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Telomestatin-induced stabilization of the human telomeric DNA quadruplex monitored by electrospray mass spectrometry

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Electrospray mass spectrometry (ESI-MS) was used to monitor the kinetics of duplex formation between the human telomeric DNA quadruplex and its complementary strand; the complexation of telomestatin to the G-quadruplex delays the unwinding of the quadruplex structure and formation of the duplex.

Chromosomal ends are protected from fusion events by telomeres, the end of which consists of a 3′ single strand overhang with the sequence repeat $(GGGTTA)_n$. Telomere length is maintained by telomerase, a reverse transcriptase enzyme that is active in 85–90% of human tumors, but not in most normal somatic cells. Telomerase has therefore become an important target for anticancer drug design.[1] An interesting approach for telomerase inhibition is to stabilize the telomeric G-rich strand into a folded, inactive structure. The human telomeric strand $(G_3T_2A)_3G_3$ can fold into a G-quadruplex structure (Fig. 1A and C) that has been found to inhibit telomerase activity.[2] The search for ligands that stabilize the folded G-quadruplex structure is of particular importance in telomerase inhibition strategies.[3,4]

The detection of drug–quadruplex complexes at equilibrium by ESI-MS has already been reported.[5–8] Here we describe an electrospray mass spectrometric assay of the kinetics of hybridization of the human telomeric sequence, mimicking the binding to the RNA template of telomerase. The telomeric G-rich strand $(GGGTTA)_3GGG$ is mixed with its complementary strand $(CCCAAT)_3CCC$, which may adopt an i-motif conformation (Fig. 1C), and the formation of the complementary duplex is monitored as a function of time. The reaction kinetics are compared in the absence and in the presence of the quadruplex-binding drug telomestatin[9] (Fig. 1B), which is a potent telomerase inhibitor.[10]

Fig. 2(A) shows the ESI mass spectra of an equimolar mixture (5 μM + 5 μM) of the telomeric sequence $(G_3T_2A)_3G_3$ and the complementary strand $(C_3A_2T)_3C_3$ at two different reaction times.† After 200 s (top) the mass spectrum shows peaks corresponding to the telomeric quadruplex (noted "G") at the charge state of 5^- and 4^- as previously described,[6] and of the i-motif $(C_3A_2T)_3C_3$ (noted "i") at *m*/*z* 1239.2, 1549.1 and 2065.4 ($[i]^{5-}$, $[i]^{4-}$ and $[i]^{3-}$, respectively). The duplex formed by the strands G and i at *m*/*z* 1835.3 and 2141.2 (charge states 7^- and 6^-) is already present. The intensities of the peaks of the C-rich strand are much higher than the G-rich one even at equimolar concentration, due to the higher hydrophobicity of the cytosine bases compared to the hydrophilic guanines.[11] At *t* = 2000 s, the duplex is the most abundant species. The relative

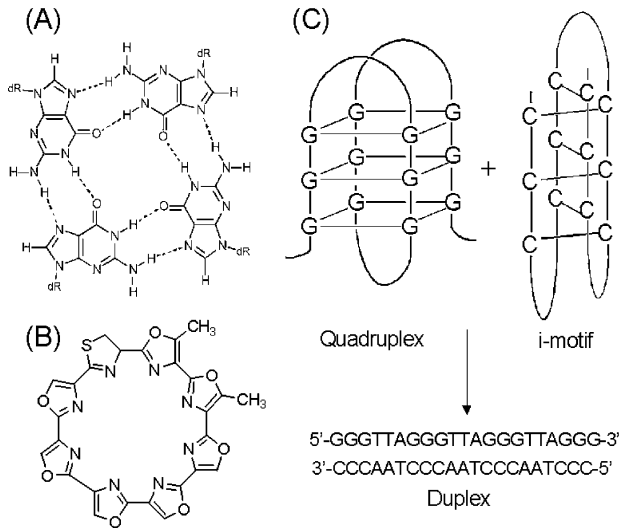

Fig. 1 (A) Structure of a guanine quartet. (B) Structure of telomestatin. (C) Design of the experiment: the G-quadruplex $(G_3T_2A)_3G_3$ is mixed with its complementary strand $(C_3A_2T)_3C_3$, adopting an i-motif structure. The hybridization of the two strands gives a Watson–Crick duplex.

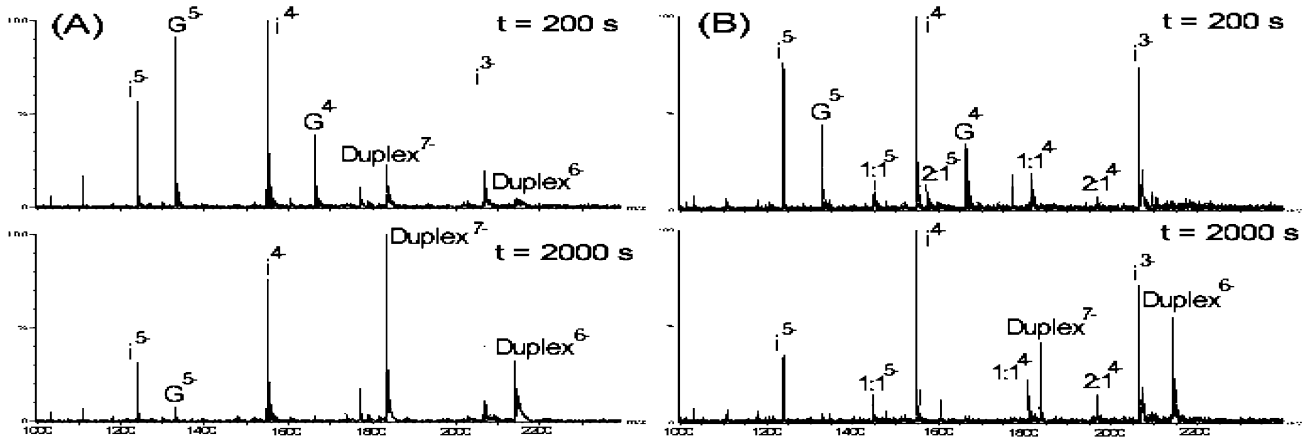

Fig. 2 (A) ESI-MS spectra of a mixture of 5 μM $(GGGTTA)_3GGG$ ("G") and 5 μM $(CCCAAT)_3CCC$ ("i") after 200 s (top) and 2000 s (bottom). (B) ESI-MS spectra of a mixture of 5 μM "G", 5μM telomestatin, and 5 μM "i" after 200 s (top) and 2000 s (bottom). "Duplex" stands for "G·i"; "1 : 1" stands for "telomestatin·G"; "2 : 1" stands for "2telomestatin·G".

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intensities of free G and of the duplex are plotted as a function of time to determine the association kinetics (Fig. 3A). All charge states are summed up for calculation of the intensities. The first 100 s are not accesible due to the time needed for mixing, injection, and obtaining a stable electrospray signal. The data were fitted to a single exponential curve and the measured time constant τ of duplex formation is 532 ± 11 s.

Telomestatin is a drug that binds specifically to quadruplex DNA.[5] The mass spectrum of an equimolar mixture (5 μM + 5 μM) of quadruplex and telomestatin was recorded (data not shown). The equilibrium binding constants of the telomestatin:quadruplex complexes calculated using the method previously described[12] are $K_1 = 1.2 \times 10^5$ M^{-1} and $K_2 = 3.8 \times 10^5$ M^{-1}. Under our experimental conditions, 53% of the drug is bound to the quadruplex. The same order of magnitude for the two binding constants suggests that the binding sites are equivalent. Then 5 μM of the complementary strand "i" was added to the (5 μM + 5 μM) quadruplex:telomestatin mixture, and the reaction kinetics followed as a function of time by ESI-MS. Fig. 2B shows the ESI mass spectra obtained after 200 s (top) and 2000 s (bottom). No telomestatin complex could be observed either with $(C_3A_2T)_3C_3$ or with the duplex, confirming the high selectivity of telomestatin for the G-quadruplex structure. The relative abundances of the different forms of the G-strand (free G, 1 : 1 and 2 : 1 complex, and duplex) are plotted as a function of time in Fig. 3B.

The relative intensity of the free G-quadruplex disappears with a time constant of 540 ± 30 s, which is the same as when no telomestatin drug is present, within experimental error. However, the relative intensities of the 1 : 1 and 2 : 1 complexes do not decrease as fast as free G, indicating that the displacement of the complexation equilibria is much slower than the hybridization of G with i to form the duplex. The relative intensity of 2 : 1 remains steady for 500 ns, then it slowly converts into 1 : 1 by the loss of one drug. The relative intensity of the 1 : 1 complex starts to decrease after *ca.* 1000 s, to give free G-quadruplex which is believed to be immediately hybridized. The global time constant for duplex formation (τ = 1010 ± 90 s) is increased two-fold in the presence of telomestatin, due to the slow dissociation of the complex [eqn. (1)].

$$2:1 \overset{\text{slow}}{\rightleftharpoons} 1:1 \overset{\text{slow}}{\rightleftharpoons} \text{free G} \xrightarrow{\text{fast}} \text{duplex} \qquad (1)$$

Compared to other methods which allow study of the kinetics of the quadruplex-to-duplex transition (circular dichroism, NMR, or fluorescence resonance energy transfer),[13,14] ESI-MS has the great advantage of monitoring each species individually, which is of prime importance for study of the effect of drug binding on the reaction kinetics. The present study clearly shows that the selective complexation of telomestatin slows down the hybridization of the quadruplex. The rate-limiting step is the dissociation of the complexes with telomestatin. It therefore appears that drugs that bind selectively to quadruplex structures, and which are characterized by a high affinity constant and a low dissociation rate constant would effectively inhibit telomerase activity.

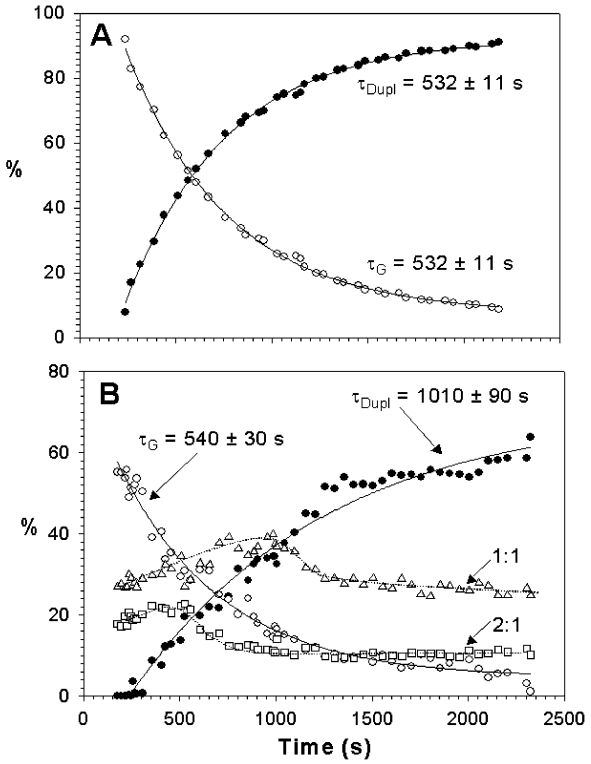

Fig. 3 Relative abundances of the different forms of the G-strand as a function of time. The complementary strand (5 μM) is added to a solution (5 μM) of preformed $(GGGTTA)_3GGG$ quadruplex alone (A) or in the presence of 5 μM telomestatin (B). ●: duplex; ○: free G-strand; △: 1 : 1 complex with telomestatin; □: 2 : 1 complex with telomestatin.

Notes and references

† Single stranded oligonucleotides were purchased from Eurogentec (Belgium). The quadruplex solution was prepared in 50 mM NH_4OAc, pH 6.5. The solution was heated to 80 °C for 5 min and then slowly cooled to 20 °C to form the quadruplex structure. These conditions are slightly different from our previously published protocol[6,7] because we needed to slightly destabilize the quadruplex structure to allow online observation of duplex formation in a reasonable time. The quadruplex melting temperature is reduced from 58 °C in 150 mM NH_4OAc[7] to 39 °C in 50 mM NH_4OAc (not shown). Experiments were performed on a Micromass Q-TOF Ultima Global apparatus operated in the negative ion mode. 15% methanol was added just before injection. The source parameters were: cone voltage: 35 V, RF lens 1: 70 V, source and desolvation temperatures: 70 and 100 °C respectively, collision energy: 10 eV.

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