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Bioorganic & Medicinal Chemistry Letters 13 (2003) 3923–3926

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Triethylene Tetraamine: A Novel Telomerase Inhibitor

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Received 30 June 2003; revised 31 August 2003; accepted 4 September 2003

Abstract—Telomerase inhibition can be achieved by stabilization of G-quadruplex structure. Triethylene tetraamine, a small linear molecule, has been identified as a potent telomerase inhibitor. It stabilizes both intra- and inter-molecular G-quadruplexes and shows a good differential between potent telomerase inhibition and acute cytotoxicity.

Human telomerase is a specialized ribonucleoprotein with an intrinsic RNA component. It is able to utilize its endogenous RNA template to synthesize telomeric repeats and maintain stable telomere length. Telomerase activity has been detected in some 80–90% of all human cancers but not in adjacent normal cells.[1] Consequently, telomerase is an essential factor in cellular immortalization and tumorigenesis[2,3] and has been proposed as a potentially highly selective target for the development of a novel class of anticancer agent. Different approaches to telomerase inhibition currently consist of targeting the RNA component of telomerase with antisense oligonucleotides,[4] peptide nucleic acids,[5] ribozymes[6] or disturbing the telomeric structure and blocking the telomere/telomerase interaction with G-quadruplexes affine agents.

In cells, the natural substrate for the enzyme is the single-stranded DNA 3′-overhang at the end of the telomere. The G-rich single-stranded DNA can adopt an unusual four-stranded DNA structure in solution termed quadruplexes.[7] Working pioneered by Zahler et al.[8] and later by Hurley's group[9] showed that telomerase requires access to a single-stranded region of telomeric DNA in order to bring about the required elongation. These findings suggest that telomere extension by telomerase could be inhibited if agents could be found that act to stabilize the folded G-quadruplex structure. Following these discoveries, a variety of small molecules have been devised to selectively promote the formation and/or stabilization of such higher-order structures, ranging from derivatives of anthraquinones[10] to porphyrins,[11] acridines[12] and perylene.[13]

In this report, a small linear molecule, triethylene tetraamine (TETA), exhibited interesting properties of stabilizing both inter- and intra-molecular G-quadruplex structures and telomerase-inhibitory potency. Moreover, it shows good characters of potent telomerase inhibition and acute cytotoxicity.

After DNA solutions were prepared in 10 mM Tris–HCl buffer, pH 7.4, in the presence/absence of K^+ and/or TETA, CD spectroscopy had been used to distinguish between parallel and anti-parallel G-quadruplex structures formed by two G-rich oligonucleotides, the oligonucleotide d $(TTAGGG)_4$ and d (TGGGGT). In previous studies, it has been identified that the oligonucleotide d $(TTAGGG)_4$ can form a fold-over intramolecular G-quadruplex structure in the presence of K^+.[14,15] Figure 1(a) showed a positive band around 295 nm with a shoulder around 270 nm indicating that a major fraction of molecules adopted an anti-parallel G-quadruplex structure. When TETA was added at concentration of 20 μM, the shoulder band decreased distinctively. This probably indicated the increase of anti-parallel G-quadruplex structure. Oligonucleotide d (TGGGGT) can form intermolecular parallel G-quadruplex structure.[16–18] In Figure 1(b), the positive band around 260 nm and negative band around 240 nm were typical of a parallel G-quadruplex structure. The addition of the agent increased the intensity of the band but

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0960-894X/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2003.09.004

not influences its position, which indicated there were no changes in the G-quadruplex structure.

Melting studies are widely used for determining the stability of nucleic acids and their interactions with ligands. The temperature at the mid-point of the transition from multi-stranded to single-stranded structure indicates the stability of the structure and changes in this value are used to compare the effect of drug binding. UV absorbance is the most commonly used technique for determining the melting temperature (T_m). It is a qualitative but rapid and convenient initial method to identify promising G-quadruplex ligands. In the case of intramolecular G-quadruplex formed by d(TTAGGG)$_4$, the addition of TETA increased the melting temperature of the G4 by about 5 °C at 20 μM TETA concentration. When there was no K^+, the agent could also stabilize the G-quadruplex structures. With the increase of the agent concentration from 10 to 100 μM, the value of T_m increased by about 10 °C. In the case of intermolecular G-quadruplex formed by d(TGGGGT), the effect of TETA was even more significant. When K^+ was present, the addition of TETA increased the T_m by about 8 °C. Adding increasing amounts of the agent (from 10 to 100 μM) to the oligonucleotides led to the T_m increased up to 26 °C progressively (Table 1).

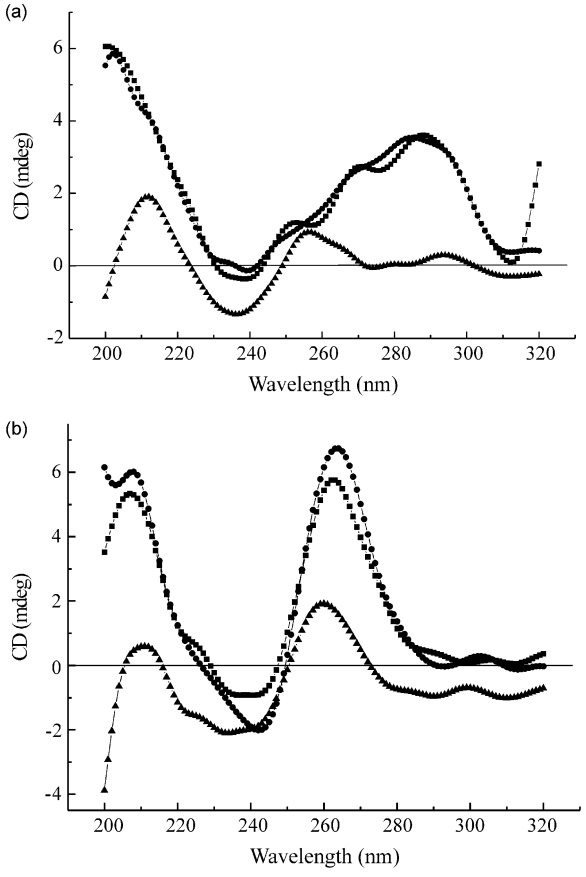

Figure 1. CD spectroscopy studies. (a) CD spectra of d(TTAGGG)$_4$ in buffers containing 100 mM KCl (square), 100 mM KCl and 20 μM TETA (circle), and no KCl or TETA (triangle). (b) CD spectra of d(TGGGGT) in buffers containing 1 mM KCl (square), 1 mM KCl and 20 μM TETA (circle), and no KCl or TETA (triangle).

Stabilizing of the G-quadruplex structures has been achieved with a range of small molecules, sharing the structural features of a planar electro-deficient chromophore and basic side chain(s), including derivatives of amido-substituted anthraquinones, acridines, and porphyrins. The planar aromatic chromophores are thought to be the key structural features.[19] However, the compound we described here has a linear structure without aromatic chromophores. Based on the simulating results of interaction between the TETA and G-quadruplex, we deduce that this agent interacting with G-quadruplex is more likely in a manner of metal ions. The distance between tetras of the G-quadruplex is 3.4–3.7 Å,[20] slightly larger than found in duplexes. While the distance between nitrogen atoms in TETA is about 3.7 Å (Fig. 2). The similar distance between tetras and nitrogen makes it possible that TETA inserts into the cavity of G-quadruplex and nitrogen sites in the two tetras. And the structural differences between parallel- and anti-parallel-G-quadruplex maybe the reasons for the different effect of TETA on G-quadruplex. However the precise binding mechanism of TETA to the quadruplex remains to be clarified. To identify the exact mode of binding to quadruplex structures, it needs additional studies specifically designed to address this question.

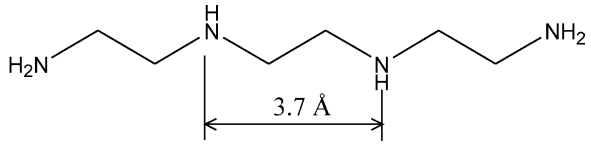

Figure 2. The chemical structure of TETA.

Table 1. Melting temperatures of G-quadruplexes

Oligonucleotide d (TTAGGG)$_4$		Oligonucleotide d (TGGGGT)	
Samples[a]	T_m (°C)	Samples[a]	T_m (°C)
100 mM K^+	57	1 mM K^+	47
100 mM K^+ + 20 μM TETA	62	1 mM K^+ + 20 μM TETA	55
0 mM K^+ + 10 μM TETA	57	0 mM K^+ + 10 μM TETA	45
0 mM K^+ + 20 μM TETA	58	0 mM K^+ + 20 μM TETA	51
0 mM K^+ + 50 μM TETA	61	0 mM K^+ + 50 μM TETA	64
0 mM K^+ + 100 μM TETA	67	0 mM K^+ + 100 μM TETA	73

[a]Samples were prepared in a 10 mM Tris?HCl buffer, pH 7.4, with KCl and TETA at indicated concentrations.

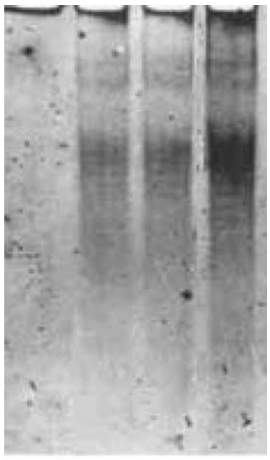

Figure 3. Telomerase inhibition of TETA by telomerase repeat amplification protocol (TRAP) assay. The reaction mixture (50 μL) contains 20 mM Tris–HCl (pH 8.3), 68 mM KCl, 1.8 mM $MgCl_2$, 0.05% Tween 20, 0.1 mM EGTA, 50 μM dNTP, 0.1 μg TS primer [d(AATCCGTCGAGC AGAGTT)], 2U Taq DNA polymerase, 1 μg of HeLa cell extract. TETA were added at the final shown concentration. After extension at 25 °C for 30 min, the reaction mixtures were subjected to denaturation for 2 min at 94 °C, and 30 s for 94 °C, 45 s for 55 °C and 90 s for 72 °C for 35 PCR cycles with 0.1 μg CX primer [d(AATCCCATTCCCATTCCCATTCCC)] added. After telomerase-extended, PCR products was separated with 10% polyacrylamide gel electrophoresis, silver staining was performed. Lane 1, negative control (lysis buffer); lane 2, cell extract plus 20 μM TETA; lane 3, cell extract plus 2.5 μM TETA; lane 4, positive control (normal HeLa cells extract).

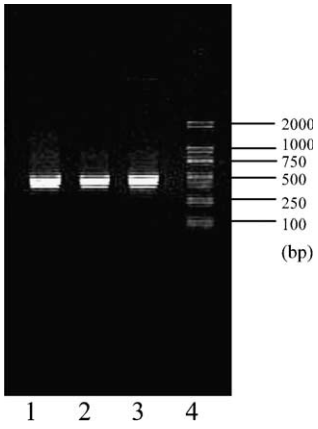

Figure 4. Taq polymerase assay. TETA at 20 and 100 μM final concentrations in a PCR 25 μL master mix contains 10 ng mammalian expression vector PET-32a(+) (TaKaLa), forward: d(CCGAATTC ATTGAGGGACGCAGCAGCCTGCTGCA ATTCAG) and reverse: d(CCGGATCCTCATTAGCAT TTCTCTGACTT) primers (200 nmol), respectively, 2.5 mM dNTP 4 μL and 1 U ExTaq. The target product about 400 bp was visualized on a 2% (wt/wt) agarose gel after amplification (30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s). Lane 1: Control (distilled water); lane 2: 20 μM TETA; lane 3: 100 μM TETA; lane 4: DL2000 Marker (TaKaLa, Japan).

To verify the effect of TETA on the proliferation of telomerase-positive cells, growth inhibition was measured in HeLa cell line using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay.[21] IC_{50} value, that is the concentration required to inhibit cell growth by 50%, was 74.8 μM. The value showed that TETA could inhibit the proliferation of HeLa cells distinctively. Moreover, at this concentration, the agent had no detectable cytotoxicity in HeLa cells and HLF cells from the LDH results, and the LD_{50} values, that is the dose required to cells lethal by 50%, for HLF cells, a normal cell line was 3.1 mM. These results demonstrated that target cells (telomerase positive cells) were more sensitive for TETA than normal cells.

The effect of TETA on telomerase activity had been examined using the modified telomeric repeat amplification protocol (TRAP) assay in HeLa cells, which express high levels of processive telomerase activity. The assay clearly showed that TETA was a potent inhibitor of telomerase activity in the micromolar range ($IC_{50} = 7.8$ μM, Fig. 3). To discriminate between telomerase elongation inhibition and Taq polymerase inhibition during the amplification steps of the assay, TETA was tested independently with Taq polymerase and a DNA substrate unable to fold into G-quadruplexes. Taq polymerase was not inhibited even at high concentration of 100 μM (Fig. 4). Therefore, telomerase inhibition, rather than Taq polymerase inhibition, is responsible for the observed effect on TRAP.

In summary, the linear small-molecule, TETA, has been identified as a potent telomerase inhibitor. It stabilizes the G-quadruplex structures not only in the presence of K^+ but also in the absence of it. This effect is more notable to intermolecular G-quadruplex. Moreover, TETA shows a good differential between potent telomerase inhibition and acute cytotoxicity.

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