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Reduced or Diminished Stabilization of the Telomere G-Quadruplex and Inhibition of Telomerase by Small Chemical Ligands under Molecular Crowding Condition

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Abstract: Telomere DNA in human cells shortens during each round of DNA replication. In cancer cells, telomere shortening is compensated by telomerase or the alternative lengthening of telomere (ALT) mechanism to maintain cell division potential. The G-rich strand of telomere DNA can fold into a G-quadruplex structure and disrupt these two processes. Therefore, stabilization of the G-quadruplex by chemical ligands is emerging as a promising anticancer strategy. So far, *in vitro* studies on such ligands are exclusively carried out in dilute solutions. However, the intracellular environment is highly crowded with biomolecules. How G-quadruplex ligands behave under molecular crowding condition is critical for their *in vivo* anticancer effect. In this work, we studied several ligands for their ability to stabilize the telomere G-quadruplex and inhibit telomerase under both dilute and crowding conditions. Surprisingly, the ligands became significantly less effective or even lost the ability to stabilize the G-quadruplex and inhibit telomerase under crowding conditions. Our data attributed this consequence to the decreased binding affinity of ligands to the G-quadruplex as a result of reduced water activity and increased viscosity of the medium associated with molecular crowding. This effect is irrelevant to and overweighs the influences from other factors such as the G-quadruplex structure, cation, and ligand species. Our work illustrates a possibility that molecular crowding inside cells may reduce or limit the potency of ligands although they may be effective in dilute solution, thus strongly arguing for the necessity of evaluating ligands under more physiologically relevant conditions and designing drugs with this concern in mind.

Introduction

Chromosomes in vertebrate cells are protected at both ends by telomere DNA. Telomere DNA shortens during each round of cell division due to the DNA end replication problem and, as a result, limits the proliferative potential of normal somatic cells.[1] In cancer cells, the erosion of telomere DNA is compensated by telomerase or the alternative lengthening of telomere (ALT) mechanism to maintain telomere length homeostasis so that these cells can undergo unlimited division.[2] Telomere DNA consists of long tandem TTAGGG repeats. In the presence of metal ions, such as K^+ and Na^+, four TTAGGG tracks can fold into a G-quadruplex structure.[3] This structure is incompatible with the telomere maintenance mechanisms. On one hand, the telomere quadruplex is not a substrate for telomerase; its formation may inhibit the binding of telomerase to a telomere substrate and promote the dissociation of telomerase from it.[4,5] On the other hand, the G-quadruplex has been reported to form preferentially at the very 3′ end of the G-rich telomere DNA strand.[6] This property should inhibit the addition of telomere repeats to the 3′ end of telomere DNA by either telomerase or the ALT mechanism where base-pairing of the 3′ end with the RNA template in the telomerase complex or a telomere C-rich strand is required.

Currently there is intense interest in exploring small chemical ligands as new anticancer drugs that can disrupt telomere maintenance by stabilizing the telomere G-quadruplex. Such ligands have been shown to inhibit telomerase activity and induce growth arrest, senescence, or apoptosis in cancer cells. G-quadruplex-forming sequences have also been found spreading throughout the genome in species ranging from human to bacteria and suggested to play roles in regulating gene expression. Manipulation of G-quadruplex formation and stability by chemical ligands in these sequences is also considered as a potential therapeutic strategy against other diseases. Over the past few years, a large number of compounds have been synthesized and screened for their ability to stabilize G-quadruplexes formed by telomere DNA and other target sequences. For recent reviews on ligands, G-quadruplexes, their interactions, and biological effects, please see refs 7−15. So far *in vitro* analysis of G-quadruplex ligands has been exclu-

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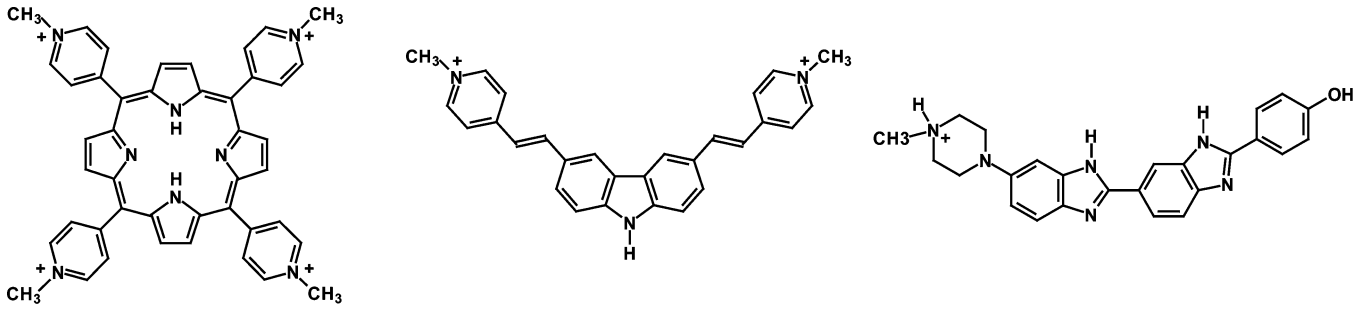

Figure 1. Structures of TMPyP4, BMVC, and Hoechst 33258.

sively performed in dilute solution. However, the cytoplasmic environment of living cells where the ligands are delivered to is highly crowded with various biomolecules, which can be as high as 0.3 to 0.4 g/mL.[16] In general, molecular crowding has a fundamental impact on the biochemical properties of biomolecules and the rates, equilibria, and mechanisms of biomolecular reactions.[17,18] Taking DNA as an example, molecular crowding can affect the structure, stability, and function of nucleic acids (for a recent review, see ref 19), processing of nucleic acids by nucleases,[20] polymerase,[21−23] and association with proteins.[24,25] Particularly, molecular crowding has been found to induce the transition of the G-quadruplex from a mixed parallel/antiparallel to a parallel topology[26−28] or from an intramolecular G-quadruplex to a long multistranded G-wire,[27] stabilize the G-quadruplex,[28,29] and even induce G-quadruplex formation without adding salt[30,31] that was once thought required for G-quadruplex formation. Given such a profound influence on the G-quadruplex, we thought it would be important to know how molecular crowding may affect G-quadruplex−ligand interaction.

In this work, we studied several ligands, i.e., TMPyP4, BMVC, and Hoechst 33258 (Figure 1), for their ability to stabilize the telomere G-quadruplex and inhibit telomerase under both dilute and molecular crowding conditions. In dilute solution, all these ligands showed significant stabilization of the telomere G-quadruplex and inhibition of telomerase activity. However, their effects were all substantially reduced in the presence of crowding agents PEG 200 and glycerol. Hoechst 33258 even completely lost the ability to stabilize the G-quadruplex and inhibit telomerase in PEG solution. The negative effect of molecular crowding can be attributed to the decreased affinity between the ligands and the G-quadruplex possibly as a consequence of reduced water activity and increased viscosity of the medium. The effect is a general environmental contribution irrelevant to and overweighing the influences from other factors such as the G-quadruplex structure, cation, and ligand species. These examples illustrate the possibility that the molecular crowding inside cells may reduce or limit the potency of ligands, although they can be effective in dilute solution. Therefore it is important that G-quadruplex ligands should be evaluated and tested under more physiologically relevant conditions and drug designing should take into account the effect of molecular crowding.

Materials and Methods

Oligonucleotides and Ligands. Oligonucleotides were purchased from Invitrogen Corporation (Shanghai, China). G-quadruplex DNA $(G_3T_2A)_3G_3$ (F21T) for human telomere and AGGGCGGTGTGGGAAGAGGGAAGAGGGGGAGG (F-*KRAS*-T) for *KRAS* gene, labeled at the 5′ end with a fluorescein (FAM) and the 3′ end with a tetramethylrhodamine (TAMRA) respectively, were purchased from TaKaRa Biotech (Dalian, China). 5,10,15,20-Tetra(*N*-methyl-4-pyridyl)porphine (TMPyP4) was purchased from Merck, and bisBenzimide H 33258 (Hoechst 33258) and daunomycin hydrochloride were purchased from Sigma. 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.

Melting Assay Based on Fluorescence Resonance Energy Transfer (FRET Melting). FRET melting experiments were carried out as described[32] in 10 mM lithium cacodylate buffer (pH 7.4)

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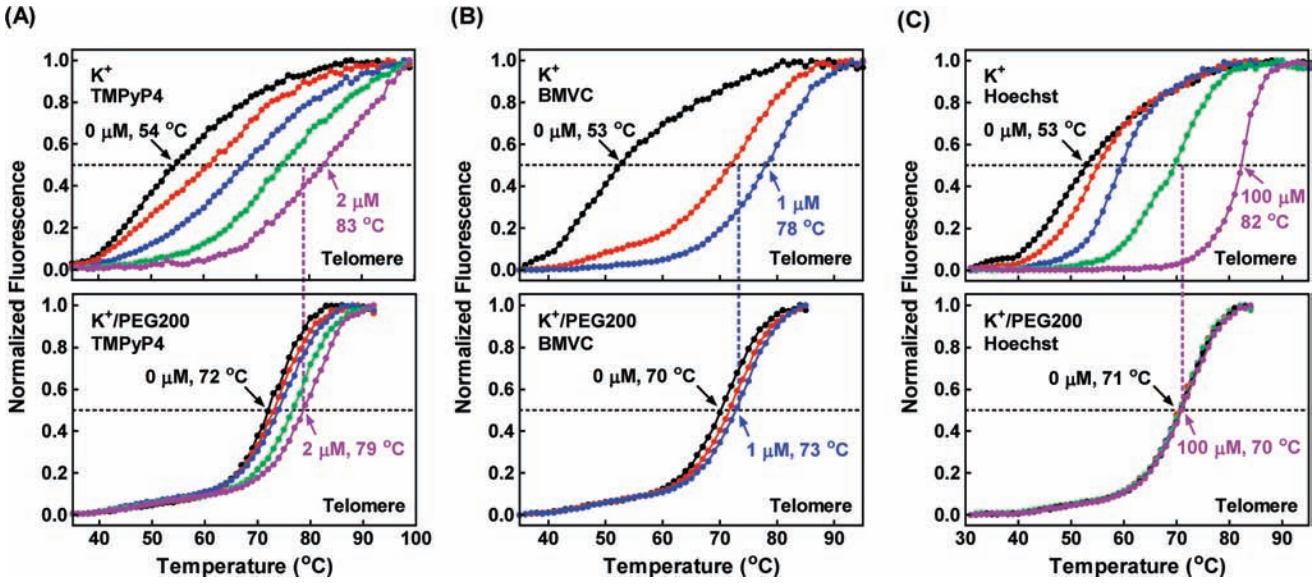

Figure 2. Effect of PEG on the stabilization of human telomere G-quadruplex by (A) TMPyP4, (B) BMVC, and (C) Hoechst 33258 in 5 mM KCl, 95 mM LiCl solution. Fluorescence thermal melting assays were carried out in the absence (top panels) and presence (bottom panels) of 40% (w/v) PEG 200. Ligand concentration used was 0, 0.25, 0.5, 1, 2 μM for TMPyP4; 0, 0.5, 1 μM for BMVC; and 0, 3, 10, 30, 100 μM for Hoechst 33258, respectively. The $T_{1/2}$ obtained without ligand or at the highest ligand concentration is indicated in the panels.

containing either 5 mM KCl plus 95 mM LiCl or 100 mM NaCl and the indicated concentration of crowding agent. Typically 18 μL samples containing 0.5 μM F21T or F-*KRAS*-T were denatured at 95 °C for 5 min before being slowly cooled down to 30 °C; then 2 μL of ligand solution were added, and the mixture was maintained at 30 °C for 30 min. Thermal melting was carried out and monitored on a Rotor-Gene 2000 Real-time Cycler. The samples were equilibrated at the starting temperature for 5 min followed by a stepwise increase of 1 °C until 99 °C. Fluorescence was recorded after stabilization for 1 min at each step.

Spectroscopic Titration. Titrations were carried out at 25 °C in 10 mM Tris-HCl buffer (pH7.4), containing 1 mM EDTA, 100 mM KCl or NaCl, and the indicated concentration of the crowding agent. UV−vis absorption was measured on a Beckman DU640 UV−vis spectrophotometer (Beckman, USA) with 2 μM TMPyP4 or Hoechst 33258 and various concentrations of G-quadruplex DNA. The wavelength was set at 424 nm for TMPyP4 and 340 nm for Hoechst 33258. Fluorescence was measured on a Spex Fluorolog-3 spectrofluorometer (HORIBA Jobin Yvon, France) with 100 nM BMVC and various concentrations of G-quadruplex DNA. The excitation wavelength was set at 460 nm, and emission at 560 nm. EC_{50} was obtained by fitting normalized absorbance or fluorescence to the G-quadruplex concentration using the sigmoidal function. For the derivation of the binding affinity constant of ligand to G-quadruplex, the spectroscopic data were fitted to the following equation:[33]

$$\Delta F = (\Delta F_{max}/2L_0)\{L_0 + nQ_0 + 1/K_A) - \sqrt{(L_0 + nQ_0 + 1/K_A)^2 - 4L_0nQ_0}\}$$

where ΔF is the increment of spectroscopic signal at each G-quadruplex concentration relative to the free ligand, ΔF_{max} the maximal ΔF at the saturating G-quadruplex concentration, L_0 the total ligand concentration, Q the added G-quadruplex concentration, n the number of binding sites per G-quadruplex, and K_A the binding affinity constant.

Telomerase Activity Assay. Telomerase activity was assayed by the telomerase repeat amplification protocol (TRAP) with modifications, using TS[34,35] or TSG4[36] primer and extract from HeLa cells. Primer extension was carried out in the presence of an internal standard (IS) and various concentrations of ligands. Then the ligands were removed by phenol extraction and ethanol precipitation. Samples were dried, dissolved in water, and added to the PCR amplification mixture. PCR products were resolved on 12% polyacrylamide gel, stained with ethidium bromide, recorded, and quantitated on a ChemiImager 5500 (Alpha Innotech, San Leandro, CA, USA). Telomerase activity was expressed as the percent of the control in which no ligand was added using the formula $(TP/TP_0) \times (IS_0/IS) \times 100$, where TP_0 and IS_0 are the integrated density of telomerase products and IS of the control, respectively; TP and IS are the corresponding integrated densities obtained in the presence of ligand. When TSG4 was used, a modified CXext primer 5′-GTGCCCTTACCCTTACCCTTACCCT-3′ was used.

Measurement of Osmolality. Measurement of osmolality for the spectroscopic titration buffer was carried out on a Wescor VAPRO 5520 pressure osmometer according to the manufacturer's instructions.

Results and Discussion

Molecular Crowding Reduced the Stabilization of the Telomere G-quadruplex by Ligands. We first analyzed three G-quadruplex ligands, i.e., TMPyP4, BMVC, and Hoechst 33258 (Figure 1), under both dilute and molecular crowding conditions created by PEG 200, a crowding agent widely used to mimic the crowded cytoplasmic environment.[37,38] These ligands have been reported to interact with the DNA G-quadruplex.[39−41] Their ability to stabilize the G-quadruplex formed by human telomere sequence $(G_3T_2A)_3G_3$ was assessed by a fluorescence melting assay in K^+ solution. 5 mM of K^+ was used to leave room for G-quadruplex stabilization. With a 5′ end fluorescein (FAM) donor and 3′ end tetramethylrhodamine (TAMRA) receptor, the formation of an intramolecular G-quadruplex of $(G_3T_2A)_3G_3$ brings the two fluorophores into close proximity and allows fluorescence resonance energy transfer (FRET) to occur between them. Thermal melting opens up the G-quadruplex, thus reducing the energy transfer from the donor to the receptor and leading to an increase in the emission intensity of the donor. The temperature ($T_{1/2}$) for the emission to reach midvalue between the minimal and maximal emission is then used to evaluate the stability of the G-quadruplex.[32] From the melting profiles in Figure 2A, TMPyP4 was very

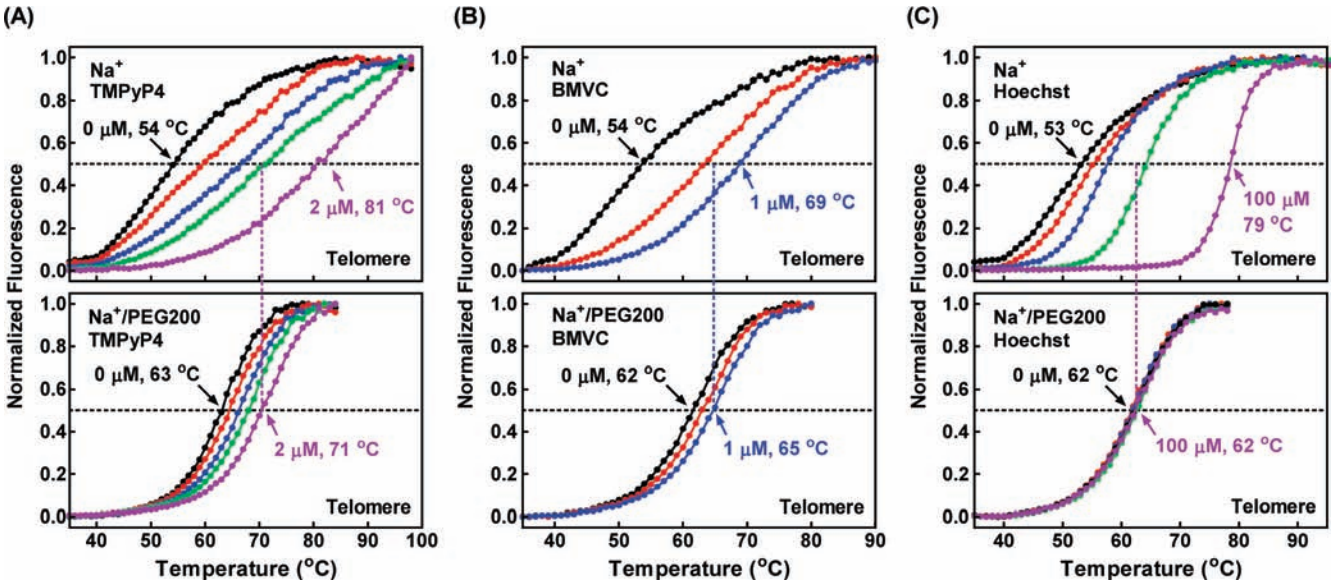

Figure 3. Effect of PEG on the stabilization of human telomere G-quadruplex by (A) TMPyP4, (B) BMVC, and (C) Hoechst 33258 in 100 mM NaCl solution. Melting assays were carried out in the absence (top panels) and presence (bottom panels) of 40% (w/v) PEG 200, using the same ligand concentrations as those in Figure 2. The $T_{1/2}$ obtained without ligand or at the highest ligand concentration is indicated in the panels.

effective at stabilizing the G-quadruplex in dilute solution. At 2 μM it increased the $T_{1/2}$ of the G-quadruplex from 54 to 83 °C resulting in a net increase of 29 °C. In contrast, 2 μM TMPyP4 increased the $T_{1/2}$ by only 7 °C in the presence of PEG. BMVC was also effective in the absence of PEG resulting in an increase in $T_{1/2}$ of 25 °C at 1 μM. In the presence of PEG, however, it increased the $T_{1/2}$ by only 3 °C (Figure 2B). Hoechst 33258 was much less effective than TMPyP4 and BMVC. At a much higher concentration (100 μM), it raised the $T_{1/2}$ by 29 °C in the absence of PEG. In contrast to TMPyP4 and BMVC, Hoechst 33258 did not stabilize the G-quadruplex at all when PEG was present (Figure 2C).

The interaction of chemical ligands with G-quadruplexes is influenced by several factors such as the structure of target DNA, the species of cation, and the properties of ligands.[42−44] Molecular crowding with PEG has been reported to turn the human telomere G-quadruplex from a mixed parallel/antiparallel hybrid-type[45−47] to a parallel-stranded structure[28] and significantly enhance its stability.[28,30,48] In agreement with these previous observations, circular dichroism (CD) analyses (Supporting Figure S1) show that the G-quadruplex changed from a mixed parallel/antiparallel[45−47] to a parallel[28] structure upon addition of PEG. The two structures were not altered by the ligands since their spectral shapes remained unchanged in the absence and presence of ligands. At this point, it is not clear whether the reduced stabilization of the G-quadruplex by the ligands was directly caused by the environmental change or the secondary results of the alterations in the G-quadruplex conformation and stability.

To address whether the reduced stabilization was associated with the specific structure or structural change of the G-quadruplex, additional melting assays were carried out with $(G_3T_2A)_3G_3$ in Na^+ solution and the G-rich sequence of the *KRAS*[49] gene in K^+ solution. In Na^+ solution the human telomere G-quadruplex remains antiparallel-stranded in both the absence and presence of PEG[28] (Supporting Figure S2). Similar to the observation in K^+ solution, PEG significantly suppressed the stabilization of G-quadruplex by the ligands (Figure 3). 2 μM TMPyP4 increased the $T_{1/2}$ by 27 °C in dilute solution but only 8 °C in PEG solution (Figure 3A). One μM BMVC elevated the $T_{1/2}$ by 15 °C in dilute solution while only 3 °C in PEG solution (Figure 3B). Hoechst 33258 also became ineffective in the presence of PEG even though it could raise the $T_{1/2}$ by 26 °C in dilute solution (Figure 3C). In K^+ solution the CD spectra suggest that the *KRAS* G-quadruplex adopted a parallel-stranded conformation in both the absence and presence of PEG featuring a negative peak near 240 nm and a positive peak near 265 nm which remained so when ligands were added (Supporting Figure S3). PEG also reduced its stabilization by the ligands. The elevation of $T_{1/2}$ was decreased from 32 to 9 °C for 2 μM TMPyP4, from 14 to 3 °C for 1 μM BMVC, and from 34 to 7 °C for 100 μM Hoechst 33258, respectively, when

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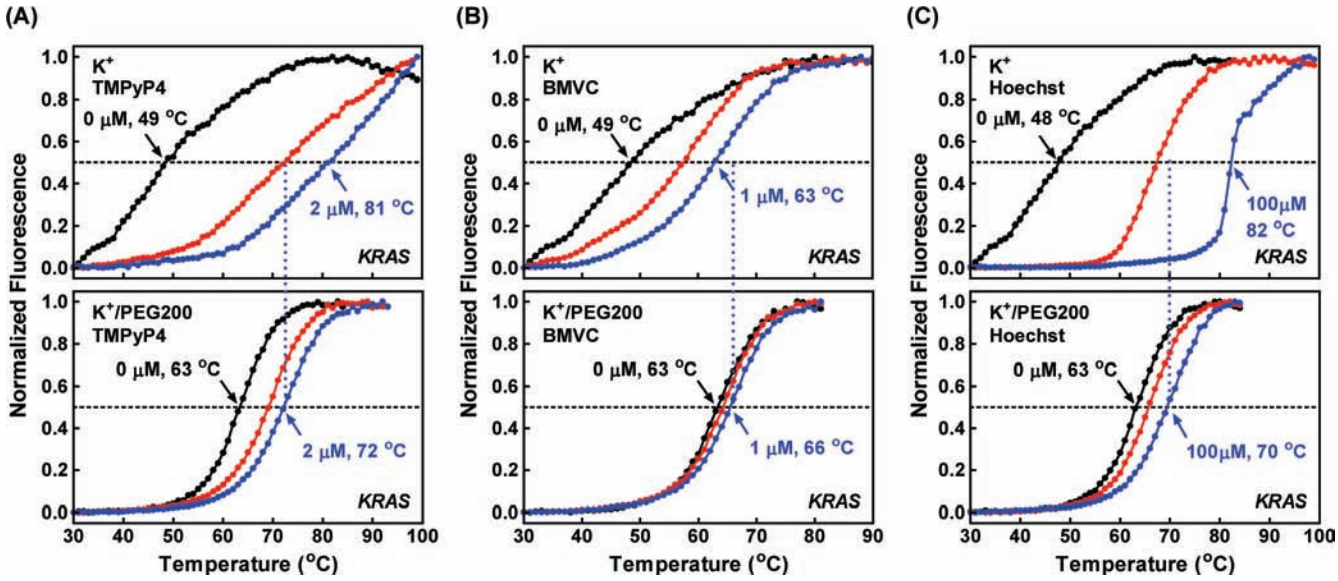

Figure 4. Effect of PEG on the stabilization of *KRAS* G-quadruplex by (A) TMPyP4, (B) BMVC, and (C) Hoechst 33258 in 5 mM KCl, 95 mM LiCl solution. Melting assays were carried out in the absence (top panels) and presence (bottom panels) of 40% (w/v) PEG 200. Ligand concentration used was 0, 1, 2 μM for TMPyP4; 0, 0.5, 1 μM for BMVC; and 0, 30, 100 μM for Hoechst 33258, respectively. The $T_{1/2}$ obtained without ligand or at the highest ligand concentration is indicated in the panels.

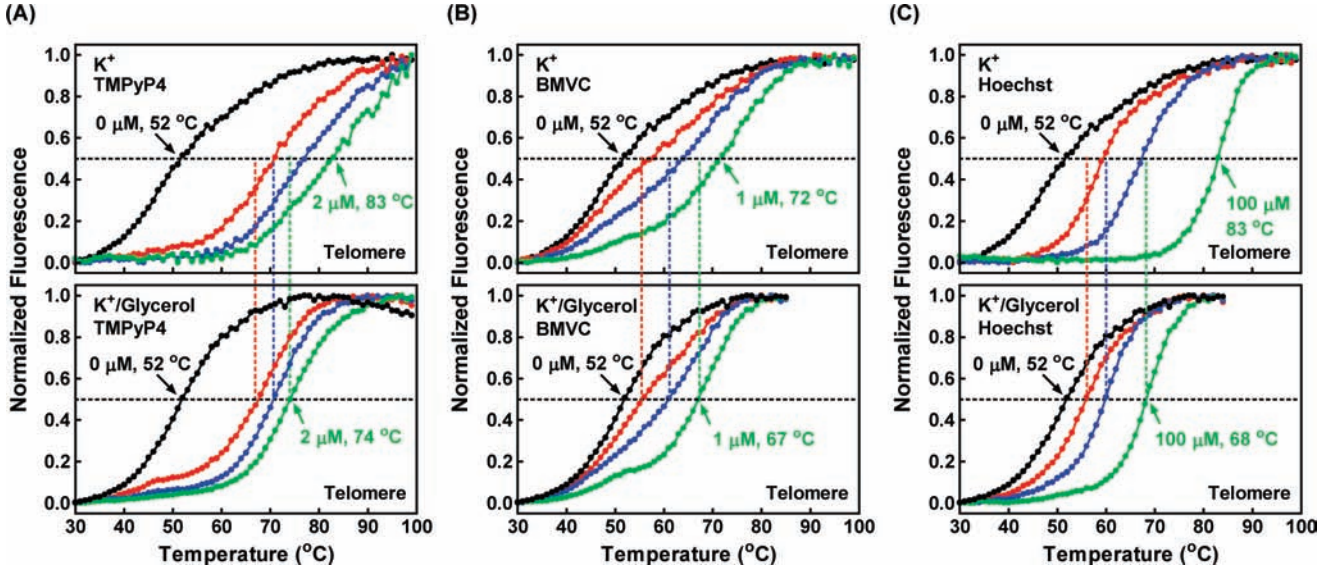

Figure 5. Effect of glycerol on the stabilization of human telomere G-quadruplex by (A) TMPyP4, (B) BMVC, and (C) Hoechst 33258 in 5 mM KCl, 95 mM LiCl solution. Melting assays were carried out in the absence (top panels) and presence (bottom panels) of 40% (w/v) glycerol. Ligand concentration used was 0, 0.5, 1, 2 μM for TMPyP4; 0, 0.25, 0.5, 1 μM for BMVC; and 0, 10, 30, 100 μM for Hoechst 33258, respectively. The $T_{1/2}$ obtained without ligand or at the highest ligand concentration is indicated in the panels.

PEG was added (Figure 4). Another ligand daunomycin also became much less effective in stabilizing the human telomere G-quadruplex in the presence of PEG in both K^+ and Na^+ solution (Supporting Figure S4). Similar observations in all these assays with different ligands and G-quadruplexes of different structures and sequences suggest that the reduced G-quadruplex stabilization is unlikely a structure-relevant event.

In the above assays, it can be noticed that without a ligand PEG substantially enhanced the stability of the G-quadruplexes (Figure 2−4). The $T_{1/2}$ of the human telomere G-quadruplex was elevated from ~53 to 71 °C in K^+, 54 to 62 °C in Na^+ and of the *KRAS* G-quadruplex from ~49 to 63 °C in K^+ solution, resulting in a net increase in $T_{1/2}$ of 18, 9, and 14 °C in each case. One then may presume that the initially more stable G-quadruplex under the crowding conditions would leave less room for further stabilization by the ligands; thus the ligands became less effective. This possibility, though it cannot be excluded, cannot explain the fact that the $T_{1/2}$ values obtained at the highest ligand concentrations in PEG solution were even lower than those obtained in dilute solution (Figures 2−4, except Figure 4B). Therefore, an effect that was independent of the stability of the G-quadruplex should be present. This reasoning is supported by the melting assays using glycerol as the crowding agent in 5 mM K^+ solution, in which the human telomere G-quadruplex was not stabilized (Figure 5) and remained in a mixed parallel/antiparallel conformation (Supporting Figure S5). At 2 μM TMPyP4, a parallel component emerged judging from the appearance of a shoulder near 260 nm. With the same initial $T_{1/2}$, the stabilization of the G-quadruplex by the three ligands was still much less effective in

Table 1. Comparison of increase in $T_{1/2}$ of G-quadruplexes by Ligands under Dilute and Molecular Crowding Conditions

			$T_{1/2}$ increase (°C)			
DNA	cation	crowding agent	TMPyP4 (2 μM)	BMVC (1 μM)	Hoechst 33258 (100 μM)	Daunomycin (100 μM)
Telomere	K^+	none	29	25	29	29
		PEG 200	7	3	0	2
	Na^+	none	27	15	26	23
		PEG 200	8	3	0	4
KRAS	K^+	none	32	14	34	
		PEG 200	9	3	7	
Telomere	K^+	none	31	20	31	
		glycerol	22	15	16	
Telomere	Na^+	none	27	16	29	
		glycerol	9	10	10	

glycerol than in dilute solution, which resulted in lower $T_{1/2}$ values relative to those in dilute solution at all the ligand concentrations tested (Figure 5). In the presence of glycerol, the $T_{1/2}$ decreased by 9 °C for TMPyP4, 5 °C for BMVC, and 15 °C for Hoechst 33258, respectively, at the highest ligand concentration analyzed. The decrease in $T_{1/2}$ was indeed notably less significant than those obtained in PEG solution, indicating that the stabilization by the ligands became less effective on the more stable G-quadruplex. This may potentially add an extra source of difficulty for the stabilization of G-quadruplex *in vivo* if it by itself becomes substantially more stable inside cells. The human telomere G-quadruplex was slightly stabilized by glycerol in Na^+ solution, and the ligands were also less effective in the presence of glycerol (Figure S6).

A summary of G-quadruplex stabilization is presented in Table 1. Taken together, our melting assays have revealed a profound negative effect of molecular crowding on the stabilization of G-quadruplexes by the ligands. The fact that the effect was observed with G-quadruplexes of different structures and sequences, different cations, and ligands of different structures indicates that this effect was a primary result produced by the environmental change that is irrelevant to and overweighs the influences from other factors such as G-quadruplex structure, cation, and ligand species.

Molecular Crowding Decreased the Inhibition of Telomerase by Ligands. Given the significantly reduced or diminished stabilization of the telomere G-quadruplex by the three ligands in PEG solution, we then analyzed how PEG would consequently affect the inhibition of telomerase activity by these ligands in K^+ solution (Figure 6) using the conventional TRAP method.[34] TMPyP4 is a cationic porphyrin compound that stabilizes the human telomere quadruplex[44,50−52] and inhibits telomerase activity.[53,54] In line with the reduced stabilization, PEG decreased the inhibition of telomerase by raising the IC_{50} from 1.44 to 2.76 μM (Figure 6A). BMVC also binds specifically to the human telomere quadruplex and inhibits telomerase activity.[40,55,56] Consistent with its reduced stabilization in PEG solution, BMVC exhibited a more dramatic reduction in inhibiting telomerase activity, resulting in an increase in IC_{50} of nearly 9-fold (Figure 6B). For Hoechst 33258, telomerase inhibition was only seen in the absence, but not in the presence, of PEG (Figure 6C), which is in good agreement with its inability to stabilize the G-quadruplex in PEG solution (Figure 2C). The above TRAP assays were carried out using the same concentration of telomerase substrate. It can be seen that telomerase inhibition by the ligands is in agreement with their ability to stabilize the G-quadruplex shown in the melting assays.

The conventional TRAP assay uses a nontelomeric sequence TS as the telomerase substrate. In such assays, G-quadruplex formation can only take place after addition of four or more telomere repeats onto the TS primer and so does its stabilization by the ligands. To improve the sensitivity, we adopted a modified TRAP assay in which the primer (TSG4) is able to form the G-quadruplex before addition of telomeric repeats.[36] In this case, Hoechst 33258 showed a much lower IC_{50} (60.8 vs 447 μM) in the absence of PEG but still had little inhibition in the presence of PEG (Supporting Figure S7). This result shows that Hoechst 33258 truly became ineffective in inhibiting telomerase in PEG solution and, in the meantime, indicates that the inhibition of telomerase by the ligand was G-quadruplex-dependent. The above assays were also performed in Na^+ solution, and a decrease in telomerase inhibition with PEG was also observed (Supporting Figure S8). Molecular crowding has been reported to affect telomerase activity by a change in the stability and conformation of G-quadruplex[28] or the stability of the DNA/RNA hybrid in the substrate/telomerase complex.[57] While the above results show that the reduced inhibition of telomerase activity by the ligands under molecular crowding conditions could be attributed to the reduced stabilization of the G-quadruplex by the ligands, there were also other possibilities by which molecular crowding might influence the effect of ligands, for instance, by changing the interaction between the DNA substrate and RNA template in the telomerase.[57]

Molecular Crowding Decreased the Affinity between Ligands and G-quadruplex. To elucidate the mechanism that contributed to the reduced stabilization of the G-quadruplex and inhibition of telomerase by the ligands under molecular crowding conditions, we analyzed the interaction between the ligands and the human telomere G-quadruplex by spectroscopic titration in the absence and presence of PEG. The spectral changes of the ligands upon binding to DNA allow us to assess their affinity to the DNA target and how it can be affected by PEG. In Figure 7, the absorbance or fluorescence intensity as a function of G-quadruplex concentration was presented. The human telomere G-quadruplex adopts different conformations in the absence and presence of PEG in K^+ solution.[28] This may change both the numbers of binding sites on the G-quadruplex and their affinity with the ligands. To simplify the comparison, a single parameter EC_{50} was derived as an overall assessment of the affinity. Here the EC_{50} presents the amount of DNA required to induce half of the maximal spectral change, or an approximation to bind half of the ligand being titrated. Our results show that the three ligands all had reduced affinity toward the human telomere G-quadruplex in the presence of PEG in both K^+ (Figure 7) and Na^+ (Supporting Figure S9) solution. This decreased affinity well explains the reduced stabilization of the G-quadruplex and inhibition of telomerase by the ligands in PEG solution. In

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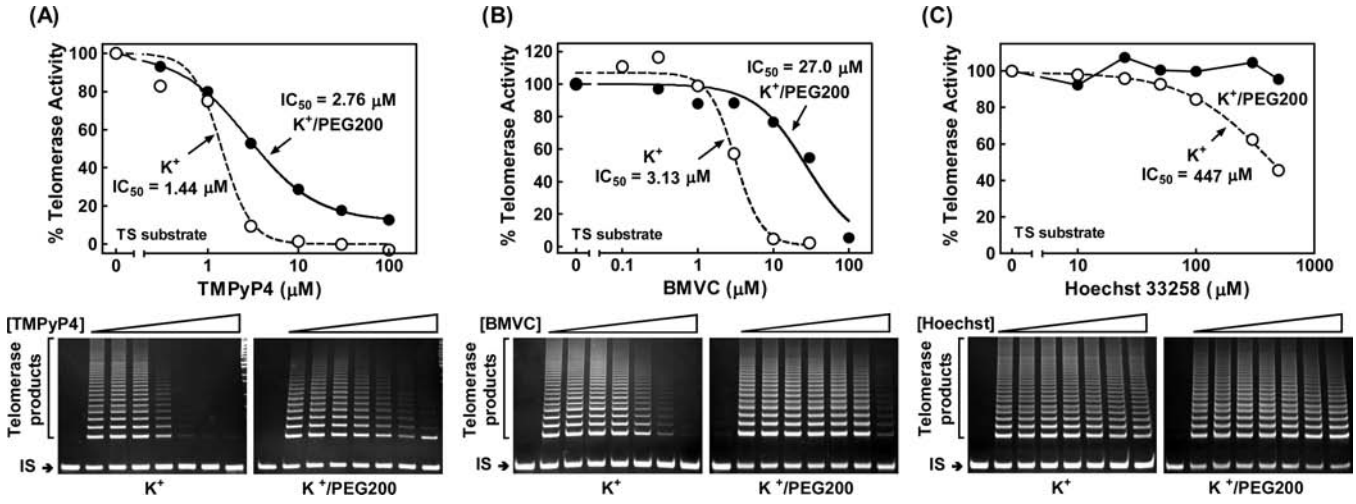

Figure 6. Inhibition of telomerase activity by (A) TMPyP4, (B) BMVC, and (C) Hoechst 33258 in 63 mM KCl solution in the absence and presence of 40% (w/v) PEG 200 assayed by the TRAP method using TS substrate. Telomerase activity (top panel) was quantitated as the percent of the corresponding control sample containing no ligand (lane 2 of each gel). The bottom panel shows a ladder of amplified extension products stained with ethidium bromide after gel electrophoresis; the first lane is the negative control using heat-inactivated telomerase without ligand; IS indicates the bands of internal standard.

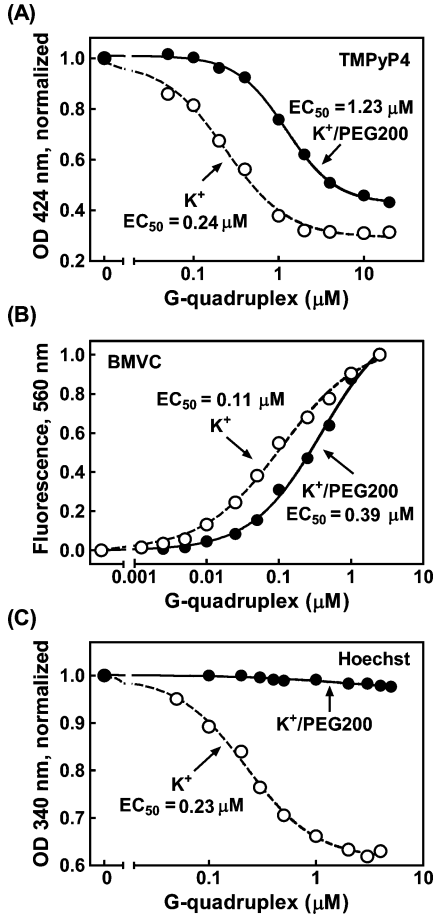

Figure 7. EC_{50} of human telomere G-quadruplex for binding with (A) TMPyP4, (B) BMVC, and (C) Hoechst 33258 in 100 mM KCl solution in the absence and presence of 40% (w/v) PEG 200 measured by spectroscopic titration. Ligand concentration was 2 μM for TMPyP4, 0.1 μM for BMVC, and 2 μM for Hoechst 33258, respectively.

particular, Hoechst 33258 showed little binding to the G-quadruplex in the presence of PEG (Figure 7C and Supporting Figure S9C), which explains its inability to stabilize the G-quadruplex (Figure 2C and 3C) and inhibit telomerase activity (Figure 6C, Supporting Figures S7 and 8C) in PEG solution.

It is noticed that in the absence of PEG, Hoechst 33258 displayed a similar affinity as TMPyP4 to the G-quadruplex (Figure 7A vs 7C), which seems to disagree with a much weaker effect of this ligand in stabilizing the G-quadruplex as compared to TMPyP4 (Figure 2A vs 2C). Since the DNA exists in both the G-quadruplex and single-stranded form in the melting assays, G-quadruplex stabilization will depend on the selectivity of the ligand between the two DNA structures. Hoechst 33258 has been reported to have a binding affinity constant of 1.4×10^6 M^{-1} in a UV absorbance titration and 5×10^6 M^{-1} in a fluorescence titration for the G-quadruplex formed by the G-rich sequence from the promoter region of human *c-myc*[41] and a comparable binding affinity constant of 4.3×10^6 M^{-1} for single stranded DNA.[58] The lack of selectivity of Hoechst 33258 may be responsible for its inefficiency in stabilizing the G-quadruplex. In contrast, TMPyP4 had been shown to have good selectivity for the telomere G-quadruplex over single-stranded DNA.[35]

Environmental Parameters Affecting G-Quadruplex/Ligand Interaction. The introduction of PEG can bring changes in several aspects to a solution. These include a decrease in dielectric constant[59] and water activity,[60] an increase in viscosity,[61] and excluded volume.[62] Any of these factors may affect the ligand−G-quadruplex interaction. Among them, the decrease in dielectric constant should not be responsible for the decreased affinity of the ligands to the G-quadruplex because the ligands are all positively charged, while the G-quadruplexes are negative. The decrease in dielectric constant brought by PEG is expected to enhance their interaction since the strength of charge interaction is inversely related to the dielectric constant of the medium.

Water plays important roles in biomolecular processes. Molecular crowding decreases water activity and compromises

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the hydration of biomolecules. Any equilibrium involving changes in bound water molecules upon complex formation is sensitive to change in water activity. This can be probed by osmotic stress produced by a low molecule weight cosolute to perturb water activity.[63−68] Acquisition of water molecules (around ten to several tens) upon ligand/DNA complex formation has been reported for a variety of ligands such as Hoechst 33258,[63] adriamycin, daunomycin,[64−66] 4′,6-Diamidino-2-phenylindole (DAPI), netropsin, pentamidine,[67] propidium, proflavine, 7-aminoactinomycin D,[65] and actinomycin-D.[68] As a result, their affinity to DNA was reduced when an inert cosolute was added to reduce water activity. Although the above studies were not performed with G-quadruplex DNA, one recent report showed that binding of berberine to the human telomeric G-quadruplex, which acquired 13 ± 2 water molecules, decreased with increasing cosolute concentration.[33]

To investigate how the change of water activity could affect the G-quadruplex−ligand interaction, spectroscopic titrations were performed under various concentrations of glycerol, PEG 200, and PEG 1000 for TMPyP4. As the most extensively studied ligand, different binding stoichiometries have been reported for TMPyP4 with the human telomere G-quadruplex, which ranged from 1 to up to 4 binding sites per G-quadruplex.[39,69,70] We found our data could be reasonably best fitted with two binding sites (Supporting Figure S10). The affinity constant extracted was 3.2×10^6 M^{-1} in K^+ and 1.6×10^6 M^{-1} in Na^+ solution in the absence of a crowding agent, which is in agreement with published values for the same interaction.[69,71] In Figure 8A, the natural logarithm of the binding affinity constant was plotted against osmolality as described in the standard method of hydration analysis.[33,63−68] It can be found that the binding affinity decreased as the osmolality was increased (water activity decreased) by the crowding agents. The negative slope of the three curves suggests that the formation of the TMPyP4/G-quadruplex complex acquired water. The linear relationship in glycerol solution allowed us to estimate that an average uptake of 30 ± 3 water molecules occurred when TMPyP4 bound to the G-quadruplex, using the following equation:[65]

$$\partial \ln(K_S/K_0)/\partial[\mathrm{Osm}] = -\Delta n_W/55.5$$

where K_s and K_0 are the binding affinity (K_A) in the presence and absence of a crowding agent, [Osm] is the osmolality of the medium, and Δn_w is the difference in the number of bound water molecules between the ligand/G-quadruplex complex and the free reactants, respectively. This number of acquired water molecules is twice the number of water molecules acquired by the berberine/telomere G-quadruplex.[33] The titrations in Na^+/glycerol solution also produced similar results (Supporting Figure S11), which yielded an acquisition of 28 ± 2 water molecules for TMPyP4 binding in glycerol solution. As a result, the binding of TMPyP4 with the G-quadruplex was not favored by the decrease in water activity caused by the crowding agents.

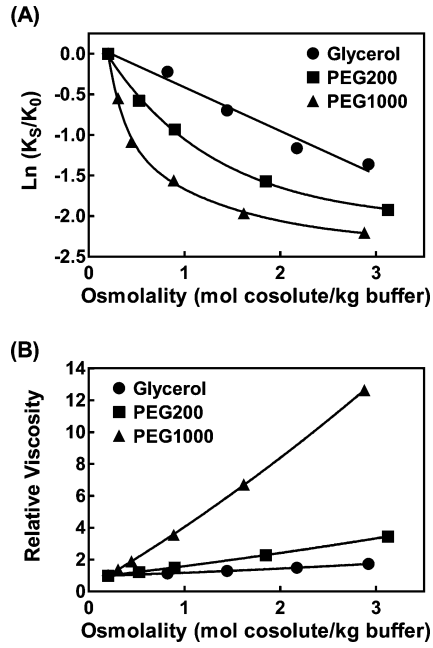

Figure 8. (A) Dependence of G-quadruplex binding affinity of TMPyP4 on medium osmolality. K_0 and K_S are the binding affinity constant in the absence and presence of crowding agent, respectively. They were obtained by titrating 2 μM TMPyP4 with human telomere G-quadruplex in 100 mM KCl solution. (B) Medium viscosity (relative to buffer without crowding agent) as a function of medium osmolality. Viscosity data were from refs 61 (PEG) and 72 (glycerol).

The lower affinity and nonlinear $\ln(K_S/K_0)$−osmolality relationship obtained in PEG solution suggest that other factors also significantly influence the binding of TMPyP4. Due to their higher molecular weight, much more PEG 200 and 1000 than glycerol had to be added to reach comparable osmolality. This would result in a higher viscosity, larger excluded volume, and lower dielectric constant in PEG solutions. As the viscosity increased, the diffusion of reactants might become rate-limiting. In Figure 8B, a more profound decrease in affinity in PEG 200 and 1000 solution correlated with a more significant increase in viscosity, suggesting the medium's viscosity might have a negative effect on the G-quadruplex−TMPyP4 interaction. Since the formation of the TMPyP4/G-quadruplex complex acquired water, its excluded volume might become larger than that occupied by the two free reactants and thus be suppressed by the more crowded medium. These contributions, as well as the decrease of dielectric constant, conformational change of the G-quadruplex induced by PEG could bring additional complexity into the G-quadruplex−TMPyP4 interaction and distort the linearity of the $\ln(K_S/K_0)$−osmolality relationship. More detailed studies are apparently required to explicitly clarify their contribution.

Summary

Stabilization of G-quadruplex by chemical ligands is emerging as a promising strategy against cancer and other diseases. For such ligands to function *in vivo*, it is important that they are still effective when delivered into cells. Molecular crowding is an important characteristic of the intracellular environment. Our present work demonstrates that molecular crowding can sub-

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stantially decrease the stabilization of G-quadruplexes and inhibition of telomerase by such ligands. This effect is a primary result directly associated with the environmental change that reduces the affinity between ligands and the G-quadruplex and is likely general because the effect was seen on G-quadruplexes of different structures (antiparallel, mixed antiparallel/parallel, and parallel) and sequences (telomere and *KRAS*), in different cation solutions (K^+ and Na^+), with different ligands (TMPyP4, BMVC, Hoechst 33258, and daunomycin), and crowding agents (PEG and glycerol). These *in vitro* findings suggest that the molecular crowding inside cells may potentially impose a negative effect on the intended ligand−G-quadruplex interaction and reduce its therapeutic potency. In addition to this, if G-quadruplexes are significantly stabilized by molecular crowding *in vivo*, then they may not be further stabilized by ligands as much as they can in dilute solution. For these reasons, it may be necessary to consider the intracellular molecular crowding reality in drug design and potency evaluation for G-quadruplex ligands.

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Supporting Information Available: Additional experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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