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Recognition of Chelerythrine to Human Telomeric DNA and RNA G-quadruplexes

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A study on binding of antitumor chelerythrine to human telomeric DNA/RNA G-quadruplexes was performed by using DNA polymerase stop assay, UV-melting, ESI-TOF-MS, UV-Vis absorption spectrophotometry and fluorescent triazole orange displacement assay. Chelerythrine selectively binds to and stabilizes the K⁺-form hybrid-type human telomeric DNA G-quadruplex of biological significance, compared with the Na⁺-form antiparallel-type DNA G-quadruplex. ESI-TOF-MS study showed that chelerythrine possesses a binding strength for DNA G-quadruplex comparable to that of TMPyP4 tetrachloride. Both 1 : 1 and 2 : 1 stoichiometries were observed for chelerythrine's binding with DNA and RNA G-quadruplexes. The binding strength of chelerythrine with RNA G-quadruplex is stronger than that with DNA G-quadruplex. Fluorescent triazole orange displacement assay revealed that chelerythrine interacts with human telomeric RNA/DNA G-quadruplexes by the mode of end- stacking. The relative binding strength of chelerythrine for human telomeric RNA and DNA G-quadruplexes obtained from ESI-TOF-MS experiments are respectively 6.0- and 2.5-fold tighter than that with human telomeric double-stranded hairpin DNA. The binding selectivity of chelerythrine for the biologically significant K⁺-form human telomeric DNA G-quadruplex over the Na⁺-form analogue, and binding specificity for human telomeric RNA G-quadruplex established it as a promising candidate in the structure-based design and development of G-quadruplex specific ligands.

Telomeres are the specialized and functional DNA-protein complexes at the end of all eukaryotic linear chromosomes which provide protection against gene erosion at cell divisions, chromosomal non-homologous end-joinings and nuclease attacks¹. DNA of telomere in vertebrates consists of tandem repeats of the sequence dTTAGGG. Human telomeric DNA is typically 5–8 kb long with a 3' protruding single-stranded overhang of 100–200 nt. Each cell division leads to a 50–200 base loss of the telomere due to the end-replication problem of telomere, which eventually results in critically short telomeres and ultimately triggers programmed cell death². The intervention of telomere maintenance processes provides an attractive and potentially broad-spectrum anticancer strategy in the design of anticancer therapeutics because one of the key features of cancer cells is to circumvent telomere shortening processes^{3,4}. More than 85% of cancer cells overexpress telomerase, a reverse transcriptase that elongates the telomeres by appending additional telomeric repeats. This process makes cancer cells immortal and allows continual cell division without telomere shortening and subsequent senescence^{5,6}. It has been found that human telomere forms G-quadruplex structures *in vivo*^{7,8} and in cell extracts⁹. In addition to maintaining chromosome stability¹⁰, telomeric G-quadruplexes present obstacles for recruitment of telomerase¹¹ and translocation of the DNA replication machinery¹². Currently, telomeric G-quadruplex targeting with the aid of small organic molecules is emerging as a novel approach to discover cancer therapeutics¹³.

The single-stranded G-rich telomeric DNA sequence can fold into an intramolecular G-quadruplex in the presence of monovalent cations, such as K⁺ or Na⁺. In the presence of Na⁺ ion, the human telomeric sequence folds into an intramolecular basket-type G-quadruplex structure (Figure 1A), which has one diagonal and two lateral loops, with the guanine columns in an antiparallel arrangement¹⁴. However, the folding structure of human telomeric sequence in K⁺ solution has been characterized by NMR to be in equilibrium between two hybrid-type mixed parallel/antiparallel strands of G-quadruplex conformations, i.e. Hybrid-1 (Figure 1B) and Hybrid-2 (major conformation, Figure 1C)^{15–17}. The Hybrid-1 structure has sequential side-lateral-lateral loops with the first TTA loop adopting the double-chain-reversal conformation, whereas the Hybrid-2 structure has lateral-

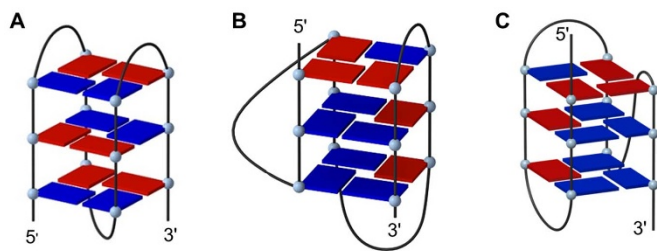


Figure 1 | The folding topologies of three types of intramolecular G-quadruplex structures for human telomeric DNA sequence. (A) Na^+ -form antiparallel G-quadruplex structure stabilized by Na^+ ion. (B) Hybrid-1 type and (C) Hybrid-2 type (major conformation) of K^+ -form mixed parallel/antiparallel G-quadruplex structure stabilized by K^+ ion. Blue and red boxes represent guanine bases in the *anti* and *syn* conformations, respectively.

lateral-side loops with the last TTA loop adopting the double-chain-reversal conformation^{15–17}. Furthermore, the K^+ readily converts the Na^+ -form conformation to the K^+ -form hybrid-type G-quadruplex¹⁵. As the intracellular K^+ concentration is much greater than that of Na^+ , the K^+ -form hybrid-type of human telomeric G-quadruplex structures are considered to be of biological significance.

Telomere is transcribed into telomeric repeat-containing RNA (TERRA)^{18,19}. These non-coding RNA molecules contain subtelomeric-derived sequences with an average of 34 rGGGUUA repeats at their 3' end²⁰. Various regulatory functions have been assigned to TERRA, such as heterochromatin regulation, telomerase inhibition, telomere length regulation and telomere protection^{21–24}. Importantly, TERRA RNA G-quadruplexes were found in living cells²⁵. TERRA is also regarded as a promising cancer therapeutic target although the functional significance of TERRA G-quadruplex is still in need of robust experimental support²⁰. It has been shown by NMR spectroscopy^{26,27} and by X-ray crystallography²⁸ that human telomeric repeat-containing RNA sequence r(UUAGGG)₄ folds into a parallel G-quadruplex in monovalent cation solution that is more stable than its DNA counterparts.

Chelerythrine (Figure 2), a natural benzophenanthridine alkaloid, possesses diverse pharmacological activities, including potent anticancer and cytotoxic activities^{29–31}. Recently, it was found that chelerythrine recognizes G-quadruplex in *c-kit* promoter with a high affinity via end-stacking mode³². G-quadruplex induction ability of chelerythrine has been studied by means of CD spectroscopy, CD-melting experiments and ESI-MS techniques. This alkaloid can transform random coil of human telomeric DNA to antiparallel G-quadruplex structure³³. To the best of our knowledge, it has never been reported in the aspect of its binding with any preformed human telomeric DNA G-quadruplex, neither the K^+ -form nor the Na^+ -form of DNA G-quadruplex structures, which is still in need of a comprehensive and comparative investigation. In addition, it still

remains unexplored in the interaction of chelerythrine with human telomeric RNA G-quadruplex. In this study, we employed DNA polymerase stop assay, UV-melting study, UV-Vis absorption spectrophotometry, fluorescent triazole orange (TO) displacement assay and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) to investigate the binding of chelerythrine to both K^+ -form and Na^+ -form human telomeric DNA G-quadruplexes. The conformation selectivity of chelerythrine to human telomeric DNA G-quadruplex was consequently examined by comparing its binding with two types of DNA G-quadruplexes. Moreover, the interaction of chelerythrine with human telomeric RNA G-quadruplex was also explored and its binding capacity with RNA G-quadruplex was compared to that with DNA counterpart. Finally, the mode of chelerythrine binding with human telomeric both DNA and RNA G-quadruplexes was studied.

Results

Binding of chelerythrine to the K^+ -form hybrid-type human telomeric DNA G-quadruplex. As a sensitive and effective method for screening G-quadruplex-interactive agents with potential clinical utility, DNA polymerase stop assay makes use of the fact that G-rich DNA templates present obstacles by formation of intramolecular G-quadruplex structures in the presence of potassium or sodium cation to DNA synthesis by DNA polymerase. The ligands which can stabilize an intramolecular G-quadruplex structure lead to enhanced arrest of DNA synthesis in the presence of monovalent cation of K^+ or Na^+ ^{34,35}.

The stabilizing effect of chelerythrine on the K^+ -form DNA G-quadruplex was firstly investigated by DNA polymerase stop assay³⁴ in the presence of K^+ cation. The experiment was carried out in 50 mM K^+ containing buffer of 10 mM Tris-HCl (pH7.50) by using DNA template tem-3 and tem-4 (Table S1) which contained 3 and 4 human telomeric repeats TTAGGG, respectively. TMPyP4, a well-studied G-quadruplex DNA binder (Figure 2), was used as a positive control. As a result (Figures 3A and 3B), neither TMPyP4 nor chelerythrine arrested DNA synthesis for template-3, because it couldn't produce a G-quadruplex structure with only three human telomeric G-rich repeats. However, chelerythrine showed the same *Taq* polymerase inhibition activity as that of TMPyP4 by stabilizing the G-quadruplex structure present in tem-4 containing four telomeric repeats in the presence of K^+ (Figure 3B). For chelerythrine, a series of concentration-dependent paused bands appeared at the very beginning of the G-quadruplex forming site, *i.e.*, the first guanine site of G-rich repeats in tem-4 (from 3' to 5'). This gel result showed that chelerythrine binds to and stabilizes the K^+ -form DNA G-quadruplex in a concentration-dependent manner.

DNA melting temperature (T_m) measurement was utilized to further study the binding behavior of chelerythrine with the preformed K^+ -form hybrid type DNA G-quadruplex formed by d[AGGG(TTAGGG)₃] in 25 mM Tris-HCl buffer (pH7.43) containing 100 mM KCl (Figure S1A–B). The melting curve of d[AGGG(TTAGGG)₃] showed a reverse “S” shape at 295 nm with a T_m value of 67.1°C, indicating the formation of K^+ -form hybrid type G-quadruplex structure. The T_m value of the K^+ -form DNA G-quadruplex was increased 6.2°C by chelerythrine (Figure S1A) in a 2:1 molar ratio of ligand-to-DNA, implying that chelerythrine provided thermal stability to the K^+ -form DNA G-quadruplex.

Binding of chelerythrine to the Na^+ -form antiparallel-type human telomeric DNA G-quadruplex. The Na^+ -form human telomeric DNA G-quadruplex is another principal secondary intramolecular four-stranded DNA structure. The stabilizing effect of chelerythrine on the Na^+ -form DNA G-quadruplex structure was also investigated by DNA polymerase stop assay in the presence of Na^+ cation. As shown in Figure 3D, the gel result in the presence of Na^+ ion is completely different from that conducted in K^+ condition

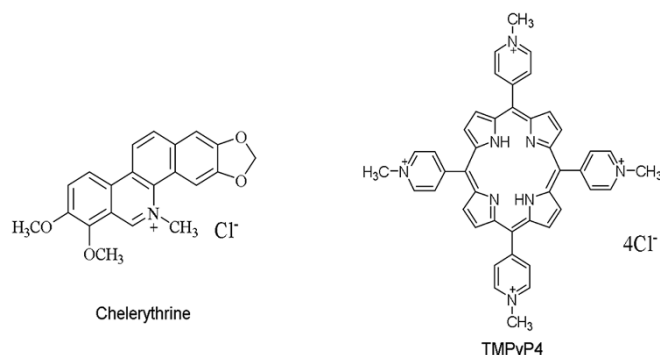


Figure 2 | Chemical structures of chelerythrine and TMPyP4.

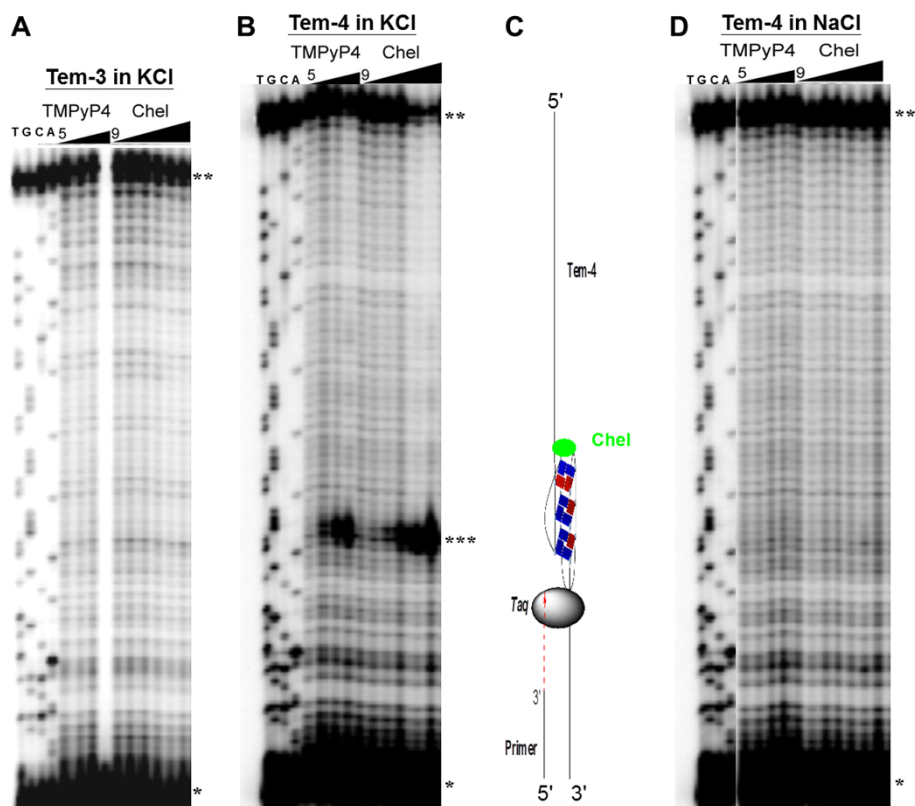


Figure 3 | Concentration-dependent inhibition of *Taq* polymerase DNA synthesis by G-quadruplex binding ligands. (A) on the DNA templates Tem-3 and (B, D) Tem-4 at 42°C in 10 mM Tris-HCl buffer (pH 7.50) containing (A, B) 50 mM KCl or (D) 50 mM NaCl. The lane markers T, G, C, and A indicate the bases on the template strand. (A) Lanes 5–8: TMPyP4 (0, 0.3, 1 and 3 μ M) and lanes 9–14: chelerythrine (0, 0.3, 1, 3, 10 and 30 μ M) for Tem-3; (B) Lanes 5–8: TMPyP4 (0, 0.1, 0.3 and 1 μ M) and lanes 9–14: chelerythrine (0, 0.1, 0.3, 1, 3 and 10 μ M) for Tem-4; (D) Lanes 5–8: TMPyP4 (0, 0.1, 0.3 and 1 μ M) and lanes 9–15: chelerythrine (0, 0.1, 0.3, 1, 3, 10 and 30 μ M) for Tem-4. * primers, ** full-length products, *** paused bands. (C) A schematic illustration for DNA polymerase stop assay with 4 telomeric repeats.

(Figure 3B). Neither TMPyP4 nor chelerythrine exhibited any significant paused band on Tem-4 at the tested concentrations (from 0 to 30 μ M for chelerythrine) in the presence of 50 mM NaCl. This suggested that chelerythrine can not sufficiently stabilize the Na⁺-form DNA G-quadruplex to cease the polymerase reaction even at a high concentration of 30 μ M, which is completely different from that of the K⁺-form DNA G-quadruplex (Lanes 12–14 in Figure 3B). The obvious difference of gel results manifested that chelerythrine selectively stabilizes the K⁺-form hybrid type human telomeric DNA G-quadruplex.

To further examine the effect of chelerythrine on the Na⁺-form DNA G-quadruplex structure, thermal denaturation profiles of d[AGGG(TTAGGG)₃] in 100 mM NaCl solution were monitored at 295 nm in the absence and presence of chelerythrine (Figure S1C–D). Apparently, there was no significant increase of T_m value of d[AGGG(TTAGGG)₃] by chelerythrine even in a 6 : 1 alkaloid-to-DNA molar ratio. This UV-melting result together with the data of polymerase stop assay conducted in 50 mM NaCl solution indicated that chelerythrine does not interact with the Na⁺-form human telomeric DNA G-quadruplex or weakly binds to the Na⁺-form G-quadruplex which can't be detected under the tested condition. The completely different binding behaviour of chelerythrine to the Na⁺-form and K⁺-form DNA G-quadruplexes revealed that this alkaloid showed binding specificity for the intramolecular human telomeric K⁺-form DNA G-quadruplex.

Binding of chelerythrine to human telomeric DNA G-quadruplex by ESI-TOF-MS. The high resolution time-of-flight mass spectrometry (TOF-MS), connected with a soft electrospray

ionization (ESI) source, has been playing a powerful and active role in the investigation of noncovalent interaction of small organic molecules with nucleic acids. It is a highly sensitive and reliable method to determine stoichiometries, relative binding affinities and equilibrium association constants of drug-DNA complexes^{36–38}. Quantitative determination of binding constants for minor groove binders with different DNA duplexes has been demonstrated³⁹, and the interaction of ligands with G-quadruplex structures has also been successfully investigated^{32,40–43}.

A remarkable difference should be taken into consideration between the gas-phase method of ESI-TOF-MS and the solution-phase methods in the preparation of complexes of the alkaloid with DNA G-quadruplex. The G-quadruplex stabilizing metal ion K⁺ cation should be replaced by NH₄⁺ ion in ESI-TOF-MS study, because NH₄⁺ ion is the best choice for ESI-MS experiment and it can also stabilize intramolecular G-quadruplex structures. CD spectroscopy was thus used to compare the conformational characteristics of chelerythrine-G-quadruplex complex between in K⁺ and NH₄⁺ cation solutions. As a result, the CD spectrum of d[AGGG(TTAGGG)₃] with chelerythrine in 100 mM NH₄OAc solution exhibited similar pattern to that in 100 mM KCl solution (Figure S2). Consequently, the complex of preformed human telomeric DNA G-quadruplex with chelerythrine in ammonium acetate solution for ESI-TOF-MS experiments is regarded as a homogeneous one with that folded in KCl solution.

ESI-TOF-MS was therefore utilized to directly observe the noncovalent complexes of chelerythrine with human telomeric DNA G-quadruplex formed by the sequences of both 22 nt d[AGGG(TTAGGG)₃] (Figure 4) and 27 nt d[(TTAGGG)₄TTA]

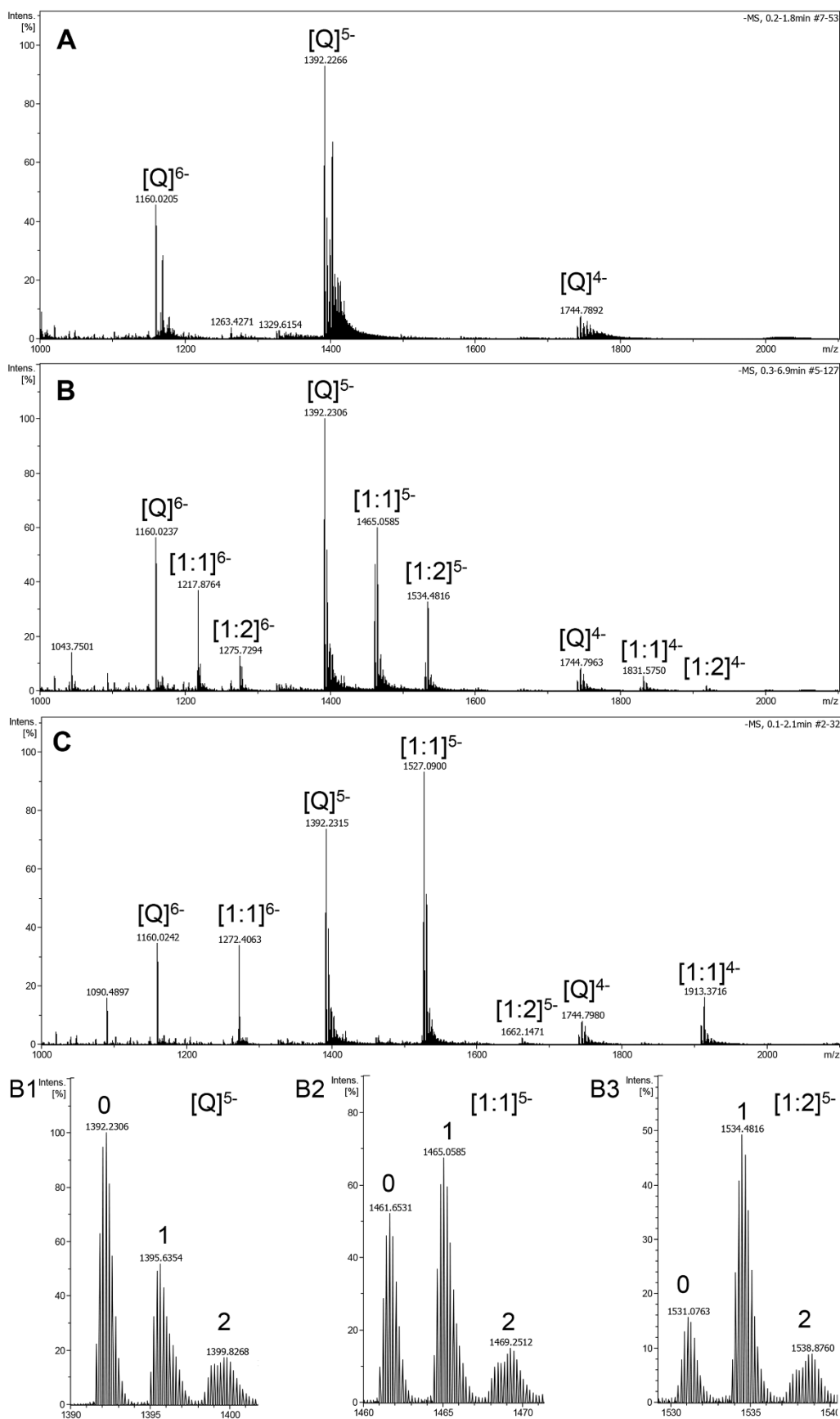


Figure 4 | Negative ESI-TOF-MS spectra of human telomeric sequence $d[AGGG(TTAGGG)_3]$ (Q). (A) without drug, (B) with equimolar of chelerythrine, and (C) with equimolar of TMPyP4 tetrachloride. B1–B3 are the enlargements of the distribution of ammonium adducts for the five charge state of the free G-quadruplex, [1:1] and [1:2] complexes of DNA with chelerythrine. The numbers represent the number of ammonium adducts.



(Figure 5). The free G-quadruplex (Q) exhibited ions mainly in the charge states of -5 and -6 in both Figure 4A and 5A. In each charge state, there were three ions corresponding to $[M - xH]^{x-}$ (the lone oligodeoxynucleotide), $[M + NH_4^+ - (x + 1)H]^{x-}$ (one NH_4^+ ion adduct), and $[M + 2NH_4^+ - (x + 2)H]^{x-}$ (two NH_4^+ ions adduct), respectively. It was found that the ammonium ions were much more stably conserved between G-tetrads of 27 nt G-quadruplex (Figure 5B1) than those of 22 nt G-quadruplex (Figure 4B1) under the same mass spectrometric conditions. Both 1:1 and 2:1 complexes of chelerythrine and DNA G-quadruplex were observed at m/z corresponding to $[M + Che - 6H]^{5-}$, $[M + NH_4^+ + Che - 7H]^{5-}$, $[M + 2NH_4^+ + Che - 8H]^{5-}$ (Figure 4B2 and 5B2) and $[M + 2Che - 7H]^{5-}$, $[M + NH_4^+ + 2Che - 8H]^{5-}$, $[M + 2NH_4^+ + 2Che - 9H]^{5-}$ (Figure 4B3 and 5B3) when an equal mole of chelerythrine was added to DNA. The complex peaks with two NH_4^+ ion adducts became more predominant than those with one NH_4^+ ion adduct or none (Figure 5B2–5B3 and Figure 53B). This indicated that the G-quadruplex structure stabilized by chelerythrine holds NH_4^+ ions inserted between G-tetrads more tightly than free G-quadruplex in the course of being introduced into the gas phase. In other words, chelerythrine-bound G-quadruplex is more stable than when it is unbound due to the great stability provided by chelerythrine⁴².

The relative binding affinity (RBA) of chelerythrine was calculated to evaluate the binding capacity of chelerythrine to human telomeric DNA G-quadruplex by the equation: $RBA = \frac{\sum \text{complex peaks areas}}{\sum \text{free G-quadruplex peaks areas}}$, and subsequently compared to that of the reference G-quadruplex binder of TMPyP4 tetrachloride⁴³ (Figure 4C and 5C). The average of RBA value was determined from three independent measurements. An equal RBA value was obtained for chelerythrine binding with both 22 nt (1.05 ± 0.04) and 27 nt (1.08 ± 0.08) human telomeric DNA G-quadruplex (Table 1). In addition, chelerythrine showed an almost equivalent RBA value to that of TMPyP4 tetrachloride [(1.15 ± 0.10) for 22 nt G-quadruplex, (1.10 ± 0.12) for 27 nt G-quadruplex] in a 1:1 molar ratio of ligand-to-DNA, suggesting that the binding strength of chelerythrine with human telomeric DNA G-quadruplex is comparable to that of TMPyP4 tetrachloride.

Binding of chelerythrine to human telomeric RNA G-quadruplex by ESI-TOF-MS. The binding of chelerythrine with human telomeric RNA G-quadruplex $r[(UUAGGG)_4UUA]$ was also studied under the same ESI-TOF-MS condition as that of DNA counterpart (Figure 6). As shown in the ESI-TOF-MS spectra of $r[(UUAGGG)_4UUA]$ (Figure 6A), the three predominant ions with m/z value of 1259.1657 ($[M + 2NH_4^+ - 9H]^{7-}$), 1469.3628 ($[M + 2NH_4^+ - 8H]^{6-}$) and 1763.4552 ($[M + 2NH_4^+ - 7H]^{5-}$) correspond to two NH_4^+ ion adducts of $r[(UUAGGG)_4UUA]$, indicating the high stability of RNA G-quadruplex in the gas phase. Similar to that of DNA G-quadruplex, chelerythrine binds to human telomeric RNA G-quadruplex with 1:1 and 2:1 stoichiometry complexes when 1:1 molar ratio of alkaloid-to-RNA was used. Moreover, it was observed that all peaks of complexes contained two NH_4^+ ions which resided between three G-tetrads of RNA G-quadruplex. The RBA value of chelerythrine was 2.51 with human telomeric RNA G-quadruplex (Table 1).

Mode of chelerythrine's binding to human telomeric DNA/RNA G-quadruplexes by fluorescent triazole orange displacement assay and UV-Vis spectrophotometry. Triazole orange (TO) is a dye which end-stacks on the two external quartets of a G-quadruplex with high affinity ($K_a = 3 \times 10^6 \text{ M}^{-1}$)⁵⁶. TO is highly fluorescent upon interaction with G-quadruplex DNA (~ 500 - to 3000-fold exaltation) whereas is completely quenched when free in solution, which ensures that the displacement of a given ligand can be easily monitored by the decrease of TO fluorescence ($\lambda_{\text{max}} = 530 \text{ nm}$) with an excitation wavelength at 501 nm^{44,45}. Therefore, fluorescent TO displacement assay was utilized to investigate the binding mode of chelerythrine with human telomeric DNA (Figure 7,

Figure S4A–C) and RNA G-quadruplexes (Figure S4D). As illustrated in Figure 7, the percent fluorescence decrease of TO was 63.1% when 0.8 equivalent of chelerythrine was added to the solution of TO-bound G-quadruplex (Table S2), indicating that most of the G-quadruplex-bound TO has been driven out by chelerythrine. This provided solid evidence that chelerythrine binds to human telomeric DNA/RNA G-quadruplex in a mode of end-stacking.

The mode of chelerythrine binding to human telomeric both RNA and DNA G-quadruplexes was subsequently investigated by means of ultraviolet-visible (UV-Vis) spectrophotometry (Figure S5). The UV-Vis absorption spectra of chelerythrine with RNA and DNA G-quadruplex in a 2:1 molar ratio displayed red shifts of 10 nm (from 268 nm to 278 nm) and 2 nm (from 268 nm to 270 nm) associated with 73.4% and 39.4% hypochromism, respectively (Figure S5). These striking spectral changes exhibited π - π stacking interactions between the chromophore of chelerythrine and the G-tetrad. Taken the results from TO displacement assay and UV-Vis spectrophotometry together, it was implied that chelerythrine binds to G-quadruplex via the mode of end-stacking. Moreover, mass spectrometry also provided supporting information on the binding mode of chelerythrine to the human telomeric DNA/RNA G-quadruplex. The complex peaks with two ammonium cations, i.e., $[Q + 2NH_4^+ + \text{Alkaloid} - 8H]^{5-}$ and $[Q + 2NH_4^+ + 2\text{Alkaloid} - 9H]^{5-}$ in Figure 5B and 6B, indicated that two molecules of chelerythrine bind to the DNA/RNA G-quadruplex by end stacking mode, as two ammonium ions were conserved between three G-tetrads^{42,46,47}. This G-quadruplex interacting mode of chelerythrine was also consistent with that of berberine, an isoquinoline alkaloid which was found to be stacked onto the two external G-tetrads determined by both X-ray diffraction⁴⁸ and molecular modeling methods⁴⁹. In addition, the UV-Vis absorption spectra of chelerythrine complexed with RNA G-quadruplex showed a more remarkable change of 8 nm bathochromic shift and 34% hypochromicity than those with DNA counterpart. This significant spectral change of chelerythrine with RNA G-quadruplex over DNA analogue provided supporting evidence that chelerythrine shows a preference for human telomeric RNA G-quadruplex over DNA counterpart.

Selectivity of chelerythrine to human telomeric G-quadruplex over double-stranded and single-stranded oligodeoxynucleotides. In order to get an insight into G-quadruplex selectivity of chelerythrine, its binding with both double-stranded and single-stranded DNA was further investigated by means of ESI-TOF-MS under the same conditions as those of G-quadruplex, since the binding of chelerythrine with double-stranded oligodeoxynucleotides has been previously reported⁵⁰. Two 27 nt oligodeoxynucleotides, $d[(TTAGGG)_2TTA(\underline{CCCTAA})_2]$ and $d[(TTAGGG)_2TTAAAATTAGGGT-TA]$ mutated from human telomeric sequence $d[(TTAGGG)_4TTA]$, were used to form double-stranded hairpin and single-stranded random coil DNA, respectively, in order to obtain similar mass spectrometric responses with oligodeoxynucleotides in the same length and close base components. Only 1:1 complexes were significantly observed for chelerythrine with double-stranded (Figure S6) and single-stranded (Figure S7) DNA, and corresponding RBA values were calculated (Table 1). As a result (Table 1), the relative binding constant of chelerythrine with RNA G-quadruplex was 6 and 12-fold tighter than that with double-stranded hairpin and single-stranded DNA, respectively. Its RBA value with DNA G-quadruplex was 2.5 and 5-fold stronger than that with double-stranded hairpin and single-stranded DNA, respectively. This indicated that chelerythrine possesses G-quadruplex specificity for human telomeric RNA and DNA G-quadruplexes.

Discussion

A study on binding of chelerythrine, an antitumor natural alkaloid, to human telomeric DNA and RNA G-quadruplex was carried out by

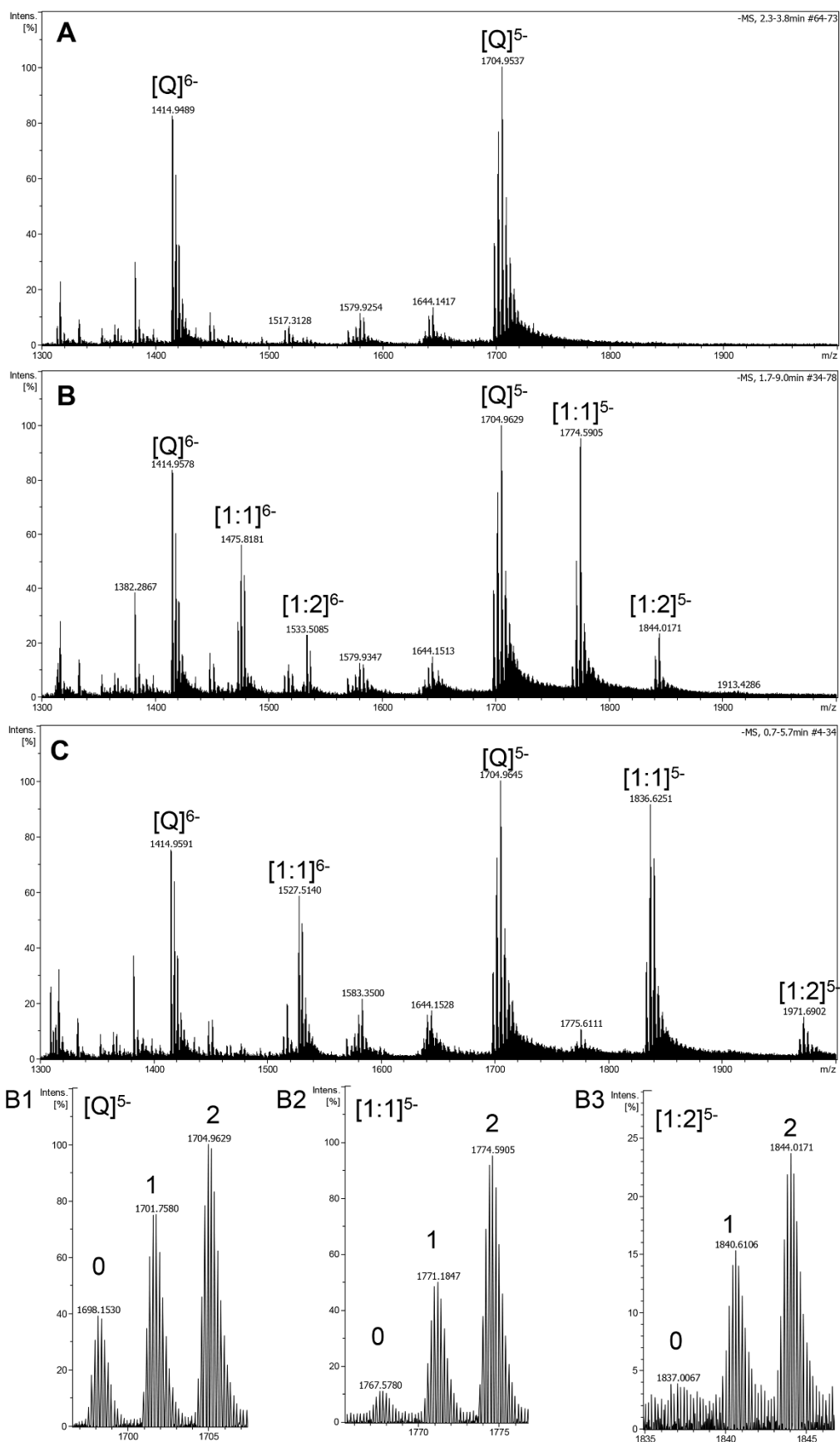


Figure 5 | Negative ESI-TOF-MS spectra of human telomeric sequence d[(TTAGGG)₄TTA] (Q). (A) without drug, (B) with equimolar of chelerythrine, and (C) with equimolar of TMPyP4 tetrachloride. B1–B3 are the enlargements of the distribution of ammonium adducts for the five charge state of the free G-quadruplex, [1 : 1] and [1 : 2] complexes of DNA with chelerythrine. The numbers represent the number of ammonium adducts.



Table 1 | Relative binding affinities of chelerythrine with the intramolecular human telomeric G-quadruplex, double-stranded and single-stranded nucleic acids obtained by ESI-TOF-MS method

Nucleic acids	RBA
r[(UUAGGG) ₄ UUA] (RNA G-quadruplex)	2.51 ± 0.13
d[AGGG(TTAGGG) ₃] (22 nt DNA G-quadruplex)	1.05 ± 0.04
d[(TTAGGG) ₄ TTA] (27 nt DNA G-quadruplex)	1.08 ± 0.08
d[(TTAGGG) ₂ TTA(CCCTAA) ₂] (ds-DNA)	0.42 ± 0.03
d[(TTAGGG) ₂ TTAAAATTAGGGTTA] (ss-DNA)	0.21 ± 0.02

using various analytical methods including DNA polymerase stop assay, UV-melting study, ESI-TOF-MS, fluorescent TO displacement assay and UV-Vis absorption spectrophotometry. This study showed that chelerythrine selectively binds to and stabilizes human telomeric K⁺-form DNA G-quadruplex which is considered to be a biologically significant DNA secondary structure, as evidenced from the appearance of a series of intensive concentration-dependent paused bands in PAGE gel, and increase of thermal stability of K⁺-form G-quadruplex by chelerythrine. In sharp contrast, chelerythrine does not show any significant stabilizing effect on the Na⁺-form DNA G-quadruplex under tested conditions. The selectivity of chelerythrine to K⁺-form G-quadruplex over Na⁺-form analogue is in agreement with that sanguinarine (sharing the same skeleton with chelerythrine) shows much larger binding affinity with K⁺-form G-quadruplex than with Na⁺-form analogue⁴⁹. ESI-TOF-MS study showed that chelerythrine exhibited a comparable relative binding affinity to that of TMPyP4 tetrachloride, a well-known G-quadruplex binder, with human telomeric DNA G-quadruplex. Both 1 : 1 and 2 : 1 stoichiometries were observed for chelerythrine complexed with human telomeric both DNA and RNA G-quadruplexes. The relative binding affinity of chelerythrine with RNA G-quadruplex was greatly higher than that with DNA counterpart. The binding specificity of chelerythrine for RNA G-quadruplex over DNA analogue was further supported by the facts of 8 nm larger bathochromic shift and 34% more hypochromicity presented in the absorption spectra of chelerythrine when complexed with RNA G-quadruplex than with DNA counterpart. The stronger interaction of chelerythrine with RNA G-quadruplex may be due to the higher stability of RNA G-quadruplex in which the C2'-OH groups make a major contribution to the increased stability of the RNA G-quadruplex over equivalent DNA analogue²⁸. Further study on chelerythrine binding to human telomeric double-stranded hairpin and single-stranded oligodeoxynucleotides revealed that chelerythrine possesses binding specificity for human telomeric RNA/DNA G-quadruplex as evidenced from that its relative binding ability with four-stranded RNA/DNA G-quadruplexes are at least 2.5 and 5.0-fold stronger than those with both double-stranded and single-stranded DNA, respectively. In addition, chelerythrine was determined to bind to the human telomeric DNA/RNA G-quadruplex by the mode of end stacking on the basis of the fact that it displaces the TO positioned on the G-tetrad and the characteristic UV-Vis absorption spectral changes of bathochromic shifts (2 nm and 10 nm, respectively) and hypochromicities (39.4% and 73.4%, respectively) of chelerythrine with addition of DNA/RNA G-quadruplex, along with a series of complex peaks of chelerythrine and DNA/RNA G-quadruplex containing two NH₄⁺ ions observed in ESI-TOF-MS spectra.

The stabilization of G-quadruplexes with small organic molecules gives rise to various effects on telomere functions, such as telomerase inhibition, shortening of telomere length, and inhibition of tumor cell growth^{51–56}. The binding selectivity of chelerythrine to human telomeric K⁺-form DNA G-quadruplex over the Na⁺-form analogue, and binding specificity for human telomeric RNA G-quadruplex establish it as a promising candidate in the structure-based design and development of G-quadruplex specific ligands.

Methods

Materials. Oligodeoxynucleotides used in DNA polymerase stop assay and UV-melting experiments were purchased from FASMAG Co., Ltd. (Japan). Human telomeric DNA/RNA and all other DNA sequences used in ESI-TOF-MS and UV-Vis absorption experiments were bought from Invitrogen (Japan). The concentrations of single-stranded oligodeoxynucleotides were determined from UV absorbance at 260 nm, supposing the molar extinction coefficients (ϵ) of A, T, G, and C to be 16,000, 9,600, 12,000, and 7,000 M⁻¹cm⁻¹, respectively. The DNA and RNA were annealed in 100 μ M stock solutions in 100 mM KCl or NaCl or ammonium acetate solution, heating to 95 °C for 5 minutes, then slowly cooling down to room temperature. The annealed solution were stored at 4 °C for at least 24 hours before the use. The 5' Texas red-labeled DNA primer and human telomeric template sequence in PAGE grade were obtained from JBioS Co., Ltd. (Japan) and dissolved in Milli Q water to be used without further purification. Acrylamide/bisacrylamide solution and ammonium persulfate were purchased from Bio-Rad, and N,N,N',N'-tetramethylethylenediamine was purchased from Fisher. r*Taq* DNA polymerase and dNTPs were purchased from TaKaRa (Japan). Sequence marker kit was purchased from TOYOBO Co., Ltd. (Japan). TMPyP4 tetratosylate was purchased from Sigma-Aldrich (USA), and converted to the tetrachloride salt by an Amberlite® IRA-400 (Cl) ion exchange resin column⁴³. Stock solutions of chelerythrine (10 mM) and TMPyP4 were made in either DMSO or Milli Q water. Further dilutions to working concentrations were made with Milli Q water immediately prior to usage.

DNA polymerase stop assay⁴⁷. A reaction mixture of template DNA (0.15 μ M) and 5' Texas red-labeled 20-mer primer (0.1 μ M) was heated to 95 °C for 3 min in a *Taq* (TaKaRa) reaction buffer (pH 7.5, final concentration of 10 mM Tris-HCl, 50 mM KCl or 50 mM NaCl, 1.5 mM MgCl₂) and left to cool to ambient temperature over 30 min. The desired amount of chelerythrine (0, 0.1, 0.3, 1, 3, 10 and 30 μ M) (TMPyP4 tetratosylate as a reference compound in the concentrations of 0, 0.1, 0.3, 1 and 3 μ M) was added to the reaction mixture and kept at ambient temperature for 1 hour before the addition of polymerase. Then *Taq* polymerase and dNTPs were put into the reaction mixture, which was further incubated at 42 °C for 30 min. The reactions were terminated by adding three-fold volume of ethanol-3 M sodium acetate mixture (30 : 1 V/V) and subsequently loaded on a 6% sequencing PAGE gel, then analyzed by a Hitachi SQ5500E automated sequencer. The sequence markers were prepared according to the TOYOBO sequence Protocol.

UV-melting study^{42,57}. UV melting profiles of G-quadruplexes were measured at 295 nm using a UV-2700 spectrometer (Shimadzu, Japan) equipped with a Shimadzu TMSPC-8 temperature controller. Before measurement, DNA was annealed in 100 mM KCl or NaCl solution at 95 °C for 5 min and cooled to room temperature, then incubated at 4 °C overnight. The G-quadruplex DNA (5 μ M) samples were heated from 4 °C to 98 °C with a heating rate of 1 °C/min in the absence and presence of chelerythrine or TMPyP4 tetrachloride (10 and 30 μ M for the K⁺-form and Na⁺-form G-quadruplex, respectively). Each absorbance profile of G-quadruplex at 295 nm was recorded using all the same substances except the DNA as a blank control. The melting temperature (T_m) was calculated by the median method from the average of three independent measurements.

CD spectroscopy⁴⁷. CD spectra were recorded in a J-725 spectropolarimeter (Jasco, Japan) equipped with a 1 cm path length quartz cuvette. CD spectra for the preformed DNA G-quadruplex (5 μ M) were determined in the absence and presence of chelerythrine (25 μ M) in either 100 mM KCl solution or 100 mM ammonium acetate solution between 200 and 400 nm at 25 °C. A buffer blank correction was made for all spectra.

ESI-TOF-MS spectrometry^{43,58}. All ESI-TOF-MS experiments were conducted on a Bruker maXis impact mass spectrometer in the negative mode with the same method as previously described⁴³. The source parameters were set as follows: capillary voltage of +3500 V, nebulizer of 1.0 bar, dry gas flow of 4.0 L/min at 120 °C, and end plate offset voltage of 500 V. The analyzer was operated at a background pressure of 5 × 10⁻⁷ mbar. The rate of sample injection into the mass spectrometer was 3 μ L/min. The mixture of a final concentration of 10 μ M chelerythrine (TMPyP4 tetrachloride⁴³ as a reference DNA G-quadruplex binder) and 10 μ M various nucleic acids in 40 mM ammonium acetate solution (pH 7.0) was directly injected into mass spectrometer. Each sample of nucleic acid-drug complex solution was prepared in triplicate. Each mass spectrum was an average of at least 50 scans. Data were analyzed with the software of Compass DataAnalysis. The oligonucleotide sequences are shown as follows:

- (i) human telomeric DNA (22 nt): d[AGGG(TTAGGG)₃] ($M = 6966.6$ Da)
- (ii) human telomeric DNA (27 nt): d[(TTAGGG)₄TTA] ($M = 8496.6$ Da)
- (iii) double-stranded hairpin DNA: d[(TTAGGG)₂TTA(CCCTA)₂] ($M = 8273.1$ Da)
- (iv) single-stranded DNA: d[(TTAGGG)₂TTAAAATTAGGGTTA] ($M = 8447.0$ Da)
- (v) human telomeric RNA: r[(UUAGGG)₄UUA] ($M = 8789.3$ Da)

Fluorescent intercalator displacement assay⁴⁵. Competitive triazole orange (TO) displacement experiments were carried out with chelerythrine on a Cary Elipse

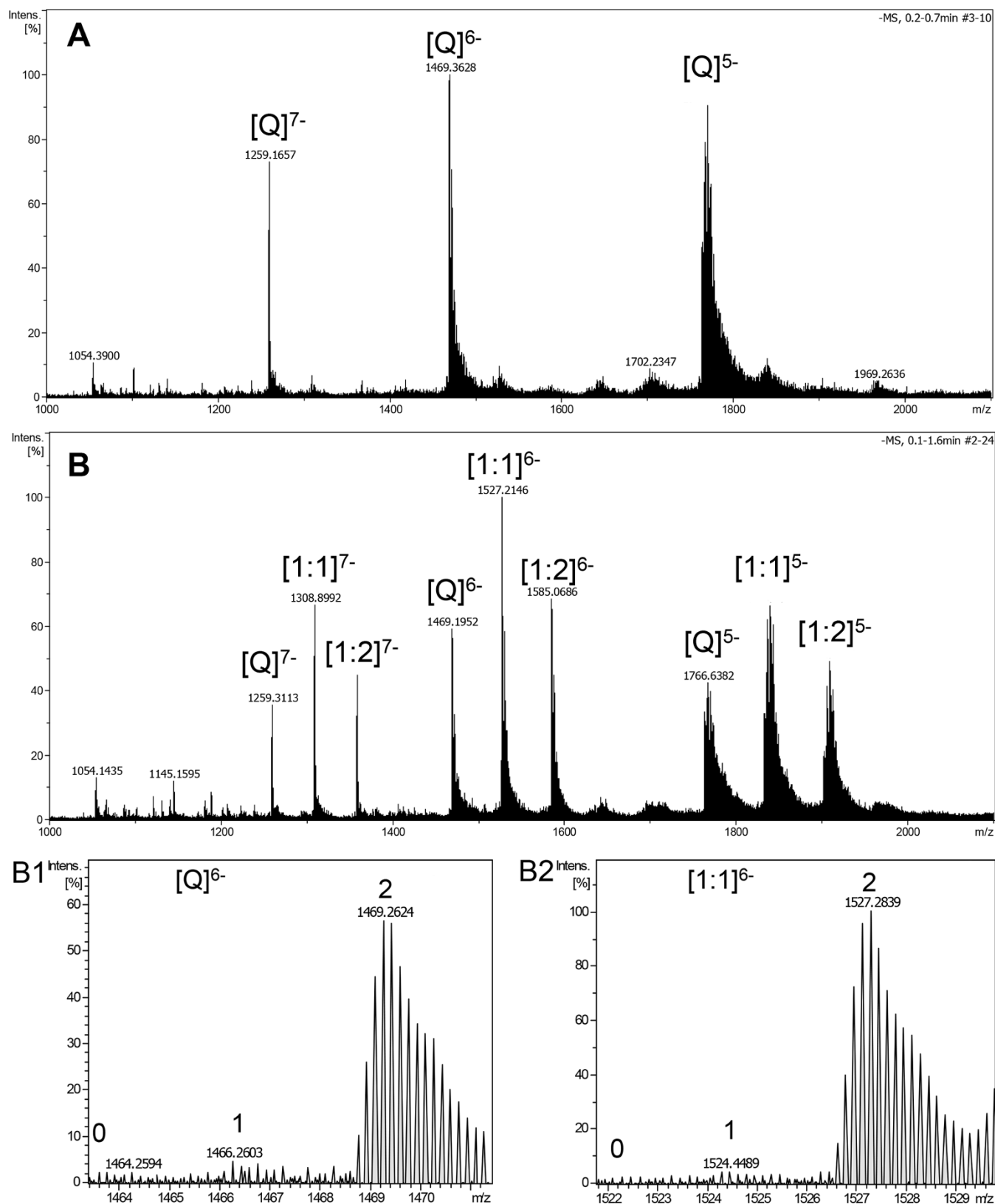


Figure 6 | Negative ESI-TOF-MS spectra of human telomeric sequence $r[(UUAGGG)_4UUA]$ (Q). (A) without drug, and (B) with equimolar of chelerythrine. B1–B2 are the enlargements of the distribution of ammonium adducts for the six charge state of the free G-quadruplex and [1 : 1] complex of RNA with chelerythrine. The numbers represent the number of ammonium adducts.

Fluorescence Spectrophotometer (Agilent Technologies) according to the method described in the literature⁴⁵. Aliquots of the chelerythrine (2.0×10^{-4} M) solutions containing 22 nt DNA G-quadruplex $d[AGGG(TTAGGG)_3]$ or 27 nt RNA G-quadruplex (0.25×10^{-6} M) and TO (0.5×10^{-6} M) were added into 400 μ L

solution of G-quadruplex (0.25×10^{-6} M) and TO (0.5×10^{-6} M) in 25 mM Tris-HCl buffer (pH7.43) containing 100 mM KCl. This operation ensured that the concentrations of chelerythrine increased gradually from 0 to 0.40 and 0.80 μ M, while the concentrations of DNA/RNA and TO were kept constant. An equilibrium

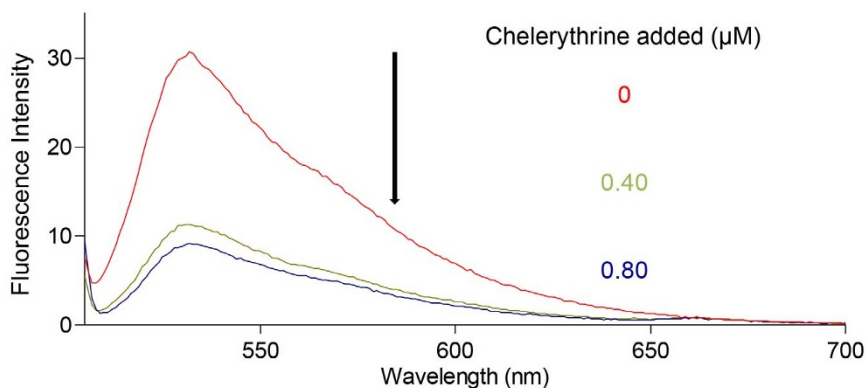


Figure 7 | Fluorescence spectra of triazole orange (0.5 μM) with d[AGGG(TTAGCG)₃] (0.25 μM). The spectra with the excitation at 501 nm were recorded in the presence of 0, 0.40 and 0.80 μM of chelerythrine, respectively, in 25 mM Tris-HCl buffer (pH 7.43) containing 100 mM KCl.

period of 5 minute for constant stirring of the mixed solution was allowed before measuring each spectrum. The fluorescence spectra were recorded with an excitation wavelength at 501 nm at room temperature, and the fluorescence intensities of maximum emission of bound TO at 530 nm were used to calculate the percent fluorescence decrease induced by competitive binding of chelerythrine.

UV-Vis absorption spectrophotometry^{47,50}. UV-Vis absorption spectra were recorded with a UV-2700 ultraviolet-visible spectrophotometer (Shimadzu, Japan) over the spectral range of 200–800 nm. Spectrophotometric titrations were carried out by keeping the concentration of chelerythrine constant while gradually increasing the RNA/DNA concentration. To a solution of chelerythrine (5×10^{-6} M, 700 μL) in 25 mM Tris-HCl buffer (pH 7.43) containing 100 mM KCl was added aliquots (0, 10 and 20 μL) of preformed RNA or DNA G-quadruplex (1.775×10^{-4} M) solution containing the same concentration of chelerythrine (5×10^{-6} M) in 100 mM KCl solution. An equilibrium period of 5 min for constant shaking of the mixed solution was allowed before measuring each spectrum. The UV-Vis absorption spectra of chelerythrine after each titration were determined by deducting the contribution of the added RNA/DNA. The absorption spectra of chelerythrine after 0 and 10 μL titration of RNA and DNA (2.5×10^{-6} M) were shown in Figure S5 for a clear comparison of spectral changes caused by two species of G-quadruplexes.

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Author contributions

Z.H.J., L.P.B. and K.N. designed experiments. L.P.B. and M.H. conducted experiments. L.P.B. and Z.H.J. analyzed data. L.P.B. and Z.H.J. wrote the paper.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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