Identification of a quinoxaline derivative that is a potent telomerase inhibitor leading to cellular senescence of human cancer cells

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Telomere maintenance is essential for the continued proliferation of dividing cells, and is implicated in chromosome stability and cell immortalization. Telomerase activity allows cells to maintain their telomeric DNA and contributes to the indefinite replicative capacity of cancer cells. Telomerase is expressed in most cancer cells, but not in normal somatic cells, suggesting that telomerase is an attractive target for cancer chemotherapy. Here we screened a chemical library for inhibition of human telomerase, and identified 2,3,7-trichloro-5-nitroquinoxaline (TNQX) as a potent inhibitor. TNQX showed a potent inhibitory effect, with 50 % inhibition at ~1.4 μ M, and did not inhibit DNA and RNA polymerases, including retroviral reverse transcriptase. A series of enzyme kinetic experiments suggested that TNQX is a mixed-type

non-competitive inhibitor, with an inhibitor-binding site distinct from the binding sites for the telomeric substrate (TS) primer and the dNTPs. Long-term cultivation of the MCF7 cell line with a drug concentration that did not cause acute cytotoxicity resulted in progressive telomere erosion followed by an increased incidence of chromosome abnormalities and induction of the senescence phenotype. The results presented here indicate that TNQX is a highly potent and selective anti-telomerase agent with good potential for further development as a promising anti-cancer agent.

Key words: anti-cancer agent, chemical small-molecule library, inhibitor, senescence, telomerase, telomere.

INTRODUCTION

Telomeres are DNA sequences at the ends of linear chromosomes that enable cells to distinguish chromosome ends from double-strand breaks in the genome. Without functional telomeres, the ends of chromosomes are at great risk of degradation, recombination, or fusion by the cellular DNA repair system [1–3]. All dividing cells show a progressive loss of telomeric DNA during successive rounds of replication because the lagging strand of DNA synthesis is unable to replicate the extreme 3' end of the chromosome [4–7]. Thus telomere shortening has been proposed as a regulatory mechanism that controls the replicative capacity of primary cells and cellular senescence by acting as a 'mitotic clock' [8]. Based on this, 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 re-activation of telomerase [5,9]. Telomerase is a complex ribonucleoprotein reverse transcriptase composed of an RNA component (hTR) and the catalytic subunit (hTERT) [10]. The enzyme is responsible for adding hexanucleotide (TTAGGG)_n telomeric repeats on to the 3' ends of chromosomes in dividing cells, and thus stabilizing telomere length. The ability of cells to express telomerase activity is limited by the presence or absence of hTERT, because all human somatic cells constitutively express hTR [11].

Telomerase activity has been reported in a variety of malignant tumours and germline cells, but it is not found in normal adult somatic tissues, with the exception of stem cells [12–14]. Introduction of the telomerase catalytic subunit gene into normal somatic cells prevents telomere erosion and senescence, and extends the life spans of the cells [15–17]. These findings

suggest that tumour growth is correlated with telomerase activity, and that activation of telomerase may be an important step in human carcinogenesis. Based on this hypothesis, inhibition of the telomerase enzyme would result in telomere shortening and the subsequent growth arrest of cancer cells due to the effects of sustained telomere erosion followed by senescence or cell death. In contrast with traditional anti-cancer agents, which kill cells by an acute cytotoxicity route shortly after administration, telomerase inhibitors require much longer (several weeks) before their effects become apparent, since senescence and concomitant growth arrest requires multiple cell cycles. A delayed-senescence phenotype has been demonstrated in knockout mice lacking the telomerase RNA component [18], and in cell lines that express a dominant-negative form of hTERT [19,20].

Based on the hypothesis that telomerase may represent a suitable target for specific anti-cancer therapies, several strategies for the inhibition of telomerase have been designed and evaluated. The telomerase holoenzyme complex presents multiple potential sites for the development of inhibitors. Among the inhibitors that have been tested are antisense oligonucleotides targeting functional RNA or hTR [21,22], hammerhead ribozymes [23], reverse transcriptase inhibitors [24] and dominant-negative hTERT-derived proteins [19,20]. The growing family of potent telomerase inhibitors is being identified through the screening of chemical small-molecule libraries using a telomerase assay [25,26]. These inhibitors include isothiazolone derivatives [27], rhodacyanine derivatives [28] and BIBR1532 {2-[(*E*)-3-naphthalen-2-yl-but-2-enoylamino]-benzoic acid} [29]. Unlike the direct-acting telomerase inhibitors, some small-molecule inhibitors have been reported to inhibit telomerase activity by interacting with G-quadruplex DNA [30–33].

Abbreviations used: hTERT, catalytic subunit of human telomerase reverse transcriptase; hTR, RNA component of human telomerase; TS, telomeric substrate; TRAP, telomeric repeat amplification protocol; TRF, terminal restriction fragment; TNQX, 2,3,7-trichloro-5-nitroquinoxaline; RT-PCR, reverse transcriptase-PCR; PD, population doubling.

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Although telomerase inhibitors lead to the cellular senescence of cancer cells by reducing telomeres to crisis lengths, they may also induce the growth of other tumour subclones by enhancing genomic instability [34]. Thus the conflicting effects of telomere dysfunction and telomerase activation should be considered carefully when developing anti-telomerase drugs, and mechanistic evaluation of the action of novel drugs is important. In the present study we have identified a novel structural class of chemical compound as an inhibitor of human telomerase through the rapid screening of small molecules. The results indicate that the compound is a highly potent and selective telomerase inhibitor *in vitro*, with good potential for further development as a promising anti-cancer agent.

MATERIALS AND METHODS

Chemical compounds

The chemical small-molecule library screened for the inhibition of telomerase in the present study was obtained from LeadGenex, Inc. (Taejeon, Korea), and contained molecules with M_r values of approx. 400. To confirm the chemical identity of the lead telomerase inhibitor, the compound was re-synthesized, and the chemical structure was confirmed by proton NMR spectroscopy. M_r was determined by MS.

Cell lines

The mammary carcinoma cell line MCF7 and the fibrosarcoma cell line HT1080 were cultured in Dulbecco's medium, and the cervical cancer cell line HeLa was cultured in RPMI medium supplemented with 10 % (v/v) fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin in 5 % CO₂ at 37 °C. For long-term exposure, the cells were grown in 100-mm plastic dishes and exposed to 2,3,7-trichloro-5-nitroquinoxaline (TNQX) at a concentration of 1 µM dissolved in 0.1 % DMSO. Every 3–4 days, the cells were trypsinized, counted using a haematocytometer, and reseeded at a density of 2×10^5 cells/dish. Control cells were treated with corresponding DMSO concentrations. Cell growth inhibition was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay [35].

Preparation of partially purified telomerase

HeLa cells were washed twice in ice-cold PBS and then were lysed for 30 min on ice in a CHAPS lysis buffer [10 mM Tris/HCl, pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 0.5 % (v/v) CHAPS, 10 % (v/v) glycerol and 0.5 M NaCl]. The lysate was then centrifuged at 20 000 g for 30 min at 4 °C. The supernatant was loaded on to a 10–40 % (v/v) glycerol gradient (in buffer containing 10 mM Tris/HCl, pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM PMSF and 5 mM β-mercaptoethanol) and centrifuged at 25 000 rev./min for 24 h at 4 °C in a Beckman SW-28 rotor. The fractions were collected from the bottom of the gradient and assayed for telomerase activity using the telomeric repeat amplification protocol (TRAP) assay. The protein concentration was measured using a Bradford protein assay kit (Bio-Rad Laboratories).

Telomerase assay

TRAP was used for the analysis of telomerase inhibition, as described previously [13], with minor modifications. The conditions

for the linear phase of primer extension by telomerase were determined in the presence of saturating substrate concentrations [200 nM telomeric substrate (TS) primer and 100 µM of each dNTP] and different amounts of partially purified telomerase. A linear correlation between reaction time, enzyme concentration, and telomerase production was observed for incubations shorter than 10 min with 60 ng of partially purified telomerase (results not shown). Thus telomerase extension reactions were 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, 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 same TS primer and an 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 and TSNT primers were added to the PCR mixture as described previously [36]. Telomerase products were resolved on a 10 % (v/v) non-denaturing polyacrylamide gel and visualized by staining with SYBR Green (Molecular Probes). The signal intensity was quantified with a LAS-1000 Plus image analyser (Fuji Film).

Determination of kinetic constants

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

Assays of DNA and RNA polymerase activities

The effects of telomerase inhibitors on DNA and RNA polymerase activities were measured. *Taq* polymerase activity was assayed as described by the manufacturer (TaKaRa). The amplified products were stained with SYBR Green and quantified by densitometric analysis using a LAS-1000 Plus image analyser. DNA polymerase and reverse transcriptase assays were performed as described by the manufacturer (Promega) using commercially available Klenow fragment and M-MLV (Moloney murine leukaemia virus) reverse transcriptase. The reactions were performed in the presence of digoxigenin-11-dUTP (Roche), and the products were transferred on to nylon membranes by slot blotting. The reaction products were detected by Western blotting with anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche), and quantified by densitometric analysis using a LAS-1000 Plus image analyser. RNA polymerase activity was measured using the T7 RNA polymerase assay as described by the manufacturer (Promega). The reaction products were treated with RQ1 RNase-free DNase, stained with RiboGreen RNA quantification reagent, and quantified using a fluorimeter (TECAN).

Terminal restriction fragment (TRF) length analysis

To measure telomere length, genomic DNA was digested with *RsaI* and *HinfI* and separated on 0.7 % (w/v) agarose gel. DNA samples were transferred to a nylon membrane (Hybond N⁺; Amersham Pharmacia Biotech) and hybridized with the probe (TTAGGG)₂₀ labelled with digoxigenin-11-dUTP (Roche) using the random priming method. Detection relies on an anti-digoxigenin antibody conjugated with alkaline phosphatase

Figure 1 Chemical structure of TNQX

(Roche). The signal intensity was detected and quantified on a LAS-1000 Plus image analyser.

Reverse transcriptase-PCR (RT-PCR) analysis

Gene expression of hTERT and hTR was analysed in MCF7 cells exposed to TNQX by RT-PCR, as described previously [37]. Briefly, total RNA was isolated with Trizol (Gibco BRL), and cDNA was synthesized from total RNA using a reverse transcriptase with random primers (Promega). hTERT mRNA was amplified using the primer pair 5'-CGGAAGAGTG-TCTGGAGCAA-3' and 5'-GGATGAAGCGGAGTCTGGA-3'; hTR was amplified using the primer pair 5'-TCTAACCT-AACTGAGAAGGGCGTAG-3' and 5'-GTTTGCTCTAGAAT-GAACGGTGGGAAG-3'. The RT-PCR products from each sample were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT-PCR signal.

Assay of senescence-associated β -galactosidase activity

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 10 h with β -galactosidase stain solution containing 1 mg/ml X-Gal (5-bromo-4-chloroindol-3-yl β -D-galactopyranoside), 40 mM citric acid/sodium phosphate, pH 6, 5 mM potassium ferrocyanide, 5 mM ferricyanide, 150 mM NaCl and 2 mM $MgCl_2$. Cells were viewed in a Zeiss light microscope and photographed.

Flow cytometry

DNA content and cell cycle phase were analysed as described previously [38] using a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson).

RESULTS AND DISCUSSION

Potent inhibition of human telomerase by TNQX *in vitro*

Telomerase is capable of providing cells with a mechanism to circumvent gradual telomere erosion by synthesizing new telomeres. Inhibition of telomerase results in telomere erosion, leading to the arrest of tumour cell growth and associated limitations in replicative life span [25]. In the present study, we screened a chemical library for inhibition of human telomerase, and identified TNQX (Figure 1) as a potent inhibitor. To examine the effects of TNQX on the activity of human telomerase, we performed the TRAP assay using partially purified human telomerase. TNQX exhibited potent inhibition of human telomerase in a dose-dependent manner, with a mean IC_{50} of 1.4 μ M (Figure 2A). A marked decrease in telomerase activity was observed at concentrations higher than 2.5 μ M TNQX. As shown with inhibitors identified previously [39,40], TNQX preferentially inhibited the synthesis of longer extension products compared with the synthesis of shorter products at concentrations greater than 1 μ M. To compare the intensities of individual extension products, dose-response curves were generated for each product. The IC_{50} values for the two short products (bands 1 and 3) were 2.5 μ M and 1.6 μ M respectively. The formation of the two longer products (band 5 and 9) was inhibited with IC_{50} values of 1.1 μ M and 1 μ M respectively (Figure 2B). These results suggest that TNQX inhibits the *in vitro* processivity of telomerase by interfering with the translocation of the enzyme or by promoting the dissociation of the enzyme upon completion of template elongation.

Since the TRAP assay involves a PCR, TNQX was evaluated in a PCR to determine whether the compound is a specific inhibitor of telomerase. There was no inhibition of the PCR at concentrations of up to 10 μ M (Figure 2C). To test the selectivity of TNQX, we examined its inhibitory effects on DNA and RNA polymerase activities, including that of reverse transcriptase, under the assay conditions described in the Materials and methods section. None of these enzymes was inhibited at TNQX concentrations exceeding the IC_{50} value for telomerase (Figure 2C), suggesting that inhibition by TNQX is highly selective for telomerase.

Mode of inhibition of telomerase by TNQX

A series of enzyme kinetic experiments was performed to characterize the mode of inhibition by TNQX. Inhibition was

Figure 2 Specific inhibition of telomerase by TNQX *in vitro*

(A) Inhibition of telomerase activity was measured in a TRAP assay. Various concentrations of TNQX were added before extension of the TS primer by telomerase. The product bands used for quantification are indicated. ITAS, internal telomerase assay standard. (B) The intensities of TRAP products were normalized to the intensities of the corresponding bands in the control cells without inhibitor and plotted against the concentration of TNQX. (C) Effects of TNQX on DNA and RNA polymerases. The activities of *Taq* polymerase (pol), DNA polymerase, RNA polymerase and reverse transcriptase (RT) were assayed as described in the Materials and methods section. TNQX was added to the reaction mixtures at the concentrations indicated. For comparison, inhibition of telomerase by TNQX is also shown.

Figure 3 TNQX is a mixed-type non-competitive inhibitor of the binding of both TS primer and dNTPs to telomerase

(A) Telomerase activity was measured with the indicated concentrations of TS primer in the absence (●) or presence of TNQX at a concentration of 0.5 μM (■) or 2.5 μM (▲), and a Lineweaver–Burk plot of the velocity curves was constructed. (B) Michaelis–Menten constants for the TS primer in the absence (K_m) and in the presence (αK_m) of 2.5 μM TNQX. (C) Affinity constants for TNQX in the absence (K_i) and in the presence (αK_i) of TS primer. (D) Telomerase activity was measured with the indicated concentrations of dNTPs as shown in (A), and a Lineweaver–Burk plot of the velocity curves was constructed. (E) Michaelis–Menten constants for dNTPs in the absence (K_m) and in the presence (αK_m) of 2.5 μM TNQX. (F) Affinity constants for TNQX in the absence (K_i) and in the presence (αK_i) of dNTPs.

measured as a function of the two substrates (TS primer and dNTPs) required for telomerase activity *in vitro*. The standard conditions for a linear correlation between reaction time, enzyme concentration and telomerase product were determined as described in the Materials and methods section. Under these conditions, velocity curves were determined for different TS primer concentrations in the presence or absence of TNQX. A clear decrease in $V_{\max}$ was observed in the presence of increasing amounts of TNQX. Lineweaver–Burk plots of these data showed inhibition by TNQX to be non-competitive of the mixed type with the TS primer (Figure 3A). The Michaelis–Menten constant of the TS primer in the absence (K_m) and presence (αK_m) of 2.5 μM TNQX was estimated as ~ 0.75 nM and ~ 2.0 nM respectively, indicating a higher affinity of the TS primer for the free enzyme than for the enzyme–TNQX complex (Figure 3B). The binding constants of TNQX were calculated in the absence (K_i) and presence (αK_i) of the TS primer. The K_i and αK_i values were estimated as ~ 0.6 μM and ~ 2.2 μM respectively, indicating a higher affinity of TNQX for the free enzyme than for the enzyme–TS-primer complex (Figure 3C).

The velocity curve and the resulting Lineweaver–Burk plot were determined as a function of dNTP concentration in the presence or absence of TNQX (Figure 3D). The K_m value (~ 17 μM) for the dNTPs increased to ~ 28 μM in the presence of 2.5 μM TNQX (αK_m), suggesting a lower affinity of dNTPs for the enzyme–TNQX complex than for the free enzyme (Figure 3E). The binding constant of TNQX in the presence of the dNTPs ($\alpha K_i \sim 3.9$ μM) was higher than in their absence ($K_i \sim 1.8$ μM) (Figure 3F), indicating a lower affinity of binding of TNQX to the enzyme–dNTP complex than to the free enzyme.

Taken together, the enzyme kinetic data clearly suggest that TNQX is a mixed-type non-competitive inhibitor of the binding of both TS primer and dNTPs. A reasonable explanation is that the

Figure 4 Long-term cultivation in the presence of TNQX induces telomere shortening

(A) Comparison of the cell-free inhibition of telomerase using the TRAP assay (○) compared with acute cytotoxicity in MCF7 cells (●) caused by TNQX at various concentrations. Values represent means $\pm$ S.D. of three independent experiments. (B) MCF7 and HT1080 cells treated with solvent or TNQX were harvested at indicated PDs, and genomic DNA was digested with *RsaI* and *HinfI*, followed by Southern blot analysis using a TTAGGG repeat as a probe. MCF7 cells were also grown in the presence of 1 μM TNQX for 191 PDs, transferred to normal medium, and cultivated for additional 140 PDs (lane indicated by +/–). (C) Representative results of RT-PCR analysis of the expression of hTR and hTERT in solvent-treated, TNQX-treated and inhibitor-depleted (lane +/–) cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

inhibitor binds at a site distinct from the binding sites for the TS primer and the dNTPs, yet influences the binding of the substrates. Presumably, these effects could be due to a conformational change in the enzyme structure, or to steric interference with regard to binding efficiency due to close proximity of the inhibitor-binding site and the substrate-binding sites. We note that the enzyme kinetic data do not support an allosteric inhibition model. Thus the hypothesis that TNQX alters enzyme conformation is less plausible.

Long-term cultivation in the presence of TNQX induces telomere shortening in cancer cells

To examine the long-term effects of TNQX on telomerase-positive cancer cells, we first determined the non-acute cytotoxic drug concentration at which telomerase could be inhibited without acute growth inhibitory effects. Short-term cell viability was determined in a 4-day cytotoxicity assay using various drug concentrations. The IC_{50} value of TNQX for MCF7 cells was 10.8 μM . This value is approximately eight times higher than the concentration (1.4 μM) required for half-maximal inhibition in TRAP assay (Figure 4A). At a TNQX concentration of 10 μM , 46% of MCF7 cells were inhibited, but TNQX had no significant effects on short-term cell proliferation at concentrations below 5 μM . Accordingly, MCF7 cells were propagated exponentially in the presence of 1 μM TNQX to investigate the cellular consequences of long-term treatment. During long-term cultivation, cells were monitored periodically by telomere-length estimation using Southern blot analysis. Control cells treated with solvent alone exhibit a heterogeneous size distribution, with an average telomere length of ~ 4 kb (Figure 4B). The same TRF length was detected in inhibitor-treated cells at early population doublings (PDs), but TRF length was progressively shortened as cells were propagated in the

Figure 5 Induction of a senescence phenotype by TNQX

(A)–(C) Phase-contrast micrographs show the cellular morphology of solvent-treated, TNQX-treated and inhibitor-depleted (+/–) MCF7 cells respectively at the indicated PDs. (D)–(F) Cells were fixed and subjected to staining for senescence-associated β -galactosidase, followed by microscopy. Arrows indicate positively stained cells.

presence of inhibitor. The average TRF length had been shortened from 4 to 2 kb at 305 PDs. In contrast, solvent-treated cells maintained a stable TRF length up to 356 PDs. Comparable telomere erosion was observed in HT1080 fibrosarcoma cells treated similarly with inhibitor (Figure 4B). This telomere erosion rate is lower than the speed of telomere erosion in the absence of telomerase (25–200 bp/PD), due largely to the end-replication problem [7]. Such a low erosion rate may be explained by an assumption that the inhibitor concentration used for long-term treatment was not high enough to inhibit telomerase activity completely during cell division. As a control for telomerase inhibitor specificity, telomerase-negative SAOS-2 cells, which exhibit the alternative lengthening of telomere (ALT) phenotype [41,42], were treated with TNQX. The results (not shown) indicated that treatment with inhibitor had no effect on telomere length.

To examine the effects of inhibitor depletion on telomere length regulation, cells were transferred to normal medium without inhibitor after growing for 191 PDs in the presence of $1 \mu\text{M}$ TNQX, and then cultivated for another 140 PDs. Examination of TRF length at 331 PDs revealed a rapid elongation of the telomeres, with an increase in TRF length to ~ 4 kb (Figure 4B), demonstrating that the inhibition of telomerase by TNQX is fully reversible. Since treatment with the specific telomerase inhibitor results in gradual telomere erosion in cancerous cells, and consequently in their senescence and death, the reversibility of telomerase inhibition and telomere erosion by TNQX may have significant implications for the pharmacodynamics of targeting telomerase in cancer therapy.

Since TNQX-induced telomere erosion is mediated by a telomerase-inhibitory mechanism, we sought to examine the

underlying mechanisms by which TNQX inhibits telomerase activity. A possible mechanism is through down-regulation of telomerase gene expression by TNQX. However, no difference in either hTERT and hTR gene expression was detected between untreated and TNQX-treated cells (Figure 4C). Another possibility is that the decrease in telomerase activity is a consequence of direct cytotoxicity [43,44]. However, we used a drug concentration ($1 \mu\text{M}$) in long-term culture well below that required for acute cytotoxicity. Alternatively, the decrease in telomerase activity in the presence of TNQX may occur via an interaction with telomeric overhangs which can form G-quadruplex structure. However, since the mode of inhibition by TNQX described here is distinct from that of inhibition by G-quadruplex-interactive inhibitors, we propose that TNQX inhibits telomerase activity through direct effects on the enzyme rather than via interaction with telomeric overhangs. This mode of inhibition has also been observed with the non-nucleoside small-molecule inhibitors BIBR1532 [39] and DPNS [3-(3,5-dichlorophenoxy)-nitrostyrene] [40].

Telomerase inhibition by TNQX induces telomere dysfunction and a senescence phenotype

TNQX-treated cells showed distinctive morphological features associated with cellular senescence. In contrast with solvent-treated cells (Figure 5A), MCF7 cells treated with TNQX exhibited an increased size and a flattened morphology at 319 PDs (Figure 5B), and also stained positively for senescence-associated β -galactosidase (Figure 5E). To determine the effects of inhibitor depletion on the senescence phenotype, TNQX-treated cells were

Figure 6 Flow cytometric analysis of MCF7 cells treated with TNQX

(A) Solvent-treated, (B) TNQX-treated and (C) inhibitor-depleted (+/–) MCF7 cells at the indicated PDs were stained with propidium iodide, followed by FACS. The percentage of total cells in each phase of the cell cycle is shown. Results are representative of three separate experiments. (D) The percentage of total cells in the aneuploidy fraction is shown graphically. Values represent means $\pm$ S.D. of three independent experiments.

transferred to normal medium without inhibitor at 191 PDs. Induction of a senescence phenotype was not observed at 349 PDs (Figures 5C and 5F).

Flow cytometric analysis showed that 61 % of solvent-treated MCF7 cells were in the G₁ phase of the cell cycle (Figure 6A). In marked contrast, treatment with TNQX resulted in a sharp increase in the population of cells with DNA content indicative of late S phase (34 %), along with a decrease in G₁ phase (35 %) (Figure 6B). In addition, there was a substantial increase in the aneuploidy fraction (Figure 6D). Inhibition of telomerase by TNQX led to telomere dysfunction due to the effects of sustained telomere erosion, as shown in Figure 4(B). Cells that lack a protective telomeric structure respond to a dysfunctional telomere by undergoing chromosomal end-to-end fusion, leading to the subsequent senescence and growth arrest of cancer cells. In the presence of TNQX, cells arrest initially in late S phase due to incomplete DNA replication because of chromosomal end-to-end fusion, which results in dicentric chromosomes that are generally unstable in mitosis. This genomic instability greatly increases the frequency of translocation and rearrangement, as well as of anaphase bridges, which probably results in non-dysjunction and changes in chromosome number [45]. Similarly, telomeres in cells expressing the dominant-negative form of TRF2 become dysfunctional and generate end-to-end fused chromosomes that can be detected as dicentric and multicentric chromosomes [46]. When TNQX was depleted in cultures as described above, the cell cycle profile was quite similar to that of solvent-treated cells (Figure 6C). These results are consistent with the notion that cells with chromosomal end-to-end fusions and higher DNA contents underwent irreversible growth arrest associated with senescence, and were eventually eliminated during long-term cultivation. These effects of TNQX on the cell cycle were

accompanied by decreases in telomere length over the same period of drug treatment.

Conclusions

In conclusion, the large-scale screening of a chemical small-molecule library identified TNQX as a novel structural class of telomerase inhibitor. This compound fulfils many of the required criteria for a telomerase inhibitor. First, addition of TNQX led to progressive telomere shortening with each division. Secondly, TNQX decreased telomerase activity, but did not initially affect cell growth rates. Thirdly, addition of TNQX eventually caused cells to undergo growth arrest associated with senescence. Therefore this compound could be potentially useful as a lead molecule for further experiments on the molecular mechanism of inhibition of telomerase, as well as for *in vivo* and clinical studies aimed at telomerase targeting. In addition, it will be of immediate interest to examine the effects on telomerase inhibitory activity caused by variations of the basic quinoxaline structure. The synthesis and inhibitory activity of a wide range of TNQX derivatives will be useful for the characterization of the inhibitor-binding site of telomerase, which is apparently distinct from the TS primer- and dNTP-binding sites.

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