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ORIGINAL PAPER

Interaction between GW2974 and telomeric G-quadruplex DNA: a possible anticancer mechanism

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Abstract

Human telomere consists of tandem repeats of guanines; thus, it can form an intramolecular G-quadruplex structure, which can inhibit the telomerase enzyme that is found active in more than 85% of cancer cells. The present work investigated the interactions of GW2974, a quinazoline derivative with telomeric G-quadruplex $AG_3(TTAGGG)_3$. GW2974 had shown a good affinity to G-quadruplex DNA with a binding constant of $2.41 \times 10^6\ M^{-1}$ and melting temperature shift ΔTm of 9.9 °C. Increasing the GW2974 ratio with DNA up to five times showed an inverse effect on G-quadruplex DNA stability. The stoichiometric ratio between GW2974 and TelQ (per strand) was 2:1. Results obtained from absorption, fluorescence, and CD spectroscopic techniques indicated that GW2974 interacted with G-quadruplex through different binding modes. GW2974 showed good specificity to quadruplex over duplex DNA by 4.71-fold. These results indicated that stabilizing of telomeric G-quadruplex inhibited telomerase enzyme, which could be a potential anticancer mechanism of GW2974.

Keywords Telomere · Telomerase · Quadruplex · Cancer · GW2974

Introduction

A single-stranded DNA rich in guanines can fold and form a four-stranded structure, which is supported by hydrogen bonding. This structure is known as G-quadruplex or tetraplex DNA. Guanine-rich sequences in chromosomal telomere, immunoglobulin switch regions, and gene promoters such as *c-myc*, *k-ras*, and *c-kit* can form the same structure (Burge et al. 2006; Sen and Gilbert 1988). In addition, telomeric sequences from *Oxytricha* and *Tetrahymena* can form a G-quartet structure that includes four guanosine residues in a square-planar array attached through hydrogen bonding (Williamson et al. 1989). G-quadruplex can be formed under physiological conditions, unlike other non-classical DNA structures that exist under unusual conditions.

Telomeres, the ends of eukaryotic chromosomes, are non-coded for genetic information, and the major part (5–15 kilobases) consists of double-stranded DNA, whereas the minor part at the 3′ end (100–200 bases) consists of single-stranded DNA. Telomere length is associated with the ability of the cell to undergo a large number of cell divisions. The telomeric DNA in normal somatic cells is shortened by 50–200 nucleotides upon each cell division, which controls their proliferative capacity. After several cell divisions, the telomere reaches a critical length, which affects its stability and enters the senescence stage and then apoptosis (Greider and Blackburn 1985). Based on previous reports, the telomerase enzyme re-elongates the telomeres in the stem, germ, and embryonic cells. In addition, this enzyme is active in more than 85% of cancer cells, which explains the indefinite division of cancer cells, and cancer cells can multiply limitlessly because the enzyme maintains telomere length (Kim et al. 1994). Therefore, telomeres have gained considerable attention because folding of their overhang single stands into the quadruplex structure can inhibit telomerase activity. Stabilizing the telomeric quadruplex structure using small molecules or specific antibodies could indirectly inhibit telomerase enzyme activity (as the telomere end is the template substrate for the RNA telomerase). Moreover, the formation of G-quadruplex may lead to the displacement of the telomere-binding proteins involved in telomere capping; thus, the folded telomere may be recognized by the cell as a DNA damage region (Salvati et al. 2007). Recently, G-quadruplex stabilization has been an attractive strategy for

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cancer therapy (Burger et al. 2005). Thus, molecules with a high preferential affinity toward DNA quadruplexes can stabilize G-quadruplex structures and inhibit/stop cancer proliferation, and such molecules could be used for developing new effective and selective genetic anticancer therapeutic agents (Marchetti et al. 2018).

Aminoanthraquinone derivatives are the first reported synthetic compounds that show considerable inhibition for telomerase enzyme (Sun et al. 1997), and then, several small molecules have been reported in the literature as G-quadruplex's stabilizers, telomerase's enzyme inhibitors, and/or oncogene regulators. Quadruplex DNA binders include anthraquinones (Huang et al. 2007; Perry et al. 1998), acridines (Harrison et al. 2003; Reed et al. 2006), porphyrins (Han et al. 2001; Izbicka et al. 1999), porphyrazines (Goncalves et al. 2006), phthalocyanines (Ren et al. 2007), thymoquinone (Salem et al. 2015), carbazole, and telomestatin (Kim et al. 2002; Shin-ya et al. 2001). Some of these compounds have shown high selectivity such as 5,10,15,20-tetra-(N-methyl-4-pyridyl) porphyrin (TMPyP4), where its porphyrin ring π–π is stacked on the G-quartet at the end of the antiparallel quadruplex DNA, while the four lateral pyridinium groups are located in the groove of the quadruplex DNA (Wei et al. 2008). In addition, some metal complexes have shown high affinity and selectivity toward binding to human telomeric and *c-myc* quadruplexes such as porphyrin with manganese (II), copper (II) (Dixon et al. 2007; Evans et al. 2007), zinc (II) phthalocyanine (Ren et al. 2007), and nickel (II) salphen (Reed et al. 2006). Most of the reported quadruplex binders are composed of a planar aromatic core and protonated side chains. The aromatic planar core intercalates among the G-quartets through π–π stacking, whereas the side chains support the stability of the complex by hydrophobic or ionic interactions with the external sides or into DNA grooves (Granotier et al. 2005; Neidle 2009; Sissi et al. 2007).

N4-(1-Benzyl-1H-indazol-5-yl)-N6,N6-dimethylpyrido[3,4-d]pyrimidine-4,6-diamine (GW2974, GW), a quinazoline derivative, was synthesized by Rusnak et al. (Fig. 1). GW can inhibit EGFR and ErbB-2 tyrosine kinase receptors in tumor cells (Rusnak et al. 2001). It is a structural analog of lapatinib (GW572016). Lapatinib works as a dual tyrosine kinase inhibitor. The USA Food and Drug Administration approved lapatinib in 2007 for the treatment of breast cancer. Consequently, many research works have been conducted to study its effects on solid tumors (Cockerill et al. 2001; Wang et al. 2013). GW has shown high effectiveness and low toxicity in gallbladder carcinoma and breast cancer (Rusnak et al. 2001). In addition, it has shown considerable efficacy alone or combined with erbB2 when targeting the EGFR for preventing and treating gallbladder carcinoma in BK5.erbB2 transgenic mice (Kiguchi et al. 2010). GW can prevent oral carcinogenesis in

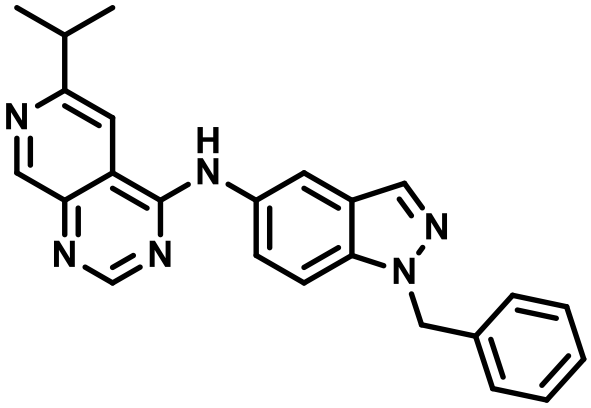

Fig. 1 Chemical structure of GW

the DMBA-induced hamster cheek pouch model at the post-initiation stage suppressing aberrant AA metabolism (Sun et al. 2008). Combining INCB3619 with GW has shown synergistic growth inhibition in MCF-7 and HER-2/neu-transfected MCF-7 human breast cancer cells (Witters et al. 2008). Giving GW orally in the diet has shown effective inhibition of skin tumor promotion in BK5.erbB2 and wild-type mice (Kiguchi et al. 2010). The interaction between GW and ATP-binding cassette (ABC) transporters show that GW, at specific concentrations, reverses ABCB1- and ABCG2-mediated MDR by blocking the drug efflux function of these transporters (Sodani et al. 2012). A combination of GW and Bcl-2 (GX15-070 or HA14-1) shows a synergistic inhibitory effect on the growth of the MCF-7, MCF/18, and MTR-3 human breast cancer cell lines (Witters et al. 2007). It shows a cardiac cell protective activity by preventing apoptosis induced by TNFα, which is detected in cardiac failure (Spector et al. 2007). Chen and coworkers evaluated the activity of 1280 compounds, including GW, on the hepatitis C virus (HCV), whereas it inhibited one or more aspects of the HCV life cycle by more than 40% (Chockalingam et al. 2010). The effect of GW on PC-3 cell growth and on NE-related markers, including NSE and chromogranin, in the androgen-independent prostate cancer cell line PC-3 was studied. The results showed that GW inhibited PC-3 cell growth and stimulated NSE and chromogranin; therefore, it may have clinical implications for the treatment of advanced prostate cancer (Terracciano et al. 2010). The interaction of GW583340 and GW compounds with ABC transporters reversed ABCB1- and ABCG2-mediated MDR by blocking their efflux function; therefore, these compounds could be used to develop a combination therapy for cancer treatment with EGFR TKIs (Sodani et al. 2012). GW showed a dual inhibition of EGFR and HER2 in glioblastoma multiforme in vitro and in vivo at low concentration (Wang et al. 2013).

In this work, the anticancer activity of GW was studied through the interaction between this compound and

telomeric G-quadruplex, 5′-AGGG(TTAGGG)$_3$–3′ (TelQ), using several techniques, including UV–Vis absorption, fluorescence, fluorescence quenching, and circular dichroism (CD) spectroscopic techniques, and calf thymus DNA (ctDNA) as a control. The interaction parameters, including binding affinity, binding constant, binding sites, melting temperature, and binding selectivity toward quadruplex over DNA duplex, were also studied.

Materials and methodology

Materials and reagents

All chemicals and reagents were used without further purification. N4-(1-Benzyl-1H-indazol-5-yl)-N6,N6-dimethyl-pyrido[3,4-d]pyrimidine-4,6-diamine (GW), ethylenediaminetetraacetic acid (EDTA), potassium chloride, ethylene glycol, tris(hydroxymethyl)aminomethane hydrochloride, and calf thymus DNA were purchased from Sigma-Aldrich, USA. HPLC purified telomeric DNA sequences 5′-AGGGTTAGGGTTAGGGTTAGGG-3′, fluorescein-labeled telomeric quadruplex (5′-Fl-AGG GTTAGGGTTAGGGTTAGGG-3′), and 3′-TCCCAATCC CAATCCCAATCCC-5′ were purchased from AlphaDNA, Canada. All experiments were conducted using Millipore deionized water.

Instrumentation

Absorption measurements were taken on Agilent 8453, Austria matched with 1-cm quartz cells. Fluorescence spectra were recorded on Cary Eclipse model-3 spectrofluorometer equipped with Xenon flash lamp (Varian, Austria) and matched with a 1-cm path length quartz cell. Circular dichroism measurements were taken using Jasco J-815 spectrometer (Jasco, USA) matched with a 1-cm path length quartz cell. pH measurements were taken using Orion-401-Plus pH meter supported with Orion glass electrode.

Preparation of standard solutions

Buffer solution

A Tris buffer solution containing 100 mM KCl (pH 7.4, 10 mM, 1.0 L) was prepared using Tris–hydroxymethyl aminomethane hydrochloride (10 mM, 1.576 g), EDTA (1 mM, 0.3077 g) and KCl (100 mM, 7.455 g) in deionized water. pH of the prepared buffer solution was adjusted using the glass electrode.

GW solution

Stock solution (2 mM) of GW was prepared in 100% ethylene glycol. The stock solution was used to prepare diluted solutions (4.0 mL) having concentrations of 1.0, 0.5, and 0.1 mM using deionized water.

DNA solutions

Calf thymus DNA (ctDNA)

A stock solution (1000 µg/mL) of calf thymus ds-DNA was prepared. Ten milligrams of ctDNA was dissolved in 10.0 mL Tris buffer containing 100 mM KCl at pH 7.4. The mixture was gently shaken upside down and was not sonicated or stirred to prevent shearing of the large genomic DNA; then, the mixture was kept in the fridge at 4.0 °C in order to completely solubilize the DNA. DNA solutions showed stability over several months at 4.0 °C in Tris buffer containing 100 mM KCl at pH 7–8.

Single-stranded DNA

Purchased synthetic nucleic acid primers with human telomere sequence; 5′-AGGGTTAGGGTTAGGGTTAGGG-3′, its fluorescein-labeled 5′-primer 5′-Fl-AGGGTTAGGGTT AGGGTTAGGG-3′ and its complementary strand 3′-TCC CAATCCCAATCCCAATCCC-5′ were centrifuged for 10 min at 7000 rpm to collect DNA strands at the bottom of the vials. 2.0 mL of Tris buffer containing 100 mM KCl at pH 7.4 was added and then was left overnight at 4.0 °C for rehydration. DNA solutions were vortexed for 30 s and then kept overnight at 4.0 °C. DNA solution showed stability over more than 6 months.

G-Quadruplex DNA (TelQ)

The G-quadruplex structure was prepared by gentle heating of a 2000 µL of the stock single-stranded 5′-AGGGTTAGG GTTAGGGTTAGGG-3′ primer up to 95.0 °C and incubated for 10 min. The resultant solution was left to gently cool to room temperature and then kept in fridge at 4.0 °C overnight.

Fluorescein-labeled G-quadruplex DNA (5′-Flu-TelQ) was prepared as per the previous procedure. A 10-µM solution of (5′-Flu-TelQ) was prepared as a stock solution for the next experiments.

Telomeric DNA oligonucleotides hybridization

Telomeric double-stranded DNA (100 µM) was prepared by mixing equimolar amounts of the complementary strands. 268.8 µL of 5′-AGGGTTAGGGTTAGGGTTAGGG-3′

(744 μM) was mixed with 738 μL of 3′-TCCCAATCC CAATCCCAATCCC-5′ (271 μM). Tris buffer containing 100 mM KCl at pH 7.4 was added to the solution so that the final volume was 2000 μL. The solution was vortexed for 15 s and then incubated at 95 °C for 10 min. The hot solution was left to cool to room temperature. The produced dsDNA was kept in the refrigerator at 4.0 °C for the next uses.

The concentrations of DNA stock solutions were determined in Tris buffer containing 100 mM KCl at pH 7.4 using absorbance spectroscopy at 260 and 280 nm. Concentrations in μg/mL are calculated using the following equation:

$$C_{(\mu_{g/mL})} = A_{260} \times \text{weight per OD} \times \text{dilution factor}$$

where OD is the optical density at 260 nm. The estimated purity of the purchased oligonucleotide was calculated based on the ratio A_{260}/A_{280}. Ratios ≥ 1.8 indicate high purity for the synthetic and calf thymus DNAs (JA 1995). Since single-stranded DNA is folded to form a G-quadruplex structure so G-quadruplex's concentration will be similar to that of its single-stranded DNA.

Effect of ethylene glycol

Most of the experiments in this study were done in solutions of 10% ethylene glycol so the effect of ethylene glycol on the telomeric G-quadruplex was tested using CD spectroscopy. TelQ DNA (4 μM) in Tris buffer containing 100 mM KCl at pH 7.4 was titrated with pure ethylene glycol using 1500 μL cuvette. CD intensity of TelQ was plotted against the added ethylene glycol.

GW–TelQ DNA interaction

Interactions between GW with TelQ were studied using UV absorption, fluorescence, fluorescence quenching, and circular dichroism spectroscopic techniques. DNA-drug interaction parameters including binding constant, binding stoichiometry, melting temperature, ligand selectivity toward G-quadruplex over duplex DNA, and binding mode were investigated.

Absorption titration

The interaction between GW and TelQ DNA was studied using UV–Vis spectroscopy. Successive amounts of TelQ (118 μM) were added to (50 μM) of GW. Ligand solution (50 μM) was prepared by diluting 50 μL of 1 mM into 1000 μL using Tris buffer containing 100 mM KCl at pH 7.4. The percentage of ethylene glycol was 10% in the prepared solutions. Solutions after each addition were shaken well, incubated for 3 min and then scanned using 1500-μL cuvette (1 cm path length) over the wavelength range 200–700 nm. The incubation time was increased up to one hour without a change in the absorption spectrum of the solution so 3-min incubation time was used in all measurements. The titration was stopped when no further change in absorbance intensity was observed, indicating that the interaction had reached the saturation point. The drug–quadruplex ratio [drug]/[DNA] was ranged between 20 and 0.88.

Fluorescence titration

Interaction of GW with TelQ was studied using fluorescence spectroscopy. GW was excited at 243 nm using slits width of 10 nm for both excitation and emission. Successive portions of TelQ (133 μM) were added to 2.0 mL of 5 μM GW in Tris buffer containing 100 mM KCl at pH 7.4. GW solution (5 μM) was prepared by diluting 100 μL (100 μM) into 2000 μL using Tris buffer containing 100 mM KCl at pH 7.4. After each addition, solutions were stirred for 20 s, incubated for 3 min and then scanned in the range 300–550 nm using 3500-μL cuvette, 1-cm path length. The incubation time was increased up to one hour without a change in the emission spectrum of the solution so 3-min incubation time was used in all measurements. The titration was stopped when no further change in fluorescence intensity was observed, indicating that the interaction had reached the saturation point. The ligand–DNA ratio ranged between 20 to 0.5 and ethylene glycol ranged between 10 and 9.2% at the end of the titration.

Fluorescence titration of fluorescein-labeled G-quadruplex (fluorescence quenching)

The binding affinity of GW toward human telomeric DNA was confirmed using fluorescence quenching assay. The fluorescence intensity of fluorescein-labeled quadruplex is quenched when a ligand molecule binds to a DNA molecule near the fluorescein. Successive portions of GW solutions (100 μM—1000 μM) were added to (3.0 mL, 2 μM) of fluorescein-labeled quadruplex; 5′-Fl-AGGGTTAGGGTT AGGGTTAGGG-3′ (5′-Flu-TelQ) in Tris buffer containing 100 mM KCl at pH 7.4. Drug–quadruplex ratio [drug]/[DNA] ranged between 0.2 and 5.0. Solutions after each addition were stirred for 20 s, incubated for 3 min and then scanned over the wavelength range 400–650 nm for their emission spectra. 5'-Flu-TelQ was excited at 494 nm and its emission was recorded at 518 nm with 5 and 2.5 nm excitation and emission slits width, respectively.

Circular dichroism titration of TelQ DNA

Interaction of GW with TelQ was studied using CD spectroscopy. TelQ (4 μM) was prepared by diluting 33.9 μL of TelQ (118 μM) into 1000 μL using Tris buffer containing

100 mM KCl at pH 7.4 and then titrated with successive amounts of GW solution (500 µM). Solutions after each addition were shaken well, incubated for 3 min and then scanned using 1500-µL cuvette (1 cm path length) at room temperature over the wavelength range 200–400 nm with 50 nm/min, bandwidth 1 nm, and 3 accumulations. The incubation time was increased up to one hour without a change in the obtained spectrum of the solution so 3-min incubation time was used in all measurements. Drug–quadruplex ratio [drug]/[DNA] ranged between 0.25 and 5.0. CD of the blank and cuvette was scanned first and then auto-subtracted from samples' CD.

Melting temperature curves

Melting temperature curves for G-quadruplex and its GW complex were constructed using CD spectroscopic technique. TelQ solution (1.0 mL, 2.76 µM) in Tris buffer containing 100 mM KCl at pH 7.4 was heated up in 5.0 °C increments over the range 25–95 °C and then incubated for 5 min at these temperature intervals. The experiments were repeated with 1–2 °C increments instead of 5.0 °C in the CD jump range. CD spectra were recorded in the range 200–400 nm with 50 nm/min, bandwidth 1 nm, and 3 accumulations. Solutions of TelQ-drug complexes were prepared by mixing 48 µL G-quadruplex (57.4 µM) with 27.6 µL of (100 µM) drug solution (equimolar). The solutions were made up to 1.0 mL using Tris buffer containing 100 mM KCl at pH 7.4. Quadruplex solutions were prepared similarly to their complexes' solutions by replacing drug solutions with the same quantity of ethylene glycol. Ethylene glycol was kept at 10% in all solutions. All CD spectra were auto-baseline-corrected against the blank solution. Intensities of CD peaks at 293 nm for G-quadruplex and its drug complex were recorded.

The experiments were repeated for ctDNA and its GW complex according to the previous procedure. A 1.0 mL of ctDNA (1 nM, 100 ppm) in Tris buffer containing 100 mM KCl at pH 7.4 was heated up in 5.0 °C increments over the range 25–95 °C using 5 min as incubation time intervals. ctDNA and GW complex were prepared by mixing ctDNA and GW (2.34 µM) in Tris buffer containing 100 mM KCl at pH 7.4. Ethylene glycol was kept at 10% in all experiments. Intensities of CD peaks at 283 nm for G-quadruplex and its drug complex were recorded.

Selectivity of GW toward G-quadruplex

A specific experiment was designed to study the selectivity of GW toward telomeric G-quadruplex DNA. A complex of equimolar amounts of fluorescein-labeled G-quadruplex (5'-Flu-TelQ) with GW was prepared and mixed with 0-, 10-, 50-, and 100-fold of ctDNA or telomeric duplex DNA. 5'-Flu-TelQ was excited at 494 nm, and its emission was recorded at 518 nm with excitation and emission slits width of 5 nm.

A 2.0-mL solution that is (0.5 nM) in 5'-Flu-TelQ and (0.5 M) in GW was mixed with 0-, 10-, 50-, or 100-fold of hybridized double-stranded telomeric DNA (0–50 nM) in Tris buffer containing 100 mM KCl at pH 7.4. Solutions were vortexed for 10 s, incubated for 30 min at room temperature, and scanned for their fluorescence spectra in the range 500–600 nm. The experiments were repeated using ctDNA instead of double-stranded telomeric DNA. Ethylene glycol was kept at 10% in all solutions.

Stoichiometric ratio

The stoichiometric ratio between ligands and G-quadruplex was studied using the molar ratio method. GW was titrated with TelQ as mentioned previously in the fluorescence titration experiment. The ratio between ligands and DNA ([Ligand]/[DNA]) was plotted against the resulted fluorescence.

Binding affinity

The binding affinity of GW toward telomeric G-quadruplex DNA was studied. Binding constants (K) of TelQ–ligand complexes were determined using the Scatchard model based on fluorescence titration. Ligand concentration was kept constant, and DNA was varied as described in detail in fluorescence titration of GW. In the Scatchard equation (Wei et al. 2008), $\frac{r}{C_f}$ is plotted versus r according to Eq. 1.

$$\frac{r}{C_f} = nK - Kr \tag{1}$$

where r is the mole ratio between bound ligand (C_b) to DNA quadruplex, (C_f) is the concentration of free ligand, n is several equivalent binding sites on quadruplex molecule, K is the binding constant. C_b is calculated from Eq. 2

$$C_b = C_{total} - C_f \tag{2}$$

where C_{total} is the concentration of GW at zero addition of quadruplex and C_f is calculated according to Eq. 3.

$$C_f = C_{total}(1 - \alpha) \tag{3}$$

where α is the fractionality factor and calculated using Eq. 4.

$$\alpha = \frac{F_f - F}{F_f - F_b} \tag{4}$$

where F_f and F_b are the fluorescence of the free and fully bound ligand and F is the fluorescence at any given point during the titration.

Plotting $\frac{r}{C_f}$ versus r gives a slope equals to K and intercept equals to nK.

The plot may result in a straight or curved line. In case of a curved line, the equation is rearranged to Eq. 5.

$$r = \frac{nKC_f}{\left(1 + KC_f\right)} \tag{5}$$

r is plotted against C_f using Microsoft Origin 8.0 through a nonlinear fitting.

Results and Discussion

Stability and aggregations

The stability and aggregations of GW in Tris buffer containing 100 mM KCl at pH 7.4 and 10% ethylene glycol were first examined at room temperature using UV–Vis absorption spectrophotometry. The results showed no significant change in the absorbance intensity of GW during the first 3 h, which is enough to run the experiments. GW solution in 100% ethylene glycol was kept in a fridge, and it showed stability over 4 months. However, freshly prepared solutions in 10% ethylene glycol were used throughout this study.

Effect of ethylene glycol

The probability of the interaction between telomeric G-quadruplex DNA and ethylene glycol was studied using CD spectroscopy. TelQ was titrated with ethylene glycol up to ≈ 20%, although it was lower than this value at the end of the titration between TelQ and GW. The obtained results showed that ethylene glycol does not affect the spectral profile of TelQ; therefore, ethylene glycol is a safe solvent up to 20% for quadruplex experiments. These results were consistent with reported results where up to 90% ethylene glycol was considered as a safe and non-denaturing agent for DNA (Bonner and Klibanov 2000).

TelQ DNA conformation

The conformation of telomeric G-quadruplex depends on the DNA sequence and its environment. G-quadruplex $AGGG(TTAGGG)_3$ forms an antiparallel structure in Na^+ solution; however, it forms a mixture of parallel and antiparallel (hybrid) forms in K^+ solution (Rezler et al. 2005; Xu et al. 2006). Circular dichroism (CD) is a well-established method for determining the presence and the conformation of G-quadruplex. Therefore, CD experiments were conducted to study G-quadruplex conformation in Tris buffer containing 100 mM KCl at pH 7.4. The results showed that TelQ has a negative band centered at 235 nm and two positive bands at 253 and 293 nm in Tris buffer containing 100 mM KCl at pH 7.4 (Fig. 4a), indicating that TelQ formed a hybrid structure, which is consistent with the reported structure in K^+ solution (Xu et al. 2006).

Absorption titration

The availability of several nitrogen atoms in GW structure, as a source of hydrogen bonding, may affect its binding affinity and binding mode. The interaction between GW and G-quadruplex was first studied using UV–Vis absorption spectroscopy. Three absorption bands were identified in the absorption spectra of GW in Tris buffer containing 100 mM KCl at pH 7.4 and 5% ethylene glycol, and two strong bands were identified at 243 nm (ε = 6100) and 312 nm (ε = 5500) in addition to a weaker band at 421 nm (ε = 2600).

Figure 2 shows a decrease in the absorption intensity (hypochromicity of more than 35%) at 421 and 312 nm without a redshift or blueshift, and the absorbance at 243 nm may increase because of the overlapping with the absorbance at 260 nm of the added DNA. Wei and coworkers found that the intercalation binding mode required a redshift of more than 10 nm, whereas groove binding and terminal stacking required less than 10 nm (Wei et al. 2008). Therefore, GW could interact with G-quad through groove binding or terminal stacking.

Fluorescence titration

Fluorescence titrations were conducted to confirm the interaction between GW and TelQ. Furthermore, the resulting data were used to estimate the binding constant, number of binding sites, and binding mode of the interaction. GW was excited at 243 and emitted at 410 nm. Figure 3 shows that the resulted fluorescence intensity of GW at 410 continuously decreases (hypochromicity of 65.6% at the end of titration) with the sequential addition of DNA. In addition, two isosbestic points were observed at 304 and 361 nm, indicating

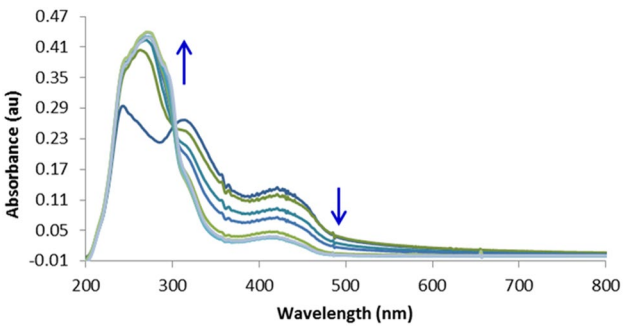

Fig. 2 Absorbance titration spectrum of GW (50 μM) titrated with TelQ (118 μM) in Tris buffer containing 100 mM KCl at pH 7.4

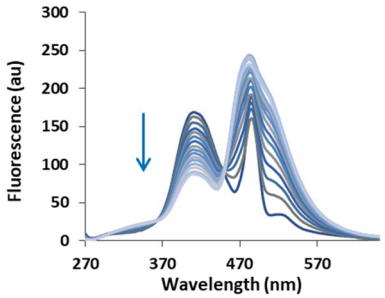

Fig. 3 Fluorescence spectra of GW (5 µM), 10% ethylene glycol, titrated with increasing amounts of TelQ in Tris buffer containing 100 mM KCl at pH 7.4

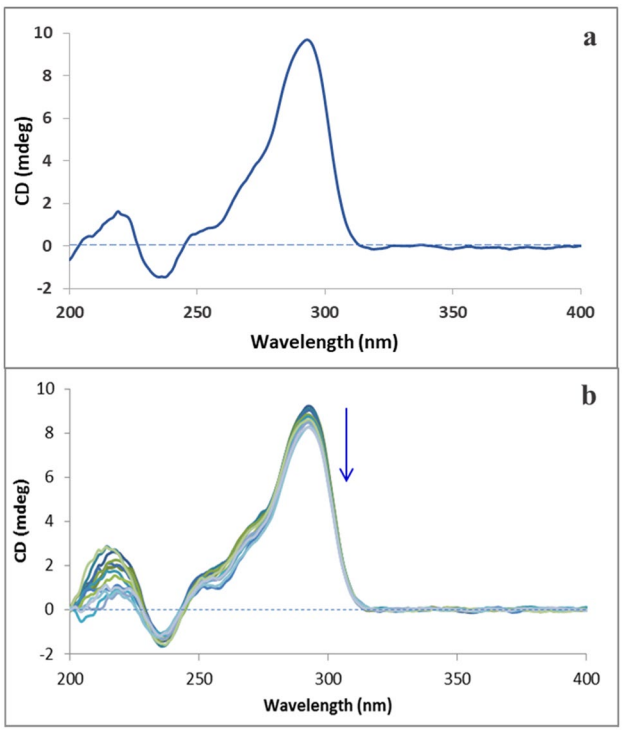

Fig. 4 **a** CD spectrum of the telomeric quadruplex (4 µM) in Tris buffer containing 100 mM KCl at pH 7.4. **b** CD spectra of TelQ (4 µM) in Tris buffer containing 100 mM KCl at pH 7.4 titrated with GW (500 µM)

that GW interacted with TelQ DNA. The isosbestic point at 448 nm was due to the scattering peak at 486 nm.

Circular dichroism (CD) titration

CD spectroscopy is an effective technique used to study the interaction between drugs and DNA. The CD spectrum of TelQ DNA titrated with GW (Fig. 4b) showed a gradual decrease in CD intensity at 293 nm with the sequential addition of GW. Furthermore, the bands' position and shape at the end of the titration are not changed, indicating that the hybrid conformation of G-quadruplex has not changed during the titration. Based on previous reports, the changing pattern in CD intensity of DNA during titration may indicate the binding mode between the ligand and DNA. Decreasing CD intensity indicates that the ligand binds to DNA through the intercalation mode. By contrast, if CD intensity increases, the ligand binds to DNA through the groove binding mode (Dash et al. 2012). Therefore, the slight decrease in CD intensity of TelQ without band shift and shape with the obtained results from UV–Vis absorption and fluorescence experiments indicates that GW may interact with TelQ DNA through different binding modes including intercalation, groove, and stacking with terminal G-quartet.

Fluorescence quenching titration

The DNA–drug interaction was further confirmed by fluorescence quenching titration experiments. Based on previous reports, if a ligand molecule binds to fluorescein-labeled DNA at a position close to the fluorescein, the tag fluorescence intensity will be quenched/decreased. The quenching intensity depends on the number of molecules bound to DNA molecules (Padirac et al. 2012). In this experiment, 5′-Flu-TelQ, excited at 494 nm and emitted at 518 nm, was titrated with GW (Fig. 5). A decrease/quenching in fluorescence intensity of 5′-fluorescein-labeled TelQ is shown with the sequential addition of GW, thereby confirming the previous results. In addition, these results indicate that GW might interact with TelQ in the proximity of fluorescein.

Binding stoichiometry

The stoichiometry of the interaction between GW and TelQ (per strand) was estimated using the molar ratio method. GW was titrated fluorometrically with TelQ DNA, and then, the [drug]/[TelQ] ratio was plotted against the corresponding fluorescence (Fig. 6). The obtained stoichiometric ratio of

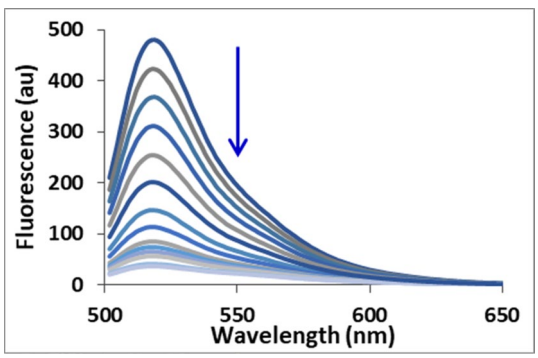

Fig. 5 Fluorescence spectra of 5′-Flu-TelQ (2 µM) in Tris buffer containing 100 mM KCl at pH 7.4 titrated with GW (100–1000 µM). 5′-Flu-TelQ was excited at 494 nm and emitted at 518 nm

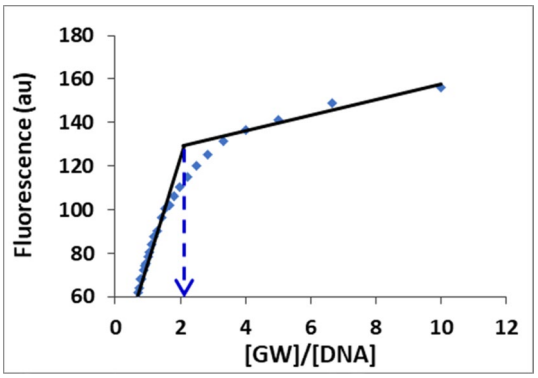

Fig. 6 Stoichiometric ratio between GW and G-quadruplex (using the molar ratio method). GW (5 µM) in Tris buffer containing 100 mM KCl at pH 7.4 was titrated with successive amounts of TelQ solution using fluorescence spectroscopy. Titration was ended at saturation

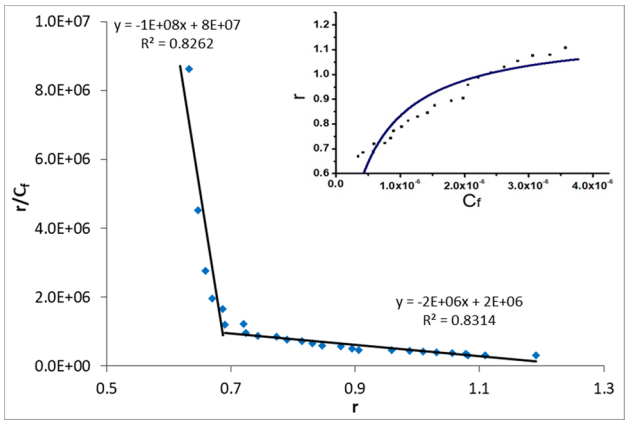

Fig. 7 Scatchard plot for GW (5 µM), 10% ethylene glycol, titrated with increasing amounts of TelQ in Tris buffer containing 100 mM KCl at pH 7.4 using fluorescence spectroscopy. The inset shows the Scatchard plot using nonlinear regression

GW:TelQ was 2:1, indicating that two GW molecules interacted with the G-quadruplex molecule.

Binding affinity

The binding affinity of GW toward DNA was studied through the binding constant and melting temperature values. In this work, the Scatchard plot was constructed to estimate the binding constant (K) between GW and TelQ and the number of binding sites (n) in the DNA molecule available for drug molecules. The Scatchard plot might be a linear or a curved line based on Eq. 1. The straight-line plot indicates that only one type of binding sites available in the DNA molecule for the ligand molecules or ligand molecules can interact with DNA molecules through different types of binding sites, including independent and equivalent sites. The nonlinear or curved line plot indicates that more than one type of dependent binding sites is available, thereby indicating that the first bound ligand in a site may encourage or suppress the next bound ligand in the second site, which is known as the neighbor exclusion effect (Boyer 2000; Wei et al. 2010). The obtained Scatchard plot between r and C_f for GW and TelQ using Eq. (1) showed an upward concave line (Fig. 7), suggesting that more than one type of dependent binding sites was available for these molecules in the G-quadruplex molecule. Analysis of the obtained plot showed two straight lines, indicating that GW interacted with G-quadruplex through two types of binding sites. The first binding site showed a high binding affinity with a binding constant (7.9×10^7 M^{-1}) and a number of binding sites (n = 0.7). The second site showed a low binding affinity toward G-quadruplex molecule with a binding constant (2.1×10^6 M^{-1}) and a number of binding sites (n = 1.3). The binding plot was reconstructed according to Eq. (5) with nonlinear regression using Microsoft Origin pro 8 (Fig. 7, inset). GW has shown a considerable binding affinity toward the examined telomeric G-quadruplex with a binding constant (2.41×10^6 M^{-1}). The number of binding sites for GW was 1.18. The results may indicate that GW interacted with G-quadruplex through two types of dependent binding sites with different binding modes. The first binding site has a high binding affinity, and the other site has a low binding affinity. It is possible that one ligand binds to groove and the second stacks with terminal G-quartet.

The interaction of the reported ligands with TelQ, $AGGG(TTAGGG)_3$, is varied. Perylene derivative containing 1,3-diamino moieties in 10 mM phosphate buffer and 100 mM KCl at pH 7.0 has shown a binding constant ($1.16 \pm 1.61 \times 10^6$ M^{-1}) slightly less than that of GW (Chitranshi and Xue 2011). Anticancer drugs such as epirubicin and adriamycin in 10 mM phosphate buffer containing 100 mM KCl at pH 7.0 interacted with TelQ through two independent binding sites with a binding affinity of 2.5×10^5 and 5.2×10^5 M^{-1}, respectively (Raje and Barthwal 2019).

Melting temperature

Melting temperature, Tm, is the temperature at mid-transition of DNA duplex, triplex, or quadruplex to the single-stranded DNA (Blackburn et al. 2006). Tm indicates the stability of the DNA–ligand complex and binding affinity of the ligand toward DNA (Blackburn et al. 2006). In addition, the comparison between ΔTm of the TelQ–drug complex and that of the ctDNA–drug complex indicates the selectivity of the drug toward TelQ over ctDNA. Melting temperatures for TelQ, ctDNA, and their complexes with GW were estimated using CD spectroscopy (Figs. 8 and 9).

Melting curves showed a melting temperature shift ΔTm by 9.9 °C for TelQ/TelQ-GW (Table 1). On the contrary, the melting temperature shift (ΔTm) of ctDNA/ctDNA-GW

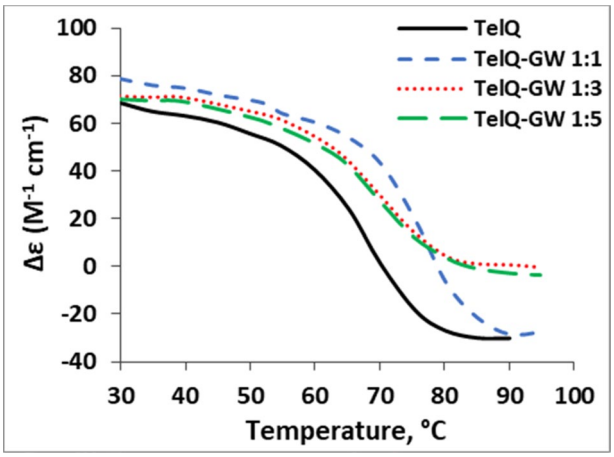

Fig. 8 Normalized melting curves of TelQ and TelQ–GW complexes at 1:1, 3:1, and 5:1 [Drug]/[TelQ] ratios

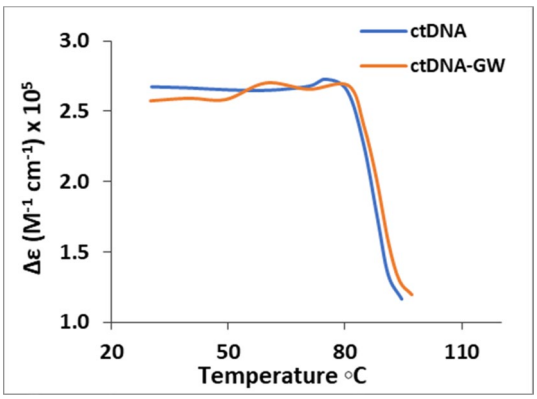

Fig. 9 Normalized melting curves of ctDNA and ctDNA–GW complexes

was 2.1 °C. ΔTm of TelQ/TelQ-GW, indicating that GW showed a considerable stabilization effect to the telomeric G-quadruplex molecule. In addition, GW has shown a preference for G-quadruplex over ctDNA by 4.71-fold.

Perylene derivative containing 1,3-diamino moieties in 10 mM phosphate buffer and 100 mM KCl at pH 7.0 has shown a melting temperature shift ΔTm of TelQ by 5.2 °C, which is less than that of GW (Chitranshi and Xue 2011). However, epirubicin and adriamycin have shown a binding constant less than that of GW, and they showed high thermal stability by 13.0 °C and 11.6 °C, respectively (Raje and Barthwal 2019).

The melting temperature experiment was repeated at different GW concentrations. The [drug]/[DNA] ratio = 1:1 showed the highest melting temperature shift (ΔTm) of 9.9 °C. In addition, the results showed that increasing GW concentration up to five times of G-quadruplex decreased DNA stability (ΔTm) to 3.5 °C. The majority of the reported ligands were able to confer stabilization to G-quadruplex DNA; however, some ligands induced destabilization at high concentrations. For instance, Mela et al. found that triarylpyridine derivative induced the unfolding of the G-quadruplex at concentrations around 50 μM (Mela et al. 2012). Kaluzhny and his colleagues found that anthrathiophenedione derivatives at high concentrations caused a partial conformational perturbation of the telomeric G-quadruplex (Kaluzhny et al. 2011). O'Hagan, Galan, and coworkers found that stiff-stilbene derivative induced the unfolding of the hybrid structure of the sodium form of telomeric G-quadruplex, whereas the same ligand stabilized the potassium form of the same sequence. They suggested that the ligand targeted the G-quadruplex grooves before intercalating the G-quartets, causing a disruption of the hydrogen bond network of the G-quartets (O'Hagan et al. 2019).

Selectivity test

Another experiment was designed to study the specificity of GW toward telomeric G-quadruplex DNA in the presence of ctDNA or Tel-duplex. Figure 10 shows the fluorescence intensity (quenching) of the complex 5′-Flu-TelQ-GW in the presence of 0-, 10-, 50-, and 100-fold of ctDNA or Tel-duplex DNA. The figure shows a slight increase (less quenching) in the fluorescence intensity of both complexes with the addition of ctDNA or Tel-duplex, indicating that GW showed reasonable selectivity toward TelQ over ctDNA. These results are consistent with the results obtained from melting temperature experiments.

Finally, the obtained results have shown that GW interacted with G-quadruplex DNA with a considerable affinity and preference; therefore, the interaction between GW and telomeric quadruplex DNA could be a potential anticancer mechanism of GW.

Table 1 Melting temperature and melting temperature change of TelQ–GW complexes at different [drug]/[DNA] ratios. The melting temperature of TelQ is 65.5 °C

	[GW]/[TelQ] = 1:1		[GW]/[TelQ] = 3:1		[GW]/[TelQ] = 5:1	
	Tm (°C)	ΔTm (°C)	Tm (°C)	ΔTm (°C)	Tm (°C)	ΔTm (°C)
TelQ-GW	75.4	9.9	69.7	4.2	69.0	3.5

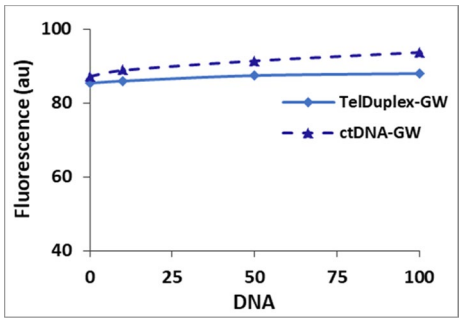

Fig. 10 Selectivity of GW toward G-quadruplex (5′-Flu-TelQ, 0.5 nM) in the presence of 0-, 10-, 50-, and 100-fold of Tel-duplex DNA and ctDNA in Tris buffer containing 100 mM KCl at pH 7.4

Conclusion

The present work investigated the interactions of GW, a quinazoline derivative, with telomeric G-quadruplex $AG_3(TTAGGG)_3$ in Tris buffer containing 100 mM KCl at pH 7.4 and 10% ethylene glycol using UV absorption, fluorescence, and CD spectroscopic techniques. Ethylene glycol was considered as a safe solvent up to 20% for quadruplex experiments. Experimental studies indicated that GW showed a good affinity to G-quadruplex DNA with evident preferences to GW as indicated in the binding constant and melting temperature shift (ΔTm) values. The results also showed that increasing the GW ratio with DNA up to five times led to a decrease in G-quadruplex DNA stability. The stoichiometric ratio between GW and TelQ (per strand) was 2:1. The obtained results have proven that GW interacted with G-quadruplex through the different binding modes. In addition, GW has shown good specificity to quadruplex over duplex DNA by 4.71-fold. These results indicated that the interaction between GW and telomeric quadruplex DNA could be a potential anticancer mechanism of GW.

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Declarations

Conflict of interest The author declares no competing interests.

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