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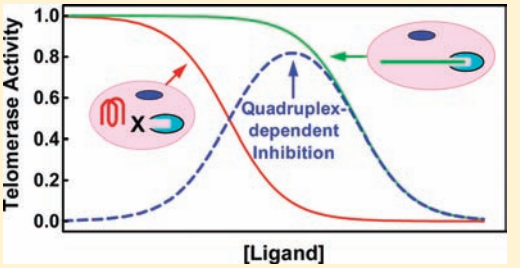
Contribution of Telomere G-Quadruplex Stabilization to the Inhibition of Telomerase-Mediated Telomere Extension by Chemical Ligands

Chang-yue Chen,[†] Quan Wang,[‡] Jia-quan Liu,[†] Yu-hua Hao,[‡] and Zheng Tan*[,†,‡]

[†]Laboratory of Biochemistry and Biophysics, College of Life Sciences, Wuhan University, Wuhan 430072, P. R. China

[‡]State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, P. R. China

ABSTRACT: Inhibition of telomerase activity through stabilizing telomere G-quadruplex with small chemical ligands is emerging as a novel strategy for cancer therapy. For the large number of ligands that have been reported to inhibit telomerase activity, it is difficult to validate the contribution of G-quadruplex stabilization to the overall inhibition. Using a modified telomere repeat amplification protocol (TRAP) method to differentiate the telomere G-quadruplex independent effect from dependent ones, we analyzed several ligands that have high affinity and/or selectivity to telomere G-quadruplex. Our results show that these ligands effectively inhibited telomerase activity in the absence of telomere G-quadruplex. The expected G-quadruplex-dependent inhibition was only obvious for the cationic ligands at low K^+ concentration, but it dramatically decreased at physiological concentration of K^+. These observations demonstrate that the ligands are much more than G-quadruplex stabilizers with a strong G-quadruplex-irrelevant off-target effect. They inhibit telomerase via multiple pathways in which stabilization of telomere G-quadruplex may only make a minor or neglectable contribution under physiologically relevant conditions depending on the stability of telomere G-quadruplex under ligand-free conditions.



INTRODUCTION

Telomere length homeostasis is a prerequisite for the unlimited growth potential of cancer cells. Due to the end-replication problem, telomere DNA in animal cells shortens in DNA replication at each cell division. In more than 85% of cancer cells, telomere length homeostasis is maintained by telomerase, a ribonucleoprotein that synthesizes telomere repeats onto telomere ends to compensate the loss of telomere sequence. Human telomere DNA terminates with a 3′ single-stranded G-rich overhang that can fold into a four-stranded G-quadruplex structure and, as a result, inhibit telomerase activity. For this reason, telomere G-quadruplex stabilization by small chemical ligands is being enthusiastically explored as a novel chemotherapeutic strategy against cancer. A growing number of synthetic or natural ligands have been identified to stabilize G-quadruplex and inhibit telomerase-mediated telomere extension. Such ligands have also been shown to induce growth arrest, senescence, or apoptosis in cancer cells (for recent reviews, see refs 1 and 2).

Chemical ligands can be characterized by spectroscopic[3] or calorimetric[4] melting assays and inhibition of enzyme-catalyzed reactions[5−7] regarding their G-quadruplex-stabilization ability. As to their eventual effect on telomerase-mediated telomere extension, a telomere repeat amplification protocol (TRAP)[8,9] or a modified version (TRAP-G4),[10] and a direct primer extension method[11] have been used to measure telomerase activity in the presence of ligands. Even though a ligand is intended to stabilize telomere G-quadruplex, it may, however, target at other components of the telomere extension machinery. These assays quantitate the overall effect of a ligand on the in vitro telomere extension by telomerase, but they provide no information on how telomere G-quadruplex stabilization contributes to the inhibition. As a result, it is difficult to validate G-quadruplex stabilization as a source of telomerase inhibition and judge whether a ligand is acting in a way it is designed to.

In order to evaluate the contribution of telomere G-quadruplex stabilization, we modified the conventional TRAP by comparing inhibition in the presence and absence of telomere G-quadruplex and analyzed several ligands that have been reported to have high affinity and/or selectivity to telomere G-quadruplex and efficiently inhibit telomerase-mediated telomere extension. Unexpectedly, our data showed that all the ligands produced similar inhibition regardless of the presence of telomere G-quadruplex, suggesting that either the method is unable to discriminate G-quadruplex-dependent inhibition or such inhibition is overweighed by G-quadruplex-irrelevant ones. At this end, we established a more stringent single-tube/two-color TRAP (ST/TC-TRAP) method that simultaneously assays inhibition on the extension of two substrates with or without the ability to form G-quadruplex by a same telomerase carrying a mutated RNA template that adds nontelomeric repeats to the substrates. In addition to confirming the G-quadruplex-irrelevant inhibition in the above assays, the ST/TC-TRAP assays detected obvious G-quadruplex-dependent inhibition at low K^+ concentration

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Table 1. Primers Used in the Conventional TRAP Assay[a]

telomerase	RNA template (3′-5′)	downstream primer sequence (5′-3′)
WT	CAAUCCCAAUC	GCGCGGCTTACCCTTACCCTTACCCTAACC
CUA	CAACUACAACU	GCGCGGCCTACATCTACATCTACATCAACA
CAC	CAAUCACAAUC	GCGCGGCTTACACTTACACTTACACTAACA

[a] TS (5′-AATCCGTCGAGCAGAGTT-3′) was used as substrate for both the wild-type (WT) and mutated (CUA or CAC) telomerase, as well as upstream primer for PCR amplification. Underlined residuals show mutation introduced to the RNA template of telomerase to abolish G-quadruplex formation in extended repeats.

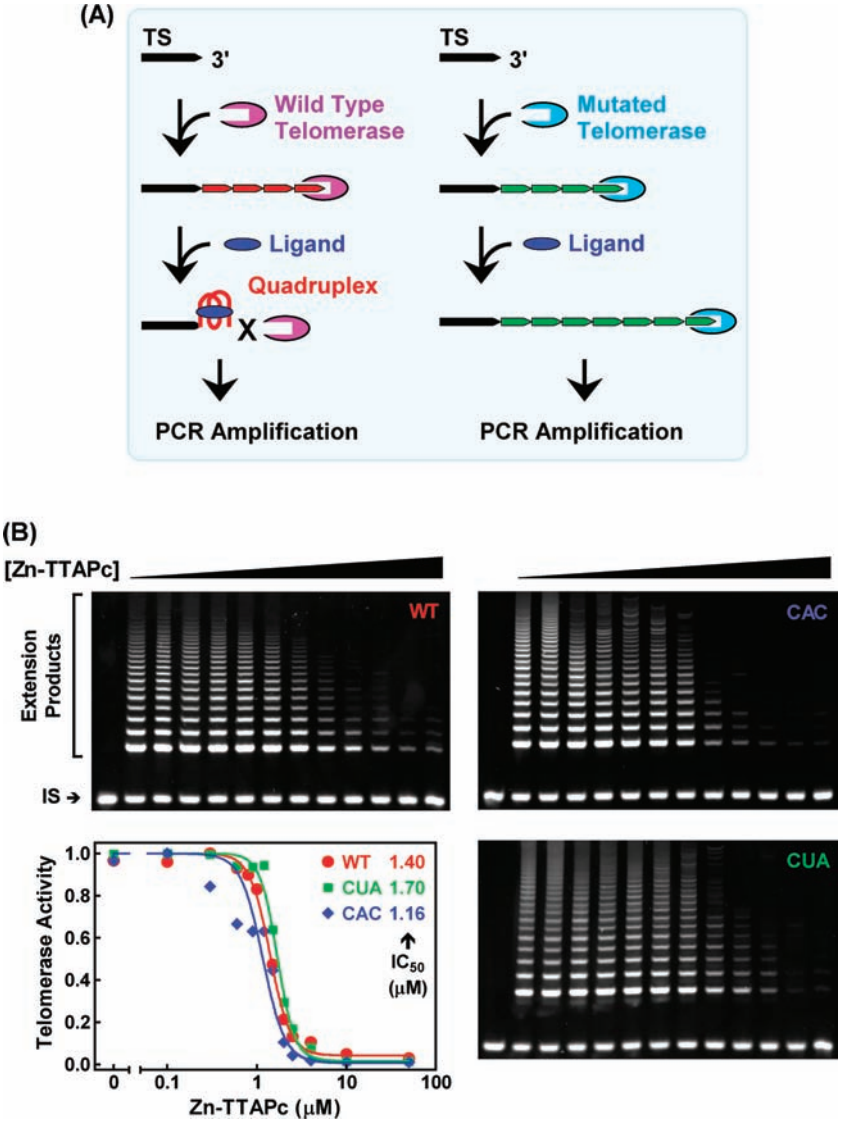

Figure 1. (A) Illustration of analysis of telomere G-quadruplex stabilization by ligand on inhibition of telomerase-mediated primer extension by TRAP. Linear substrate TS was extended by wild-type (left) or mutated (right) telomerase. Inhibition via G-quadruplex stabilization only occurs to the wild-type telomerase after addition of four or more telomeric repeats, but not to the mutated telomerase because its extension products do not form G-quadruplex. (B) Inhibition of Zn-TTAPc on primer extension by wild-type (WT) or mutated (CUA, CAC) telomerase. TS (330 nM) was extended using normal dNTPs and amplified by PCR in the presence of an internal standard (IS). Telomerase activity was expressed as the fraction of the control sample containing no ligand (lane 2 of each gel). The first lane in each gel is a negative control using heat-inactivated telomerase without ligand. Extension buffer contained 63 mM K^+.

for the ligands that could stabilize G-quadruplex. Such inhibition was, however, dramatically reduced at physiological concentration of K^+ and the G-quadruplex-irrelevant effect became the major source of inhibition. These results indicate that the ligands can inhibit telomerase via multiple pathways among which stabilization of telomere G-quadruplex may only make a minor or negligible contribution depending on the stability of telomere G-quadruplex under ligand-free conditions.

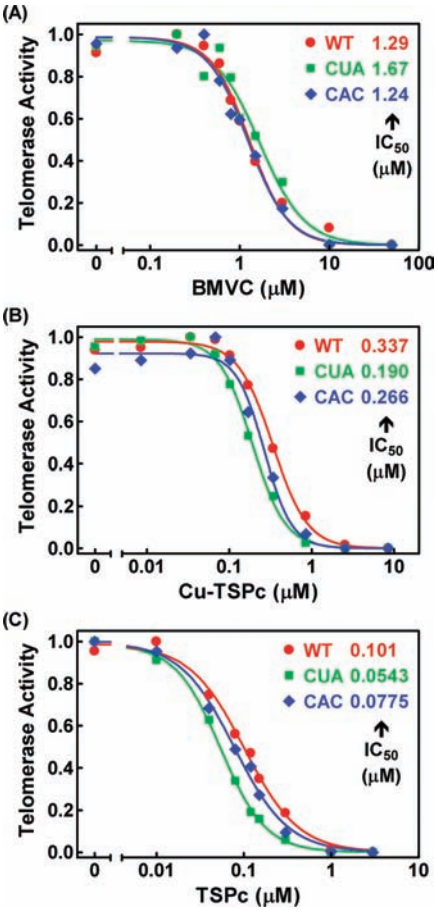

Figure 2. Inhibition of (A) BMVC, (B) Cu-TSPc, and (C) TSPc on primer extension by wild-type (WT) and mutated (CUA, CAC) telomerase analyzed by TRAP. Conditions were the same as in Figure 1B.

MATERIALS AND METHODS

Oligonucleotides and Chemical Ligands. Oligonucleotides were purchased from Invitrogen (Shanghai, China), and fluorescently labeled oligonucleotides from TaKaRa Biotech (Dalian, China). Phthalocyanine tetrasulfonate hydrate (TSPc) (product # P4374) and copper phthalocyanine-3,4′,4″,4‴-tetrasulfonic acid tetrasodium (Cu-TSPc) (product # 245356) were purchased from Sigma-Aldrich. 3,6-Bis(1-methyl-4-vinylpyridinium)carbazole diiodide (BMVC) was a generous gift from Dr. T.C. Chang at the Institute of Atomic and Molecular Sciences Academia Sinica, Taipei, Taiwan, ROC, and tetrakis(2-trimethylaminoethylethanol) phthalocyaninato zinc tetraiodine (Zn-TTAPc) from Dr. Xiang Zhou at the College of Chemistry and Molecular Sciences, Wuhan University, Wuhan, China. Solutions of ligand were prepared at 10 mM in DMSO and kept at −20 °C in dark. Further dilution was freshly made in buffer containing 100 μg/mL BSA before use.

Reconstitution of Wild-Type and Mutated Human Telomerase. Mutations were introduced to the plasmids pUC119-hTR-(+1-451) at the hTR template (Table 1) by site-directed mutagenesis. Transcription of hTR RNA was carried out using the plasmid pUC119-hTR(+1-451) or its mutants and the T7 transcription kit (Fermentas, Lithuania) as described.[12] Expression of hTERT and its reconstitution were carried out as described.[13]

TRAP Assay. Conventional assays were carried out as described[7,9,14] with modifications. TS primers were incubated with ligands at 30 °C for 30 min; extension was initiated by addition of telomerase

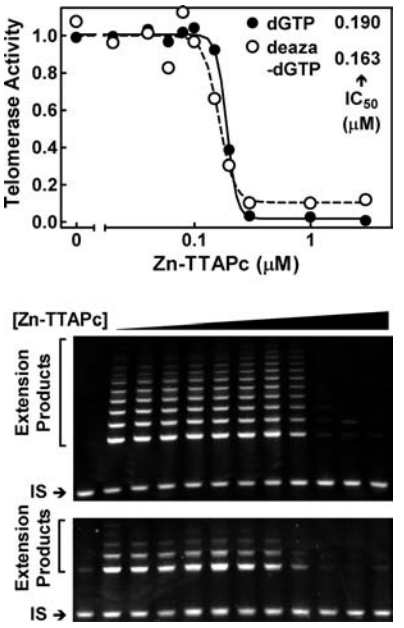

Figure 3. Inhibition of Zn-TTAPc on primer extension by wild-type telomerase analyzed by TRAP using either normal dGTP or 7-deaza-dGTP to substitute dGTP in the extension step. 7-deaza-dGTP prevented G-quadruplex formation in the extension product. Other conditions were the same as in Figure 1B except that extension was conducted with 10 nM TS at 0.5 mM K^+. Top gel: extension with normal dGTP. Bottom gel: extension with 7-deaza-dGTP.

(WT, CUA, or CAC), normal dGTP or 7-deaza-dGTP, and the other three normal dNTPs. The reaction was maintained at 30 °C for 10 min before being heat inactivated at 94 °C for 10 min. Ligands (Zn-TTAPc, BMVC) were removed by phenol extraction and ethanol precipitation to avoid interference in the PCR step.[15] Cu-TSPc and TSPc could not be extracted so that the extraction was omitted for these two ligands. They did not affect PCR at the concentration we used, as was reported previously (where TSPc was designated as APC).[16] Extension products were amplified with 330 nM of primer pair (Table 1) in the presence of a TSNT internal standard, resolved on 12% polyacrylamide gel, stained with SYBR Green, scanned on a FLA 7000 imager (GE Healthcare, America), and quantitated using the software ImageQuant 5.2. The theoretical curve and IC_{50}, that is, total ligand concentration required to reach half maximal inhibition, were obtained by fitting telomerase activity to total ligand concentration using the Hill function:[17]

$$A = A_{\min} + \frac{A_{\max} - A_{\min}}{1 + 10^{H\left[\log(L/IC_{50})\right]}}$$

where A denotes the activity of telomerase, $A_{\min}$ and $A_{\max}$ the minimal and maximal activity of telomerase, respectively, H the Hill coefficient, and L ligand concentration.

Single-Tube/Two-Color TRAP (ST/TC-TRAP) Assay. This assay followed the same procedures as the conventional TRAP assay except that different primers and internal standard (Table 3) were used. Two primers, GTS and MTS, labeled at the 5′ end with Cy3 and Cy5, respectively, were mixed together and extended in the same tube by the mutated telomerase CUA at the indicated K^+ concentration. Extended products (2.5 μL) were amplified using Cy3-GTS and Cy5-MTS, and a shared downstream primer (SDP), at 400 nM each in 25 μL volume. A modified internal standard (SIS) was coamplified by two primers labeled at the 5′ end with Cy3 and Cy5, respectively. KCl used in the PCR of the conventional TRAP assay was replaced by LiCl to prevent the GTS primer from folding into G-quadruplex. PCR products were resolved on

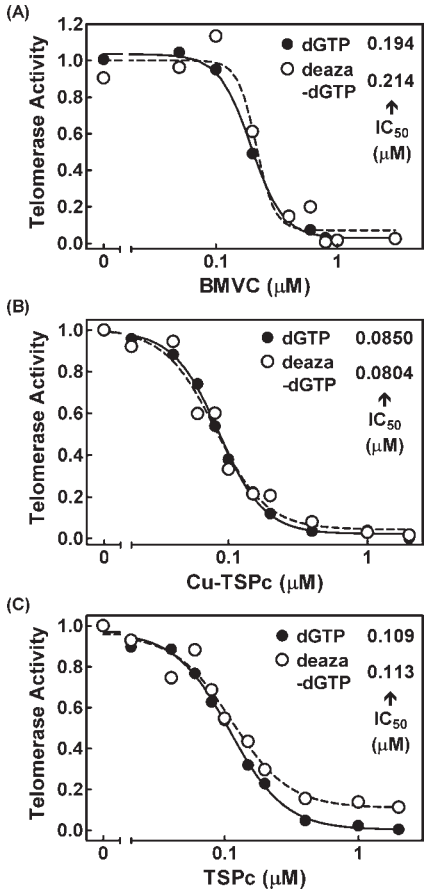

Figure 4. Inhibition of (A) BMVC, (B) Cu-TSPc, and (C) TSPc on primer extension by wild-type telomerases analyzed by TRAP using either normal dGTP or 7-deaza-dGTP to substitute dGTP in the extension step. Other conditions were the same as in Figure 3.

12% polyacrylamide gel, scanned on a Typhoon 9400 imager (GE Healthcare, America), and quantitated using the software ImageQuant 5.2. The theoretical curve and IC_{50} were obtained as in the conventional TRAP assay.

Melting Assay Based on Fluorescence Resonance Energy Transfer (FRET Melting). FRET melting experiments were carried out as described[14,18] in 20 mM lithium cacodylate buffer (pH 7.4) containing 0.5 μM $(G_3T_2A)_3G_3$, labeled at the 5′ end with fluorescein (FAM) and the 3′ end with tetramethylrhodamine (TAMRA), 0.5 μM ligand, and 0.5 mM or 150 mM KCl on a Mx3000P real-time PCR system (Stratagene).

RESULTS

Failure to Detect Telomere G-Quadruplex-Dependent Inhibition by TRAP Using Wild and Mutated Telomerase. The conventional TRAP uses a nontelomeric TS primer as substrate for telomerase extension.[9] We first attempted to differentiate G-quadruplex-dependent inhibition from G-quadruplex-irrelevant effects using reconstituted telomerases, in which the RNA template was mutated such that the extension repeats were unable to form G-quadruplex (Figure 1A and Table 1). Any inhibition to these mutants would be irrelevant to telomere G-quadruplex. In contrast, the extension by reconstituted wild-type telomerase permitted G-quadruplex formation when four or more telomeric repeats were added to the primer.

Table 2. Increase in $T_{1/2}$ of Telomere G-Quadruplex Induced by Ligands[a]

	$T_{1/2}$ increase (°C)			
K^+ (mM)	Zn-TTAPc	BMVC	Cu-TSPc	TSPc
0.5	8	7	0	0
150	2.5	6.5	~1	~1

[a] Each melting assay used 0.5 μM FAM-5′-$(G_3T_2A)_3G_3$-3′-TAMRA and equimolar ligand. The $T_{1/2}$ was 42 and 69 °C at 0.5 and 150 mM K^+, respectively, in the absence of ligands.

Stabilization of G-quadruplex by ligands in this case would result in G-quadruplex-dependent inhibition in addition to the G-quadruplex-irrelevant ones. With this additional source of inhibition, a greater inhibition to the wild telomerase than to the mutated telomerase would be expected.

Based on this strategy, we tested four ligands, Zn-TTAPc,[19,20] BMVC,[21] Cu-TSPc,[16] and TSPc,[16] all of which have shown to have high affinity and/or selectivity to human telomere G-quadruplex and effective inhibition on telomerase activity. Figure 1B shows the results obtained from the wild-type (WT) and two mutated telomerases (CUA and CAC) in the presence of increasing concentrations of Zn-TTAPc. Zn-TTAPc is a cationic phthalocyanine with four ammonium groups on the periphery of a planar structure.[20] Due to their excellent shape complementarity to the G-quadruplex quartet plane, phthalocyanines have a dissociation constant (K_d) to G-quadruplex at the nanomolar level that is hundreds to thousands fold smaller than that to tRNA and corresponding cDNAs.[22,23] However, Zn-TTAPc displayed similar inhibitions to all three telomerases despite its high affinity to G-quadruplex. In Figure 2, the effect of the three other ligands, BMVC, TSPc, and Cu-TSPc, is presented. BMVC is a cationic carbazole derivative that has a K_d of a few nanomolar to human telomere G-quadruplex[24] and has been used to selectively stain human telomere G-quadruplex among other forms of DNA structures.[25] As is seen in Figure 2A, BMVC produced almost identical inhibition to the three telomerases. Being positively charged, Zn-TTAPc and BMVC can potentially interact with DNA primer through electrostatic attraction regardless of its structural form. This may compromise their selectivity to G-quadruplex while bringing high affinity to their targets. For this concern, anionic phthalocyanines Cu-TSPc and TSPc have recently been explored and shown to bind telomere G-quadruplex with high selectivity and inhibit telomerase activity in the presence of excessive DNA duplexes.[16] Therefore, we further tested whether they can better inhibit the wild-type telomerase. Like Zn-TTAPc and BMVC, Cu-TSPc and TSPc also produced similar inhibition to the three telomerases (Figure 2B,C).

The inhibition to the mutated telomerases indicates the presence of telomere G-quadruplex-independent inhibition by the four ligands. The similar IC_{50} values for both the native and mutated telomerases suggest that telomere G-quadruplex stabilization was not a dominant source of inhibition. This is also supported by the fact that the IC_{50} values to the native telomerase are far different from the reported K_d values for the ligands/G-quadruplex binding.[16,22,23] If G-quadruplex stabilization were the dominant source of inhibition, IC_{50} values near their K_d values would be expected for these ligands. For Zn-TTAPc and BMVC, a greater IC_{50} than K_d might be explained by the presence of other nucleic acids and proteins present in the assays since the

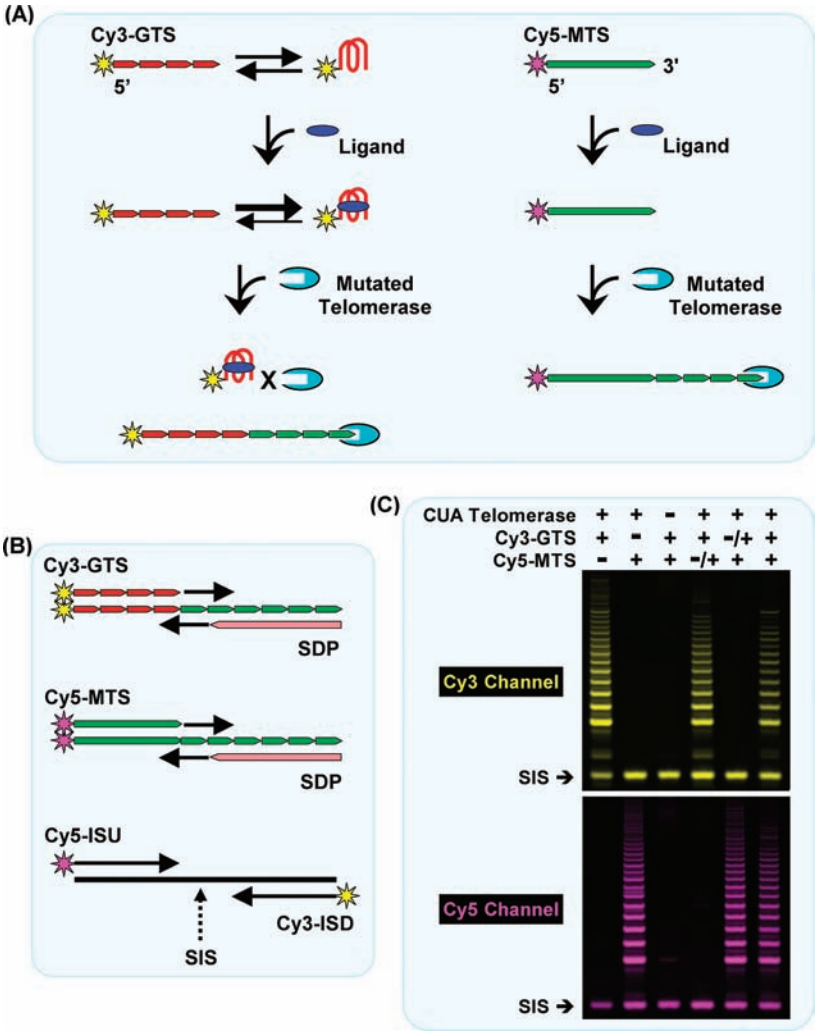

Figure 5. Illustration of the single-tube/two-color TRAP (ST/TC-TRAP) assay for analysis of G-quadruplex specific inhibition by ligands on telomerase-mediated primer extension. (A) Two fluorescently labeled primers, GTS (left) and MTS (right), with and without the ability to form G-quadruplex, respectively, were extended by a mutated telomerase (CUA) and amplified by PCR in the presence of a shared internal standard (SIS) in the same vial. G-Quadruplex formation promoted by ligand in the GTS reduces the amount of available substrate to inhibit extension. Inhibition on the linear MTS substrate reflects G-quadruplex-independent effect. The difference in inhibition between the GTS and MTS reflects G-quadruplex specific inhibition (Figure 7A,B). (B) Scheme of PCR amplification of telomerase extension products and shared internal standard (SIS). (C) Gel electrophoresis of ST/TC-TRAP products showing absence of cross amplification in PCR and fluorescence spillover in detection. Both channels were scanned from a same gel. Primers indicated by −/+ were only absent in the extension but supplied in the PCR amplification step. Extension was conducted with 10 nM Cy3-GTS or Cy5-MTS or both at 0.5 mM K^+ concentration.

telomerase was translated using a kit prepared from rabbit reticulocyte lysate. These molecules may also bind ligands and, as a result, reduce the effective concentration of ligands or increase the apparent IC_{50}. However, the smaller IC_{50} (<1 μM) than K_d (42 μM)[16] for Cu-TSPc and TSPc is physically impossible for a G-quadruplex-dependent inhibition. A smaller IC_{50} was also observed in the original work, which was attributed to possible interaction with telomerase or its related proteins in addition to telomere G-quadruplex.[16]

Failure to Detect Telomere G-Quadruplex-Dependent Inhibition by TRAP Using dGTP and 7-deaza-dGTP. The inhibition observed from the two mutant telomerases of the four ligands indicates that the ligands could directly inhibit telomerase activity in the absence of telomere G-quadruplex. G-quadruplex-irrelevant inhibition might result from their interactions with telomerase, substrate, or interference with the telomerase−substrate interaction. Failure to observe a greater inhibition to the wild-type than to the mutant telomerases suggests two possibilities. On one hand, G-quadruplex-irrelevant inhibition might significantly overweigh the effect of G-quadruplex stabilization so that the latter was invisible. On the other hand, the conventional TRAP may have an inherent limitation in that the amount of TS substrate is in excess of that of telomerase molecules. Since TS substrate is nontelomeric, ligand promoted G-quadruplex formation on a very small amount of extended TS primer is unlikely to limit the availability of TS primer to telomerase and produce meaningful inhibition.

Due to the above concerns, further TRAP assays were carried out using the wild-type telomerase (WT) with the following modifications. First of all, a lower TS primer concentration was used to avoid substrate abundance. At this concentration, the extension product began to show dependence on TS concentration in our pilot experiments. Second, the normal dGTP was

Table 3. Primers and Internal Standard Used in the ST/TC-TRAP Assay

name	sequence (5′-3′)
GTS[a]	Cy3-GGGTTAGGGTTAGGGTTAGGGTT
MTS[a]	Cy5-AGCATCCGTCGAGCAGAGTT
SDP	GCGCGGCCTACATCTACATCTACATCAACA
SIS[b]	GTCACTGCGGATTGGTCCTTAAAAGGCCGAGAAGCGAT
ISU	Cy5-GTCACTGCGGATTGGTCCTT
ISD	Cy3-ATCGCTTCTCGGCCTTTT

[a] Substrate used for extension with the mutated CUA telomerase and as upstream primer for amplifying their extension products, respectively. [b] Shared internal standard, amplified by ISU and ISD primers.

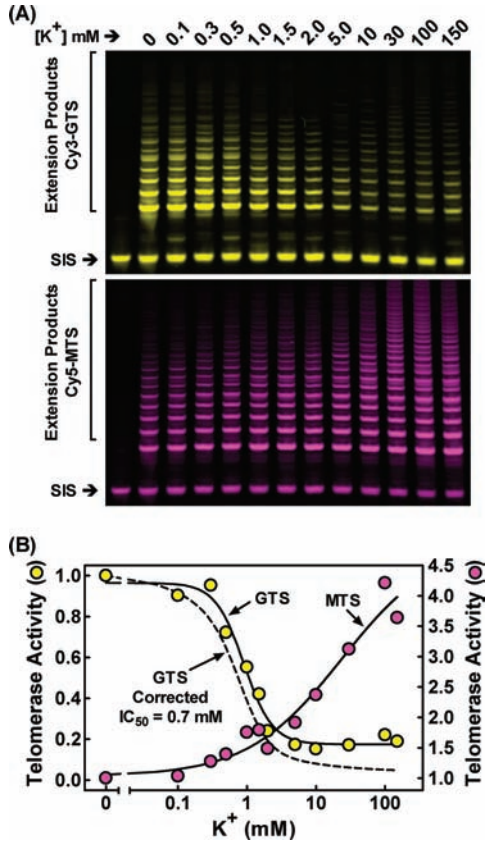

Figure 6. Effect of K^+ concentration on the extension of GTS and MTS substrates analyzed by ST/TC-TRAP. Extension was conducted with 10 nM Cy3-GTS and Cy5-MTS at increasing concentration of K^+. Both Cy3 and Cy5 channels were scanned from the same gel. Dashed curve represents the inhibition on GTS corrected for the activation effect of K^+ by dividing the GTS curve over the MTS curve.

substituted by 7-deaza-dGTP, which lacks the 7-nitrogen essential for the formation of G-quartet such that the extension products with this nucleoside analogue incorporated were unable to form G-quadruplex.[26] Lastly, a lower K^+ concentration was used in the extension reaction to reduce G-quadruplex stability to leave more room for further stabilization by ligands.

In Figures 3 and 4, all four ligands showed similar inhibition to the two sets of TRAP assays despite G-quadruplex formation being only possible in the assays using dGTP but not in those using 7-deaza-dGTP. In agreement with previous reports,[27,28] 7-deaza-dGTP substantially limited the processivity of telomerase (the ability to continuously add telomere repeats without leaving the substrate). The extension seemed to proceed normally for two additions of telomere repeat, since the first two bands are comparable to that of the corresponding bands in the assays using normal dGTP (Figure 3, gels at bottom). This suggests that deaza-dGTP only inhibited telomerase reaction after it was incorporated into two repeats in the extended primer and telomerase underwent at least one normal translocation after the first repeat addition as previously reported.[27] Thus the short extension products might truly reflect the activity of telomerase and allowed us to analyze the effect of ligands. The disappearance of longer bands suggests that the translocations followed were affected. Due to the large reduction in primer concentration, IC_{50} values in these assays, most notably for Zn-TTAPC and BMVC, decreased. Failure to detect apparent G-quadruplex-dependent inhibition supports the dominance of G-quadruplex-independent activity of the ligands.

To access stabilization of telomere G-quadruplex by these ligands, FRET thermal melting analyses were carried out in the absence and presence of ligands. The increase in $T_{1/2}$ induced by ligands (Table 2) reflects their ability to stabilize telomere G-quadruplex. It should be noted that the telomere DNA was not in fully folded form at 0.5 mM K^+ at the lowest recording temperature and thus the $T_{1/2}$ values may well be overestimated.[18] As a result, the assays under this condition serve only as a qualitative analysis. The two cationic ligands Zn-TTAPc and BMVC stabilized the G-quadruplex at both 0.5 and 150 mM K^+ concentration, but the two anionic ligands Cu-TSPc and TSPc exhibited no or little stabilization. The inability of the anionic ligands to stabilize the G-quadruplex is explained by the electrostatic repulsion between the ligands and G-quadruplex, which disfavors ligand binding with G-quadruplex. This may partially explain why their IC_{50} is less sensitive to the reduction in primer concentration relative to that for Zn-TTAPc and BMVC. However, the inhibition of Cu-TSPc and TSPc on telomerase activity confirmed the presence of G-quadruplex-irrelevant effect.

A Single-Tube/Two-Color TRAP (ST/TC-TRAP) Method. In the above TRAP assays, telomere G-quadruplex-dependent inhibition, if any, could only occur after at least four telomeric repeats were synthesized on to the TS primer. G-quadruplex formation promoted by ligands might drive telomerase off to the other TS primers. Due to the abundance of TS substrate relative to the amount of telomerase, G-quadruplex formation in a limited number of extended primers might not significantly reduce the availability of primer to telomerase (Figures 1 and 2) even when a lower primer concentration is used (Figures 3 and 4). This could explain the failure to detect the expected telomere G-quadruplex-specific inhibition. In 2002, a TRAP-G4 assay was introduced that included a telomere G-quadruplex-mimicking sequence (5′-GGGATTGGGATTGGGATTGGGTT-3′) to analyze G-quadruplex ligands.[10] In this case, G-quadruplex stabilization could take effect before the primer was extended by telomerase. To address the issues in our TRAP assays, we established a more stringent ST/TC-TRAP method to discern the contribution of telomere G-quadruplex stabilization (Figure 5A) based on the conventional TRAP[9] and TRAP-G4[10] method.

Our ST/TC-TRAP uses two substrates: one consists of native telomere repeat (GTS) so that it can form G-quadruplex, and the other is a linear nontelomeric sequence (MTS)[29] that does not form G-quadruplex (Table 3). Both substrates are extended by a same mutated telomerase (CUA) such that the extension repeats

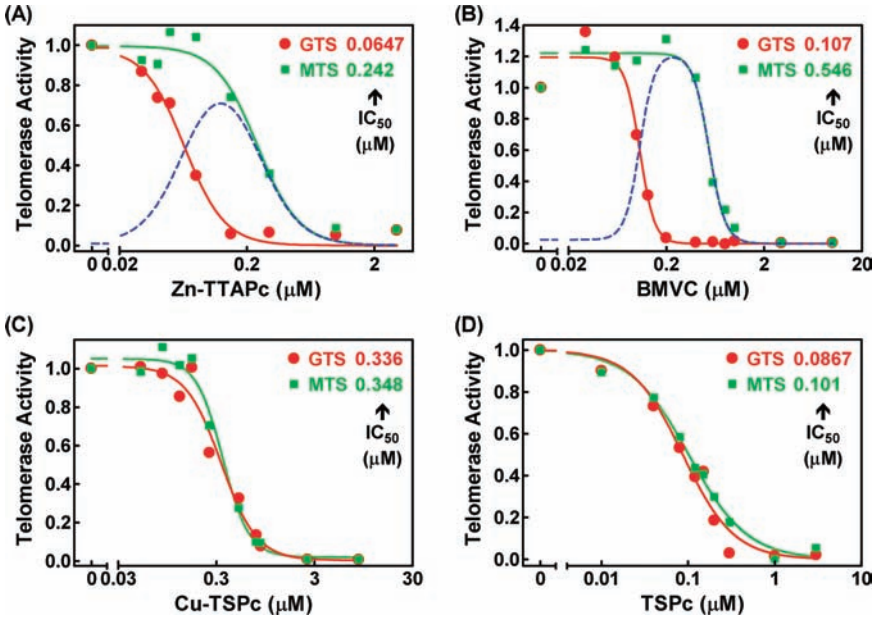

Figure 7. Inhibition of (A) Zn-TTAPc, (B) BMVC, (C) Cu-TSPc, and (D) TSPc on primer extension by the CUA telomerase mutant analyzed by ST/TC-TRAP. Other conditions were the same as in Figure 6 except that extension was conducted at 0.5 mM K^+. Dashed curve indicates G-quadruplex-dependent inhibition obtained by subtracting the GTS curve from the MTS curve.

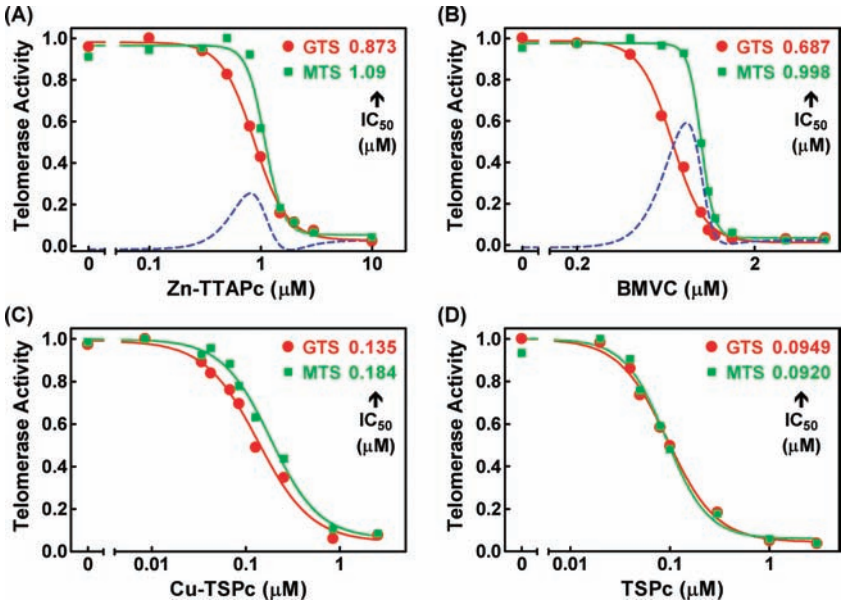

Figure 8. Inhibition of (A) Zn-TTAPc, (B) BMVC, (C) Cu-TSPc, and (D) TSPc on substrate extension by the CUA telomerase mutant analyzed by the ST/TC-TRAP. Other conditions were the same as in Figure 6 except that extension was conducted with 200 nM Cy3-GTS and 10 nM Cy5-MTS at 150 mM K^+. Dashed curve indicates G-quadruplex-dependent inhibition obtained by subtracting the GTS curve from the MTS curve.

added will not form G-quadruplex. The inhibition on the former substrate has a contribution from G-quadruplex stabilization and that on the latter substrate is G-quadruplex-independent. To ensure a completely identical assay condition, the two substrates are labeled at the 5′ end with a Cy3 and Cy5 fluorescent dye, respectively, extended (Figure 5A), and subsequently amplified with a shared downstream primer (Figure 5B) in the same vial, followed by electrophoresis and quantification on the same gel (Figure 5C). The same internal standard was also coamplified by two primers labeled at the 5′ end with Cy3 and Cy5, respectively (Figure 5B), producing a dual labeled internal standard to calibrate the extension for both substrates (Figure 5C).

To test if the ST/TC-TRAP can identify the effect of G-quadruplex stabilization from other G-quadruplex-irrelevant ones, we carried out assays under various concentrations of K^+, the most effective monovalent cation in stabilizing G-quadruplex.[30] In Figure 6, it can be seen that while increasing K^+ concentration increased the extension of the nontelomeric MTS substrate, it inhibited the extension of the telomeric GTS substrate in a concentration dependent manner. This result is in agreement with

Table 4. IC_{50} of Ligands Obtained in the ST/TC-TRAP Assays[a]

substrate	GTS					MTS				
	IC_{50} (μM)	H	IC_{50} (μM)	H	$I_{(150/0.5)}$	IC_{50} (μM)	H	IC_{50} (μM)	H	$I_{(150/0.5)}$
K^+ (mM)	0.5	0.5	150	150		0.5	0.5	150	150	
Zn-TTAPc	0.0647	2.74	0.873	2.95	13.5	0.242	2.66	1.09	5.40	4.50
BMVC	0.107	5.76	0.687	4.74	6.42	0.546	5.22	0.998	12.1	1.83
Cu-TSPc	0.336	2.40	0.135	1.52	0.40	0.348	3.22	0.184	1.67	0.53
TSPc	0.0867	1.47	0.0949	1.77	1.09	0.101	1.29	0.092	2.07	0.91

[a] IC_{50} indicates the total ligand concentration required to reach half maximal inhibition, and H the Hill coefficient. $I_{(150/0.5)}$ indicates fold increase in IC_{50} when the concentration of K^+ was increased from 0.5 to 150 mM in primer extension by telomerase.

a previous report, in which a high concentration of K^+ enhanced telomerase activity when G-quadruplex formation was prevented by supplying only dATP and dTTP as nucleotide substrates, but inhibited the processivity of the telomerase when G-quadruplex was formed.[31] We have previously shown that G-quadruplex formation in telomere DNA increases with increasing K^+ concentration.[7] Therefore, the current result indicates that the ST/TC-TRAP is able to detect the effect of G-quadruplex stabilization on telomerase activity.

Contribution of Telomere G-Quadruplex Stabilization to Telomerase Inhibition Detected by ST/TC-TRAP. Using the ST/TC-TRAP, we analyzed the four ligands and the results are summarized in Figures 7 and 8 and Table 4. Extension was first conducted at 0.5 mM K^+ to permit more room for the ligands to stabilize G-quadruplex. In agreement with their ability to stabilize G-quadruplex (Table 2), Zn-TTAPc and BMVC showed greater inhibition (lower IC_{50}) to the GTS than to the MTS substrate, indicating that G-quadruplex stabilization by the two ligands produced significant inhibition in addition to the G-quadruplex-irrelevant effect. In particular, BMVC at approximately 0.2 μM could completely inhibit extension by stabilizing G-quadruplex before the G-quadruplex-irrelevant effect showed apparent inhibition (Figure 7B). Without the G-quadruplex-stabilizing effect (Table 2), Cu-TSPc and TSPc both displayed almost identical inhibition to the two substrates (Figure 7C,D), implying that their effects were G-quadruplex-independent.

Since the concentration of K^+ inside animal cells is around 150 mM, ST/TC-TRAP assays were also carried out at 150 mM K^+ to investigate the effect of the ligands under a more physiologically relevant condition (Figure 8). Raising K^+ concentration is expected to increase the stability of telomere G-quadruplex and affect the electrostatic interaction between the ligands and their targets. To obtain similar extension for both substrates at zero ligand concentration, GTS was used at 200 nM while MTS remained at 10 nM in the extension step. For the cationic ligands Zn-TTAPc and BMVC, electrostatic attraction between them and substrates is reduced at 150 mM K^+. This seems to explain the increase in their IC_{50} for both the GTS and MTS substrate. Notably, the increase in IC_{50} was more dramatic for the G-quadruplex-forming GTS than for MTS. For example, Zn-TTAPc had an IC_{50} value 13.5-fold greater at 150 mM than at 0.5 mM K^+ for GTS, while it was only 4.5-fold greater for MTS. This fact suggests that the effect of G-quadruplex stabilization by ligand strongly depended on the original stability of the telomere G-quadruplex under the ligand-free condition. It can be imagined that a more stable G-quadruplex at higher K^+ concentration will leave less room for further stabilization by ligands. This also provides an explanation to the fact that BMVC showed less G-quadruplex-dependent inhibition in 150 mM (Figure 8B) than in 0.5 mM (Figure 7B) K^+ solution while it induced similar increase in $T_{1/2}$ in both solutions (Table 2). The relative increase in IC_{50} at 150 mM K^+ (i.e., 6.42-fold for GTS and 1.83-fold for MTS) was less significant for BMVC because it has fewer charges than Zn-TTAPc and is therefore less sensitive to charge sheltering by the increased K^+ concentration. For the anionic ligands, the increase in K^+ concentration reduces the electrostatic repulsion between the ligands and G-quadruplex, thus facilitating possible interaction between them. However, their inhibitions still remained irrelevant to G-quadruplex at 150 mM K^+.

DISCUSSION

Telomere G-quadruplex tends to form at the furthest 3′ end of telomere DNA.[32] This minimizes the size of the free 3′ tail and efficiently inhibits telomere extension by both telomerase and the ALT mechanism.[13] While directly targeting telomerase in cancer cells may potentially risk selecting for or inducing the ALT mechanism, stabilizing telomere G-quadruplex by ligands is anticipated to inhibit both pathways, thus potentially providing a more secure strategy for disrupting telomere extension. To achieve this goal, G-quadruplex-stabilizing ligands should act in the way they are intended to. However, the ligands studied in this work show that their inhibition on telomerase is, to a large portion or even almost entirely, attributed to activities independent of telomere G-quadruplex. They all inhibited telomerase activity (Figures 1−4, 7, and 8), whether they stabilized G-quadruplex or not (Table 2), or in the absence of G-quadruplex. The G-quadruplex-independent effect is best illustrated by the inhibition of the anionic ligands Cu-TSPc and TSPc that did not stabilize G-quadruplex. Since the ligands we studied were chosen for their high affinity and/or selectivity toward telomere G-quadruplex, the G-quadruplex-irrelevant effect may also present for the many other ligands in their inhibition to telomerase-catalyzed telomere extension.

In our assays, telomerases with mutated RNA template or different primers were used in order to differentiate the G-quadruplex-independent effect from G-quadruplex-dependent ones. This might alter the telomerase reaction. Native telomerase can catalyze extension on various nontelomere primers[33] in which the telomerase−primer interaction may be different. This suggests that telomerase can tolerate alterations in such interaction. Since the G-quadruplex-independent effect was seen in different assay methods with different ligands on different telomerases, we believe it was not associated with a specific interaction, but a real property of ligands on telomerase reaction. Generally speaking, potential sources of telomere G-quadruplex-irrelevant inhibition

in the in vitro assays may include interactions of ligands with the subunits of telomerase (e.g., hTERT and hTR), substrate, as well as interference to the interaction between substrate and telomerase. For example, the RNA component (hTR) of human telomerase carries a G-quadruplex prone sequence[34−36] that may also be targeted by ligands. The candidates in in vivo applications may expand further because a telomerase holoenzyme consists of tens of proteins resulting in a complex of 1000 kD or more.[37,38]

The telomere G-quadruplex-specific inhibition by the two cationic ligands Zn-TTAPc and BMVC is obvious at low K^+ concentration (Figure 7A,B), but it is dramatically decreased at high K^+ concentration (Figure 8A,B). This fact suggests that the effect of G-quadruplex stabilization by these ligands depends on the stability of telomere G-quadruplex in the absence of ligand. As is shown in Figure 6, K^+ is a robust G-quadruplex stabilizer that inhibits telomerase activity by stabilizing telomere G-quadruplex at the sub-millimolar level. Hence, our results raise a concern on how much telomere G-quadruplex stabilization by ligands can actually contribute to the inhibition of telomerase-mediated telomere extension, especially under the in vivo conditions where high K^+ concentration (150 mM) is present.

The ST/TC-TRAP assay should provide a useful tool for evaluating the specificity of telomere G-quadruplex ligands. By changing the assay conditions, it also provides insight on certain aspects of the working mechanism of ligands. For example, comparison of inhibition between low and high K^+ concentration can help evaluate the contribution of electrostatic interaction. As is shown in Table 4, the more positively charged Zn-TTAPc had a smaller IC_{50} to the GTS than that of the less positively charged BMVC at 0.5 mM K^+. The difference was reversed when the K^+ concentration was raised to 150 mM. These results suggest that the inhibition of Zn-TTAPc depends more on charge interaction, but BMVC is more selective. This may explain why BMVC showed better specificity than Zn-TTAPc (Figures 7A,B and 8A, B, dashed curves).

AUTHOR INFORMATION

Corresponding Author

z.tan@ioz.ac.cn; tanclswu@public.wh.hb.cn

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