (Fig. 5, A and B). A clear reduction of V_{max} was observed in the presence of increasing amounts of DPNS, indicating inhibition to be noncompetitive with the dNTPs. In the presence of 1 μM DPNS, the K_m value (~30 μM) for the dNTPs increased to ~110 μM (αK_m), suggesting a lower activity to the telomerase-DPNS complex for dNTPs (Fig. 5C). The binding constant of DPNS



DPNS DNS NVN

Fig. 1. Chemical structures of telomerase inhibitors DPNS, DNS, and NVN identified by screening a chemical library.

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in the presence of the dNTPs (αK_i, ~2.7 μM) was higher than in the absence of the dNTPs (K_i, ~0.6 μM) (Fig. 5D), indicating a lower binding of DPNS to the telomerase-dNTPs complex. Taken together, our enzyme kinetic data suggest that DPNS is a mixed-type noncompetitive inhibitor for the binding of both TS primer and dNTPs.

DPNS Induces Telomere Shortening and Senescence Phenotype in Telomerase-Positive Cells. To examine the long-term effect of DPNS on telomerase-positive HeLa cells, we determined the drug concentration in which telomerase could be inhibited without extensive inhibition of cell proliferation. Short-term cell viability was determined in a 4-day cytotoxicity assay using variable drug concentrations. The IC_{50} value of DPNS for HeLa cells was 37 ± 2.6 μM. At a concentration of 10 μM, 80% of HeLa cells were viable, but DPNS had no effect on short-term cell proliferation at concentrations of less than 1 μM (data not shown). Accordingly, HeLa cells were grown exponentially in the presence of 1 μM DPNS to investigate the cellular consequences of long-term treatment. Cells were monitored periodically by microscopic analysis and by telomere-length estimation using Southern blot analysis. Control cells treated with the solvent alone exhibit a heterogeneous size distribution, with an average telomere length of ~4 kb (Fig. 6A). The same terminal restriction fragment (TRF) length was detected in DPNS-treated cells at early population doubling (10 PD), but TRF length was slowly and progressively shortened as cells were propagated in the presence of an inhibitor. The average TRF length shortened from 4 kb at early PDs to 2.5 kb at the late PDs (80 to 100 PD) (Fig. 6A). In contrast, control cells treated with solvent alone maintained a stable TRF length. As a control for telomerase inhibitor specificity, telomerase-negative SAOS-2 cells, which exhibit the alternative lengthening of telomere phenotype (Bryan et al., 1995, 1997), were treated with DPNS for 80 PD. The results showed that inhibitor treatment had no effect on telomere length (data not shown). A fraction of cells treated with DPNS for 100 PD exhibited a flattened morphology with elongated cellular processes and stained positively for the senescence-associated β-galactosidase (SA-β-gal) (Fig. 6B).

Reversibility of Telomerase Inhibition by DPNS. To examine the effect of inhibitor depletion on telomere length regulation, cells were transferred to normal medium without inhibitor after growing for 130 PD in the presence of 1 μM DPNS and then cultivated for another 60 PD. An examination of TRF length at 190 PD revealed a rapid elongation of the telomeres with an increase in TRF length to ~4 to 5 kb (Fig. 7A). In contrast, when cells were grown in the presence of DPNS for 190 PD, telomeres continued to shorten, with TRF length reaching the minimal length of ~2.5 kb. These results demonstrate that the telomerase inhibition by DPNS is fully reversible. We also examined the effects of inhibitor depletion on telomerase activity. The telomerase activity was measured using the TRAP assay with cell extracts prepared from cells grown in the presence or absence of 1 μM DPNS. Cells treated with DPNS for 190 PD showed a reduced telomerase activity compared with untreated cells. When the treated cells were transferred to normal medium without inhibitor at 130 PD, telomerase activity was regained to the original level shown in untreated cells (Fig. 7B).

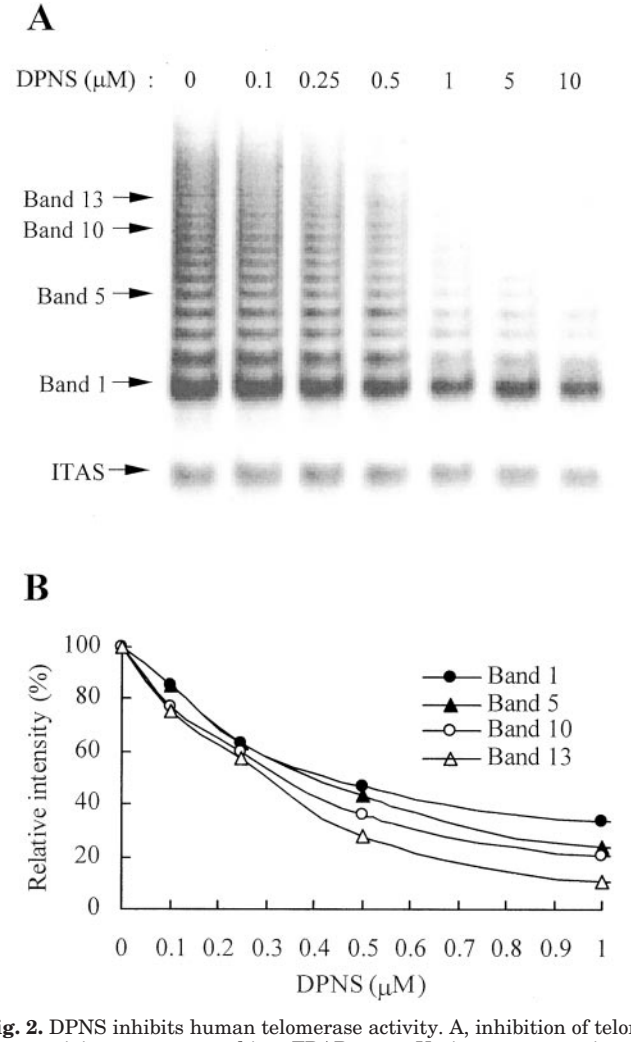

Fig. 2. DPNS inhibits human telomerase activity. A, inhibition of telomerase activity was measured in a TRAP assay. Various concentrations of DPNS were added before extension of TS primer by telomerase. The arrows indicate the product bands used for quantification. B, the intensities of TRAP products were normalized to the intensities of the corresponding bands in the control without inhibitor and plotted against the concentration of DPNS.

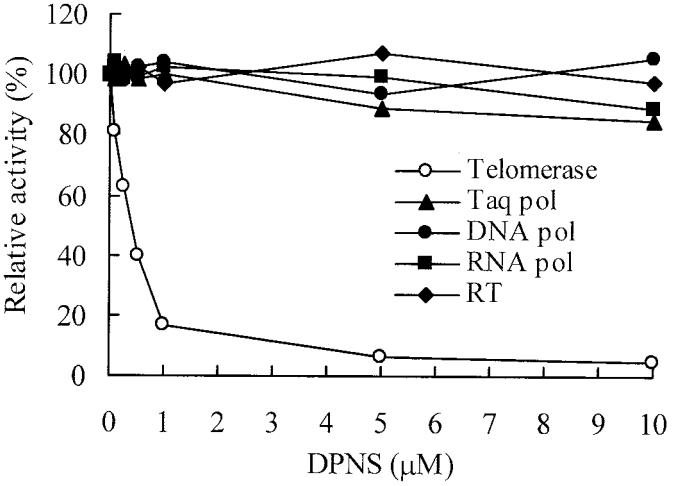

Fig. 3. Effects of DPNS on DNA and RNA polymerases. Activities of *Taq* polymerase, DNA polymerase, RNA polymerase, and reverse transcriptase (RT) were assayed as described under *Materials and Methods*. DPNS was added to the reaction mixtures at the concentrations indicated. For comparison, the inhibition of human telomerase by DPNS is also shown.

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Discussion

Telomerase is capable of providing the cells to circumvent gradual telomere erosion by synthesizing new telomeres, thereby avoiding the M1 senescence checkpoint (Bearss et al., 2000). Inhibition of telomerase results in telomere erosion, leading to the arrest of tumor cell growth and associated limitations in replicative life span (White et al., 2001). In this study, we screened a chemical library for the inhibition of human telomerase, identifying three inhibitors. All compounds contained a common nitrostyrene moiety conjugated to different side chains. One of these compounds, DPNS, showed the most potent inhibitory effect (IC_{50} = 0.4 μM). The data presented here demonstrate that DPNS is a selective, small-molecule telomerase inhibitor in vitro. Extensive propagation of cancer cell line in the presence of DPNS results in progressive telomere erosion followed by the induction of senescence phenotype in vivo.

Nitrostyrene derivatives have been reported previously to have antifungal activity as well as antitumor activity and have effects as metabolic inhibitors. For example, β-bromo-β-nitrostyrene is a wide-spectrum biocide most frequently used as a fungicide (Mikami et al., 1991). β-bromo-β-nitrostyrene also acts as an inhibitor of energy transfer in photophosphorylation by binding of the nonphosphorylated

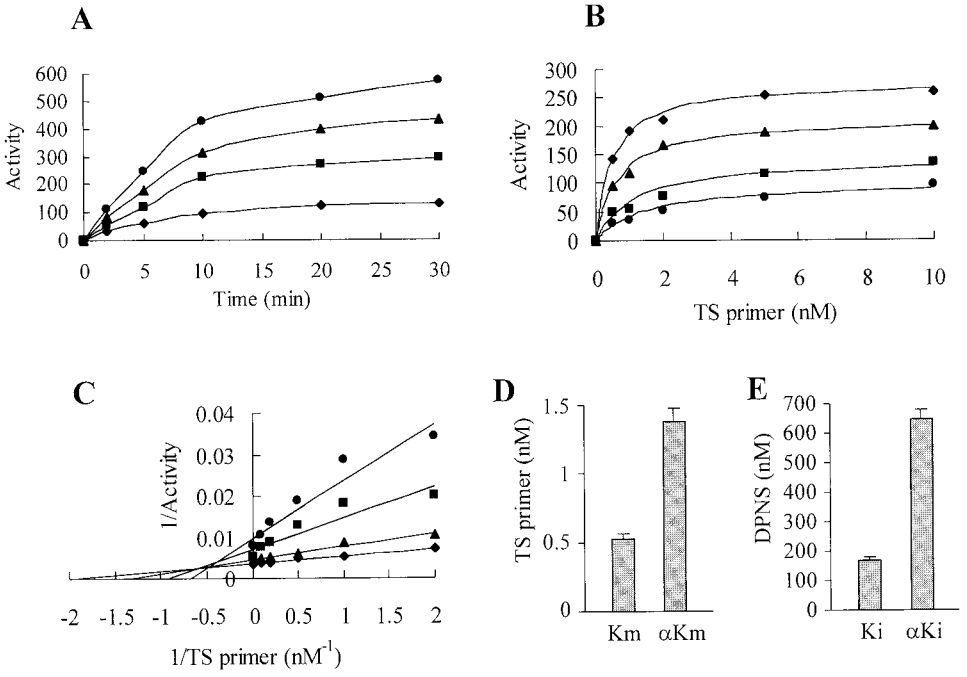

Fig. 4. DPNS is a mixed-type noncompetitive inhibitor for the binding of the TS primer. A, TRAP assays were performed with different quantities of partially purified telomerase: 15 ng (♦), 30 ng (■), 60 ng (▲), or 90 ng (●) of protein. Reactions were stopped after various time points, and products were analyzed. Telomerase activity was plotted against the reaction time. B, TRAP assays were performed for 8 min with 60 ng of partially purified telomerase in the absence (♦) or presence of DPNS at concentrations of 0.1 μM (▲), 0.5 μM (■), and 1 μM (●). Telomerase activity was plotted versus the concentration of the TS primer. C, double reciprocal plot (Lineweaver-Burk) of velocity curve was constructed. D, Michaelis-Menten constants of the TS primer in the absence (K_m) or presence (αK_m) of 1 μM DPNS. E, affinity constants of DPNS in the absence (K_i) or presence (αK_i) of the TS primer.

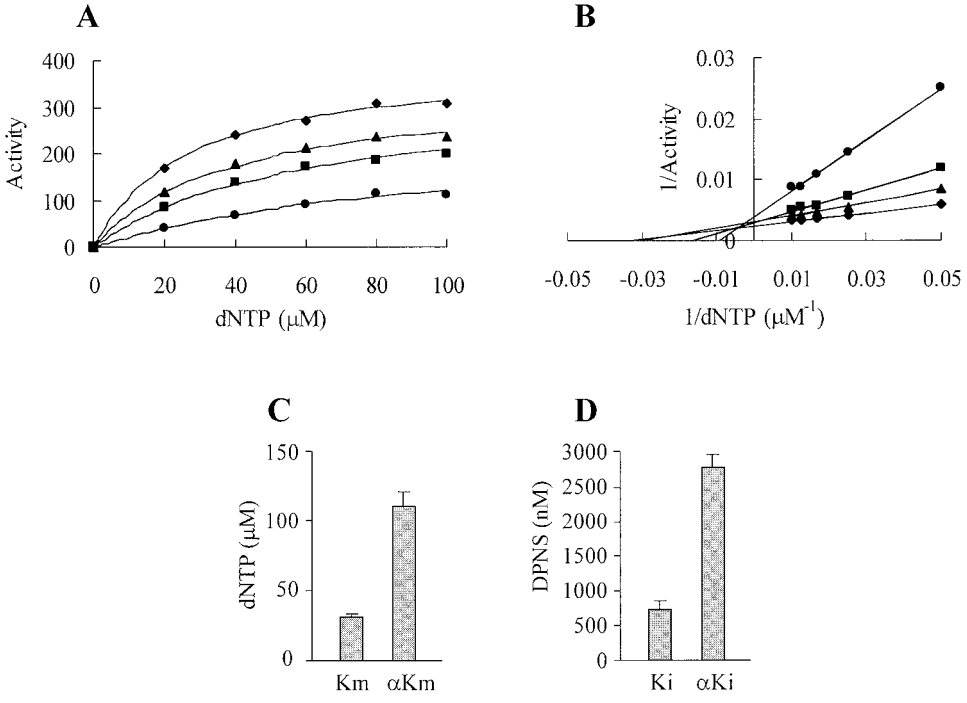

Fig. 5. DPNS is a mixed-type noncompetitive inhibitor for the binding of dNTPs. A, TRAP assays were performed for 8 min with 60 ng of partially purified telomerase in the absence (♦) or presence of DPNS at concentrations of 0.1 μM (▲), 0.5 μM (■), and 1 μM (●). Telomerase activity was plotted versus the concentration of the dNTPs. B, double reciprocal plot (Lineweaver-Burk) of velocity curve was constructed. C, Michaelis-Menten constants of the dNTPs in the absence (K_m) or presence (αK_m) of 1 μM DPNS. D, affinity constants of DPNS in the absence (K_i) or presence (αK_i) of the dNTPs.

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high-energy intermediate (Brandon, 1971). A series of sulfonylbenzoyl nitrostyrene derivatives has shown to be specific inhibitors of the epidermal growth factor receptor tyrosine protein kinase (Traxler et al., 1991). One of these derivatives showed potent antiproliferative effects on mouse epidermal keratinocyte cell line. Furthermore, treatment of nitrostyrene has a significant suppressive effect on the proliferation of the stomach cancer cell line (Carter et al., 2002). The results in this study revealed the molecular mechanism of action of nitrostyrene derivatives as telomerase inhibitors. However, further study is required to fully elucidate the relationships between the potency of telomerase inhibition and other biological effects of various nitrostyrene derivatives.

Although several strategies have been developed to inhibit telomerase activity, the growing family of potent telomerase inhibitors is being identified through the screening of chemical small-molecule libraries using a telomerase assay (Bearss et al., 2000; White et al., 2001). These include isothiazolone derivatives (Hayakawa et al., 1999), rhodacyanine derivatives (Naasani et al., 1999), and BIBR1532 (Damm et al., 2001). Unlike the direct-acting telomerase inhibitors, some small-molecule inhibitors have been reported to inhibit telomerase activity by interacting with G-quadruplex DNA (Perry et al., 1998, 1999; Izbicka et al., 1999; Gowan et al., 2001, 2002; Grand et al., 2002). The inhibition by DPNS reduced the overall rate of telomerase reaction, affecting the relative length of reaction products. The synthesis of longer products was preferentially inhibited at varying concentrations of DPNS. This suggests that DPNS inhibits the in vitro process of telomerase by interfering with the translocation of the enzyme or promoting the dissociation of the enzyme upon completion of template elongation. DPNS exhibits a noncompetitive mode of inhibition, which is distinct from the inhibition of G-quadruplex interactive inhibitors or nucleoside compounds. This mode of inhibition has also been observed with the non-nucleoside small-molecule

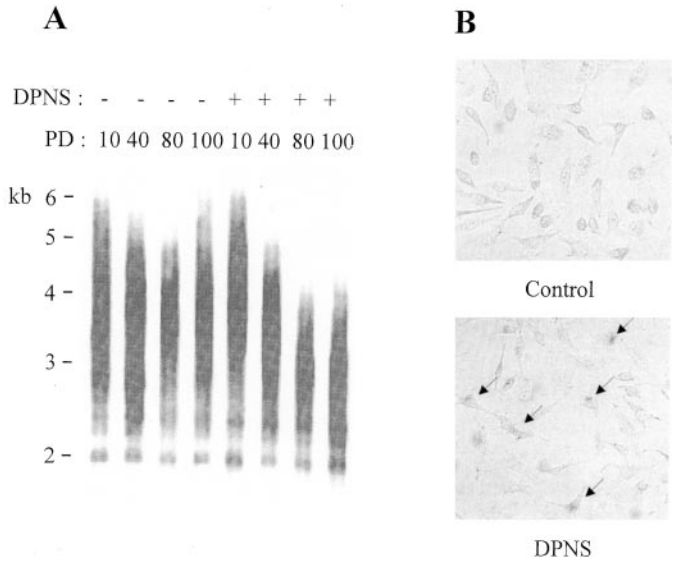

Fig. 6. Telomere shortening and cellular senescence induced by DPNS. A, HeLa cells treated with solvent alone or DPNS (1 μM) were harvested at various PDs, and genomic DNA was digested with *Rsa*I and *Hin*fI, followed by Southern blot analysis using a TTAGGG repeat as a probe. B, solvent- (control) or DPNS-treated cells at 100 PD were fixed and subjected to SA-β-gal staining followed by microscopy. Arrows indicate positively stained cells.

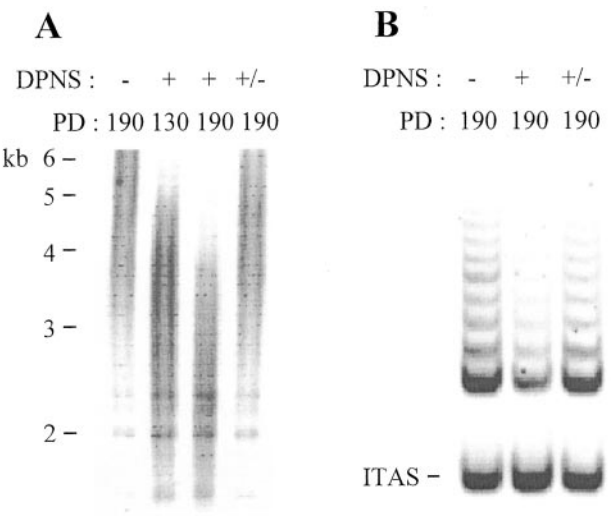

Fig. 7. Reversibility of inhibition by DPNS. A, HeLa cells were cultivated in the absence or presence of DPNS (1 μM) for indicated PDs. After growing for 130 PD, DPNS-treated cells were washed, replated in medium without inhibitor, and grown for additional 60 PD (lane indicated by +/−). Genomic DNA was assessed for TRF length by Southern blot analysis. B, TRAP assays were performed with cell lysates from cells grown in conditions described in A.

inhibitor BIBR1532 (Pascolo et al., 2002). From the observations described here, DPNS is proposed to inhibit telomerase activity through direct effects on the enzyme rather than via an interaction with G-quadruplex structures.

In an enzyme kinetic analysis, we detected a significant inhibition of the binding of the TS primer or the dNTPs to the telomerase enzyme in the presence of DPNS. Each substrate showed a 3-fold higher affinity to the free enzyme than to the enzyme-DPNS complex. Conversely, binding of the TS primer or the dNTPs reduced approximately 4-fold the affinity of the enzyme for DPNS. This mode of inhibition by DPNS corresponds to a mixed-type noncompetitive inhibition for the binding of both TS primer and dNTPs. A reasonable explanation is that the inhibitor is binding at a site distinct from the binding sites for the TS primer and the dNTPs, yet it is influencing the binding of the substrates. Presumably, these effects could be transmitted via a conformational change of the enzyme structure or a steric interference for the binding efficiency because of the close proximity between the inhibitor-binding site and the substrate-binding sites. Because the enzyme kinetic data described here do not support an allosteric inhibition mode, the hypothesis with alterations in the enzyme's conformation is less favorable.

Long-term exposure of HeLa cells to nonacute cytotoxic concentration (1 μM) of DPNS resulted in a marked reduction in telomere length and induced senescence phenotype expression of SA-β-gal. The average TRF length shortened from 4 to 2.5 kb at PD 100, corresponding to a telomere loss of 15 base pairs/PD. This telomere erosion rate is slightly lower than the speed of telomere erosion in the absence of telomerase (25–200 base pairs/PD) largely because of the end-replication problem (Lingner et al., 1995). Such a slow erosion rate may be explained by an assumption that the inhibitor concentration used for long-term treatment was not enough to completely inhibit the telomerase activity during cell division. A concomitant reduction in telomerase

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activity was detected in DPNS-treated cells using the TRAP assay. This observation suggests that DPNS-induced telomere erosion is mediated by a telomerase-inhibitory mechanism. Because DPNS has a higher affinity to the free enzyme than to the enzyme-substrate complex, the telomerase-inhibitor complexes might not be completely separated during the detergent-extraction step of the TRAP assay. Another possible mechanism for this effect is through a down-regulation of telomerase gene expression via the inhibition of telomerase activity by DPNS. However, this seems unlikely because no significant change was detected in steady-state levels of hTERT and human telomerase RNA transcripts between untreated and DPNS-treated cells (data not shown). Inhibitor depletion resulted in a rapid elongation of telomeres. When the DPNS-treated cells were cultivated in inhibitor-free medium for the prolonged period, the intracellular concentration of DPNS is gradually diluted out, and subsequently telomeres regain their original lengths. Because the treatment of a specific telomerase inhibitor results in gradual telomere erosion of cancerous cells and consequently in their senescence and death, the reversibility of telomerase inhibition and the telomere erosion by DPNS may have a significant implication on the pharmacodynamics of targeting telomerase in cancer therapy.

In conclusion, the large-scale screening of a chemical small-molecule library identified DPNS as a novel structural class of telomerase inhibitor. This compound could be useful as a lead for further experiments on the molecular mechanism of telomerase inhibition. As recently reported with the direct-acting telomerase inhibitor BIBR1532 (Damm et al., 2001) and a G-quadruplex–interactive inhibitor BRACO19 (Gowan et al., 2002), further studies on in vivo antitumor activity of DPNS are needed to determine the best candidates with an improved potential for in vivo efficacy. In addition, it will be of interest to examine the effects on telomerase inhibitory activity by variations on the basic nitrostyrene structure.

Acknowledgments

We thank S. W. Kim of Lead Genex Inc. for providing a chemical small-molecule library and S. T. Lee and J. B. Yoon for helpful discussions.

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