GW-2974 and SCH-442416 modulators of tyrosine-kinase and adenosine receptors can also stabilize human telomeric G-quadruplex DNA: Insights into their anticancer effect

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Abstract

GW-2974 is a potent inhibitor for the tyrosine kinase receptor. It inhibits EGFR and ErbB-2 in tumor cells with high selectivity towards malignant over normal cells. SCH-442416 is a potent antagonist for adenosine receptor with high selectivity towards human A2a receptor over other adenosine receptors. The two compounds were reported to possess antitumor properties. In this work, we studied the stabilization of human telomeric G-quadruplex DNA by GW-2974 and SCH-442416, as a probable underlying mechanism for their anticancer effects. Interactions of human telomeric G-quadruplex DNA - sequence $AG_3(TTAGGG)_3$ – with both compounds were studied using UV-Vis, fluorescence quenching, melting temperature, circular dichroism and molecular docking techniques.

Results obtained from absorption, fluorescence and CD indicated that GW2974 and SCH-442416 interacted with G-quadruplex through intercalation binding modes on two types of dependent binding sites. Binding affinities of 1.3×10^8 - 1.72×10^6 M⁻¹ and of 1.55×10^7 - 3.74×10^5 M⁻¹ were respectively obtained for GW-2974 for SCH-442416. An average number of binding sites between 1 and 2 was obtained for both compounds. The melting temperature curves indicated that complexations of both compounds to G-quadruplex DNA have respectively stabilized it by $\Delta T_m = 9.9$ oC and 9.6 oC, relative non-complexed G-quadruplex DNA. Increasing the molar ratios of GW-2974 or SCH-442416 relative to G-quadruplex DNA over 1:1 has shown to destabilized G-quadruplex DNA. Our selectivity experiment indicated 4.7- and 4.0-folds better selectivity for GW-2974 and SCH-442516 towards G-quadruplex DNA over ds-DNA, respectively. The two compounds have also proved to selectively bind and stabilize G-quadruplex DNA over calf thymus duplex ct-DNA. In silico molecular docking indicated exothermic intercalation bindings on two sites of the G-quadruplex DNA

These results supported our hypothesis that both GW-2974 and SCH-442416 strongly stabilize human telomeric G-quadruplex DNA in additions to modulating **tyrosine-kinase and adenosine receptors**. Consequently, stabilizing G-quadruplex DNA could contribute to the mechanism of their anticancer activity.

1. Introduction

GW-2974, N4-(1-Benzyl-1H-indazol-5-yl)-N6,N6-dimethyl-pyrido[3,4-d]pyrim-idine-4,6-diamine, is a quinazoline derivative synthesized by David W. Rusnak [1]. It is structurally analogous to GW-572016; the tyrosine kinase inhibitor approved in 2007 by FDA for treating breast cancer and known as lapatinib (Scheme 1) [2–3]. GW-2974 is a dual inhibitor of EGFR and HER2 in glioblastoma multiforme (GBM) in vitro and in vivo at low-concentration [3]. GW-2974 specifically inhibits both EGFR and ErbB-2 tyrosine kinase receptors in tumor cells. It showed better therapeutic efficacy and limited toxicity in gallbladder and breast cancers than GW-572016 [1]. Testing GW-2974 on gallbladder carcinoma in BK5.erbB2 mice revealed that targeting EGFR alone or in combination with erbB2 was effective in both prevention and treatment in BK5.erbB2 transgenic mice [4]. GW2974 was also found to prevent oral carcinogenesis in

DMBA induced hamster cheek pouch model at the post-initiation stage suppressing aberrant AA metabolism [5].

Combination of INCB3619 as a potent inhibitor for ADAM10 and ADAM17 with GW-2974 as a dual inhibitor of EGFR and HER-2/neu kinases, gave synergistic growth inhibition effect in MCF-7 and HER-2/neu-transfected MCF-7 human breast cancer cells [6]. Also combination of GW-2974 with a Met inhibitor yielded maximal growth inhibition for the treatment of non-small-cell lung carcinoma (NSCLC) than the use of any individual inhibitor [7]. Combination of GW-2974 with GX15-070 or HA14-1 inhibitors of Bcl-2 was also found to have a synergistic inhibitory effect on the growth of the MCF-7, MCF/18, and MTR-3 human breast cancer cell lines [8]. Oral administration of GW-2974, was also found to effectively inhibit skin tumor promotion in BK5.erbB2 and wild-type mice [9].

Chen et al studied the interaction of GW-2974 with ABC transporters. They reported that GW-2974 reverses ABCB1- and ABCG2-mediated MDR by blocking their efflux function. A conclusion was made that it may be useful to combine GW-2974 with EGFR TKIs for treating cancer [10]

GW-2974 was also found to have cardiac cell protective activity through protecting against apoptosis induced by TNF α , a known cytokine detected in cardiac failure [11]. Moreover, GW-2974 was reported to have inhibitory effects on the activity of hepatitis C virus (HCV) and the growth of PC-3 cell while stimulated NSE and chromogranin in androgen-independent prostate cancer cell line PC-3 [12, 13].

SCH-442416, 5-Amino-7-[3-(4-methoxy)phenylpropyl]-2-(2-furyl)-pyrazolo [4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (Scheme 1) is a pyrazolo-triazolo-pyrimidines derivative synthesized by Baraldi as a highly potent and selective human A_{2A} adenosine receptor antagonists [14–15]. The role of SCH-442416 during proliferation and osteogenic differentiation of human primary bone marrow stromal cells was investigated. Treating HPOC cultures with SCH-442416 induced osteogenic differentiating effect on CPA resulting in increased ALP activity [16]. Several derivatives of SCH-442416 have been prepared to enhance its potency and selectivity [17–21].

The effect of combination of L-DOPA with SCH-442416 on the rotational behaviors in a hemi-Parkinsonian mouse induced by unilateral 6-hydroxydopamine (6-OHDA) injection was studied. SCH-442416 was found to lower the dosage of L-DOPA in the 6-OHDA induced hemi-Parkinson mouse model [22]. Peiquan Zhao et al, reported that low concentrations of SCH-442416 may increase the mRNA expression of the Kir 2.1 and Kir 4.1 channels in Müller cells. Subsequently it may accelerate the clearance of K⁺ to protect the retinal neurons in vitro under hypoxic conditions [23–24]. Zhong et al have also investigated the effect of SCH-442416 on the expression of glutamine synthetase (GS) and glutamate aspartate transporter (GLAST) in rat retinal Müller cells at elevated hydrostatic pressure in vitro. SCH-442416 increased the expression of GLAST and GS of Müller cells and accelerated the clearance of extracellular glutamate [25].

On the other hand, G-quadruplex DNA or tetraplex structures are formed under physiological conditions by guanine rich strands in the human chromosomal telomere, immunoglobulin switch or gene promoter's

regions such as *c-myc*, *k-ras* and *c-kit*. Single or multiple strands DNA can fold into G-quadruplex structures through intramolecular or intermolecular eight Hoogsteen hydrogen bonds. Each four guanines in a plane form a layer called G-tetrads or G-quartets where Watson-Crick edge of a guanine base forms two hydrogen bonds with Hoogsteen edge of a neighbor guanine base. G-quartets are held together by π - π stacking to form the quadruplex structure and stabilized by electrostatic interactions between the guanine carbonyl groups and alkali metal cations as shown in scheme 1. [26–37].

Telomeres are the endcaps of chromosomes. They are non-coded for genetic information and consist of 5-15 kb of double stranded DNA and 100-300 single-stranded nucleotide bases at the 3' end [38]. The ability of cell division is linked to telomere's length. In normal somatic cells, telomere is shortened by 50–200 nucleotides upon each cell division and reaches a critical length at which it eventually enters in senescence stage then apoptosis [39]. Telomerase enzyme re-elongates the telomeres in stem, germ and embryonic cells. The presence of telomerase enzyme in cancer cells maintains telomere length and subsequently causes the indefinite divisions of the cells [40]. Folding of the telomere single strand overhang into G-quadruplex DNA inhibit the activity of telomerase enzyme by hiding the telomere end acting as template substrate for RNA telomerase. It also lead to displacing the telomere binding proteins involved in telomere capping which results in identifying the folded telomere as a DNA damage region by the cell [41].

Thus, stabilization of G-quadruplex structure by small molecules interfere the biological function of telomerase by inhibiting its activity and alter its maintenance which are crucial for the unlimited divisions of cancerous cell [41]. Molecules with preferential affinity towards G-quadruplexes can inhibit cancer proliferation. This has become an attractive strategy for developing new effective and selective genetic anticancer therapeutic agents [42–45].

Hundreds of small molecules have been reported as G-quadruplex's stabilizers, telomerase's inhibitors and/or oncogene regulators [46]. Anthraquinones [47–48], acridines [49–50], porphyrines [51–52], porphyrazines [53], phthalocyanines [54] and telomestatin [55–56] were reported as G-quadruplex DNA binders. Complexes of divalent selenium, manganese, nickel and copper ions with porphyrin, zinc with phthalocyanine and nickel with salphen showed high affinity and selectivity towards human telomeric and *c-myc* G-quadruplex DNA [35, 38, 357-59].

A planar aromatic core and a protonated side chain are thought to be important feature for G-quadruplex DNA's binders. The aromatic planar core intercalates in between the G-quartets through π - π stacking while the side chain support stability of formed complex by hydrophobic or ionic interactions with the external sides or into the DNA grooves [46, 60–61]. As an example, stabilization of G-quadruplex by perylene was attributed to the length of the side chain connecting the aromatic planar core to the protonated nitrogen atom and to the basicity of the system [58, 62]. For tetra (N-methyl-4-pyridyl) porphyrin (TMPyP4), the porphyrin ring was assumed to π - π stack on the G-quartet while the four lateral pyridinium groups are bound onto the grooves of G-quadruplex DNA [63]. Thus, a good G-quadruplex stabilizer is required to have aromatic planar core to intercalate by π - π stacking between the G-quartets

DNA planes, protonated side chains for face's stacking and groove binding. Partial positive charge may also be required to substitute K^+ or Na^+ cations in the guanine's center and bind to the negatively charged phosphate backbones [64].

This work aimed to study the stabilization of human telomeric G-quadruplex DNA ($AG_3(TTAGGG)_3$) by GW-2974 and SCH-442416, as a probable underlying mechanism for their anticancer effects. Interaction parameters such as binding affinity, binding constant, melting temperature and binding selectivity towards G-quadruplex over DNA duplex were studied using UV-absorption, fluorescence, fluorescence quenching, circular dichroism, melting temperature and molecular docking techniques

The results are aimed to test the hypothesis that both GW-2974 and SCH-442416 are strongly stabilizing human telomeric G-quadruplex DNA. Consequently, they may be used to inhibit telomerase activity which in turn can inhibit cancer's proliferation. This is in additions to their confirmed biological activities as tyrosine-kinase and adenosine receptors' modulators.

2. Experimental

2.1 Materials and Reagents

All chemicals were of the highest purity available (98-99%) and used without further purification. GW-2974 (N4-(1-Benzyl-1H-indazol-5-yl)-N6,N6-dimethyl-pyrido[3,4-d]pyrimidine-4,6-diamine), SCH-442416 (5-Amino-7-[3-(4-methoxy) phenylpropyl]-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine), ethylenediaminetetraacetic acid (EDTA), potassium chloride, tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), and calf thymus DNA (ct-DNA) were purchased from Sigma-Aldrich. HPLC purified telomeric DNA sequences 5'-AGGGTTAGGGTTAGGGTTAGGG-3', FI-5'-AGGGTTAGGGTTAGGGTTAG-GG-3' and 3'-TCCCAATCCCAATCCCAATCCC-5' were purchased from Alpha DNA, Canada. Millipore deionized water was used throughout.

2.2. Apparatus

Absorption measurements were carried out using Agilent-8453 UV-Vis spectrophotometer (Austria) matched with 1 cm quartz cells. Fluorescence measurements were carried in 1.0 cm quartz cell using Cary Eclipse model-3 spectrofluorometer equipped with a high intensity Xenon flash lamp (Austria). CD measurements were made using Jasco J-815 spectrophotometer (USA). pH measurements were made using Orion-401 Plus pH meter supported with Orion glass electrode.

2.3. Standard Solutions

2.3.1. Buffer solution

A 10^{-2} M Tris-KCl buffer solution pH 7.4, was prepared by dissolving 10.0 mM tris-hydroxymethylaminomethane hydrochloride (1.576 g), 1.0 mM Na_2EDTA (0.3722 g) and 100.0 mM KCl (7.455 g) into 1.0 L of deionized water. The pH was adjusted using the glass electrode.

2.3.2. GW-2974 and SCH-442416 solutions

Stock solutions (2×10^{-3} M) of GW-2974 and SCH-442416 were prepared in ethylene glycol. Solutions having lower concentrations were prepared by appropriate dilution into the buffer solution.

2.3.3. Calf thymus DNA (ct-DNA)

A 1000.0 $\mu\text{g/ml}$ ct-DNA was prepared by dissolving 10.0 mg into 10.0 ml Tris–KCl buffer pH 7.4, without sonication or stirring. The solution was gently inverted overnight at 4.00 °C to completely solubilize the DNA and prevent shearing of the large genomic DNA. Resultant solutions are stable for several months at 4.00 °C.

2.3.4. Human telomere ssDNA

Purchased synthetic human telomere DNA sequence 5'-AGGGTTAGGGTTAGGGTTAGG G-3', its fluorescein labeled 5' primer 5'-FI-AGGGTTAGGGTTAG-GGTTAGGG-3' or its complementary strand 3'-TCCCAATCCCAATCCCAATC-CC-5' were centrifuged for 10.00 min at 7000 rpm to collect the DNA in the bottom of the tube. A 2.00 ml of Tris–KCl buffer pH 7.4, was added to each tube. Solutions were left overnight at 4.00 °C for rehydration, then vortexed for 30 s. Reconstituted primers were stable for more than six months 4.0 °C.

Concentrations of resultant stock DNA solutions were determined by diluting a 10.0 μl into 1.0 ml using the Tris-KCl buffer. The solution was vortexed for 15 s and its absorbance was measured at 260 and 280 nm. Concentrations in $\mu\text{g/ml}$ were calculated using the following equation:

$$C(\mu\text{g/ml}) = A_{260} \times \text{weight per OD} \times \text{dilution factor}$$

where OD is the optical density at 260 nm. The purity of each oligonucleotide was estimated based on the ratio A_{260}/A_{280} . Ratios ≥ 1.8 were considered enough to indicate high purity [65].

2.4. Procedures

2.4.1. Formation of human telomeric G-Quadruplex DNA

G-quadruplex structure was prepared by gentle heating of a 2.0 ml of the stock single stranded 5'-AGGGTTAGGGTTAGGGTTAGGG-3' DNA up to 95.0 °C. The solution was incubated for 10.0 min and left to cool to room temperature. The solution was kept at 4.0 °C till use. Fluorescein labeled G-quadruplex DNA was prepared similarly.

2.4.2. Hybridization of human telomeric ssDNA oligonucleotides

A 1×10^{-4} M telomeric dsDNA was prepared by mixing equimolar amounts of 5'-AGGGTTAGGGTTAGGGTTAGGG-3' (268.8 μL of 7.44×10^{-4} M) with its complementary strand 3'-TCCCAATCCCAATCCCAATCCC-5' (738.0 μL of 2.71×10^{-4} M). The solution was made up to 2.0 ml using

Tris–KCl buffer pH 7.4, vortexed for 15 s and incubated at 95 °C for 10.0 min. Resultant hybridized dsDNA was left to cool to room temperature and kept in refrigerator at 4.00 °C until use.

2.4.3. Photometric titrations

Absorption titrations were conducted by successive additions of G-quadruplex (1.18×10^{-4} M) aliquots to a 1.0 ml of GW-2974 or SCH-442416 (5×10^{-5} M) in Tris-KCl buffer, pH 7.4. After each addition, the solution was shaken, incubated for 3 minutes at room temperature and scanned in the UV-Vis range 200–700 nm. Titration stopped when no change in absorbance was observed. A [drug]/[G-quadruplex] ratio ranged in 0.88 to 20.0 was obtained. Increasing incubation times to one hour was found ineffective. Solutions having ≤ 10 % ethylene glycol were maintained during titration.

For fluorescence titration, successive amounts of human telomere G-quadruplex DNA (1.33×10^{-4} M) were added to 2.0 ml of GW-2974 or SCH-442416 (5×10^{-6} M) in Tris-KCl-buffer pH 7.4. After each addition, the solution was stirred for 20 s, incubated for 3 min and its fluorescence spectra were scanned in the range 300–550 nm. Titration stopped when no change in fluorescence intensity was observed; approximately at Ligand:DNA molar ratio of 20.0. Solutions having ≤ 10 % ethylene glycol were maintained during the titration. Increasing incubation time to one hour was found ineffective. Excitation wavelengths of 243 and 268 nm were respectively used GW-2974 and SCH-442416 with excitation and emission slit width of 10.0 nm. The stoichiometric ratios G-quadruplex:ligand were estimated using the molar ratio method, through plots of fluorescence intensity versus [DNA]/[Ligand] molar ratio.

For fluorescence quenching titration, successive aliquots of GW-2974 or SCH-442416 were added to 3.0 ml of fluorescein labeled G-quadruplex (2×10^{-6} M) (5'-Fl-(AGGGTT)3AGGG-3'). After each addition, the solution was stirred for 20 s, incubated for 3 min, and scanned for fluorescence at excitation λ_{max} of 494 nm and emission λ_{max} of 518 nm. Fluorescence's quenching of Fl-G-quadruplex was recorded using slit widths of 5.0 nm and 2.5 nm for excitation and emission slits, respectively.

Changes in G-quadruplex's conformation upon interactions with GW-2974 and SCH-442416 were also followed using CD titrations. Successive amounts of 5×10^{-4} M ligand were added to 1.0 ml G-quadruplex (4×10^{-6} M) in Tris-KCl buffer pH 7.4. After each addition, the solution was shaken, incubated for 3 min at room temperature and CD scanned in the range 200–400 nm at 50 nm/min and band width of 1 nm using 3 accumulations. Collected CD spectra were corrected for blank and base line. Changes in CD intensity at 293 nm were recorded versus ligand concentrations. The ratio [ligand]/[G-quadruplex] ranged in 0.25–5.0.

2.4.4. Melting temperature curves

Melting temperature curves for G-quadruplex DNA and its GW-2974 and SCH-442416 complexes were constructed using CD spectral measurements. A 1.0 ml solution of telomeric G-quadruplex DNA in Tris-KCl-buffer (2.76×10^{-6} M) was heated up to 95 °C in an average increment of 3 °C with average incubation time intervals of 3.0 min. At each temperature, the CD spectra were recorded in the range 200–400 nm using same CD settings in section 2.4.3. Melting temperature curves for G-quadruplex DNA complexes

with GW-2974 or SCH-442416 were made by mixing equimolar amounts of G-Quadruplex DNA (48 μL of $5.74 \times 10^{-5} \text{ M}$) with GW-2974 or SCH-442416 (27.6 μL of $1 \times 10^{-4} \text{ M}$) into 1.0 mL KCl-Tris buffer. CD spectra of resultant complexes were collected and corrected for blank and baseline. Plots of CD intensity at 293 nm versus temperature were constructed.

Similarly, the melting temperature curve of ct-DNA was obtained heating up 1.0 ml of ct-DNA in Tris-KCl-buffer pH 7.4 ($1 \times 10^{-9} \text{ M}$, 100 ppm) from 25-95 $^{\circ}\text{C}$ in an average increment of 3.0 $^{\circ}\text{C}$ and applying an average incubation time intervals of 3 min. Intensity of the ct-DNA's CD peak at 283 nm was recorded and plotted against temperature. Melting temperature curves of ct-DNA complexes with GW-2974 and SCH-442416 were made by mixing ct-DNA (100 μL , $8 \times 10^{-8} \text{ M}$) with GW2974 or SCH442416 (23.4 μL , $1 \times 10^{-4} \text{ M}$). The solutions were made up to 1.0 ml using Tris-KCl-buffer pH 7.4 and CD intensities at 283 nm were recorded. Ethylene glycol up to 10.0% was adopted in all measurements.

2.4.5. Selectivity towards telomeric G-quadruplex DNA.

Binding selectivity of GW-2974 and SCH-442416 towards G-quadruplex DNA, was evaluated using telomeric dsDNA and ct-DNA as interfering species.

A solution that is $5 \times 10^{-5} \text{ M}$ in each of fluorescein labelled G-quadruplex DNA and GW-2974 or SCH-442416, was prepared by mixing the appropriate amount of each into 2.0 ml Tris-KCl-buffer pH 7.4. The solution was vortexed for 10 s, incubated for 3 min and scanned for its fluorescence in the range 500-700 nm using 494 nm as λ_{max} of excitation.

To the previous solutions, appropriate amounts of telomeric dsDNA or ct-DNA were added to obtain solutions having 10.0, 50.0 and 100.0 concentration folds relative to Fl-G-quadruplex DNA. Resultant solutions were vortexed for 10 s, incubated for 30 min at room temperature and scanned for their fluorescence in the range 500-700 nm. Ethylene glycol was maintained at 10% in all solutions.

2.4.6. Binding affinity

Binding affinities of GW-2974 and SCH-442416 towards telomeric G-quadruplex DNA were estimated using Scatchard model. Fluorescence emission at 243 or 268 nm were followed up after adding different amounts of DNA to constant amount of GW-2974 or SCH-442416 described in section 2.4.3.

In Scatchard equation, $\frac{r}{C_f}$ is plotted versus r according to equation 1 [32].

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

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

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

where C_{total} is the concentration of GW-2974 or SCH-442416 at zero addition of quadruplex and C_f was calculated according to equation 3

$$C_f = C_{total}(1 - a) \dots\dots\dots 3$$

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

$$a = \frac{F_f - F}{F_f - F_b} \dots\dots\dots 4$$

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 nK .

The plot can result in a straight or curved line. In case of curved line, equation 1 is rearranged to equation 5

$$r = \frac{nKC_f}{1 + KC_f} \dots\dots\dots 5$$

where r is plotted against C_f Values for n and K were obtained using nonlinear curve fitting in Origin 8.0 software.

2.4.7. Molecular Docking

Molecular dockings of GW-2974 and SCH-442416 onto telomeric G-Quadruplex DNA was done using the Glide software and the telomeric G-quadruplex DNA crystal structure PDB:2MS6 from the protein data bank [66]. The structure was checked for any missing atom via the MOE software package [67–68]. All solvent molecules and hetero ligands were removed and the structure was prepared for docking using the preparation wizard tool in the Maestro software [69–71]. Preparation included creating bonds, adding hydrogens, and assigning partial charge for all atoms and checking protonation state of each ionizable group. Two binding pockets were identified from the Quercetin's co-crystallized ligand. Using the Receptor Grid Generation module in Glide, a grid box was produced for each [72]. On the other hand, GW-2974 and SCH-442416 molecules were prepared in Maestro [71] via the LigPrep module [73]. Ligand's preparation included identifying the ionization states for ionizable functional groups at the pH range of 7.0 ± 2.0 .

Subsequently, the two ligands were docked into the two prepared binding pockets on G-Quadruplex DNA structure using the Glide software [72]. The extra-precision (XP) docking mode [74] was employed for conformational sampling. All produced poses were scored and ranked via the Glide-XP scoring function. The latter included terms for van der Waals, hydrogen bond, electrostatic interactions, desolvation penalty and penalty for intra-ligand contact [74].

3. Results And Discussion

Interactions of GW-2974 and SCH-442416 with human telomeric G-quadruplex DNA were evaluated using UV-absorption, fluorescence emission, fluorescence quenching and circular dichroism spectroscopies. Binding parameters that include binding constants, binding stoichiometries, binding modes, melting temperatures and selectivity towards G-quadruplex over dsDNA and ct-DNA were investigated. The above data were supported with molecular docking.

3.1. Solubility of GW-2974 and SCH-442416

GW-2974 and SCH-442416 are insoluble in water and soluble in DMSO and ethylene glycol. Solutions of GW-2974 and SCH-442416 in ethylene glycol (100%) were found stable over more than four months at 4 °C. Stabilities of GW-2974 and SCH-442416 in 10% ethylene glycol - Tris-KCl buffer were tested using UV-Vis absorption spectrophotometry. Solutions showed no significant changes in absorption over four hours. This time was considered enough to run all experiments and we adopted 10% ethylene glycol in all our measurements.

Effect of ethylene glycol on structural conformation of telomeric G-quadruplex DNA was also tested (Figure 1). Additions of ethylene glycol to G-quadruplex (up to 20%.) in Tris-KCl buffer showed no change in the shape and intensity of G-quadruplex' CD spectrum. These results indicated that 20% ethylene glycol is safe and do not change the conformation or denaturize telomeric G-quadruplex DNA. The results are consistent with Bonner and Klibanov's report stated that up to 90% ethylene glycol is safe and non-denaturizing for DNA [75]. Therefore, freshly prepared - 10% ethylene glycol buffer solutions were used throughout this study to improve the solubility of GW-2974 and SCH-442416.

3.2. Spectrophotometric Titration

UV-Vis Absorption

Figure 2 shows the UV-Vis absorption spectra of GW-2974 and SCH-442416 in Tris-KCl buffer pH 7.4. GW-2974 spectrum shows strong bands at 243 nm ($\epsilon = 6100$) and 320 nm ($\epsilon = 5500$) and a weak band at 421 nm ($\epsilon = 2600$) (Fig. 2a). The spectrum of SCH-442416 shows a strong absorption band at 267 nm ($\epsilon = 7700$) (Fig. 2b).

Changing the pH of GW-2974 and SCH-442416 solutions (10^{-5} M) indicated no significant spectral changes in the pH range 4.0–10.0. Consequently, the two compounds were considered chemically stable within this pH range and can be safely used for further investigations on their interactions with G-quadruplex DNA at the physiological pH of 7.4.

Figure 2a also shows the effect of successive additions of human telomere G-quadruplex DNA (1.18×10^{-4} M) to GW-2974 (5×10^{-5} M) in Tris-KCl buffer pH 7.4. The absorption bands at 421 and 320 nm continuously decreased (hypochromicity) without forming isosbestic points or being shifted. On contrary, the intensity of absorption band at 243 nm increased due to the overlap with the DNA absorption band at 260 nm. Figure 2b shows that additions of human telomeric G-quadruplex DNA to SCH-442416

decreased the intensity of the absorption band at 350 nm and increased the band at 267 nm. The latter can be attributed to interference with the DNA's absorption band at 260 nm (Fig. 2b).

The above hypochromic effects ($\geq 50\%$) obtained upon addition of DNA to GW-2974 or SCH-442416 suggested intercalation binding modes for both compounds with telomere G-quadruplex DNA.

Hypochromicity along with the binding stoichiometry and binding constants were used to suggest an intercalation binding via stacking on G-quartet and outside binding mode [63]. Further evidences will be obtained using fluorescence and CD spectroscopic measurements.

Fluorescence

A confirmation for binding interactions of GW-2974 and SCH-442416 to human telomeric G-quadruplex DNA were obtained by fluorescence titrations. Figure 3 shows the fluorescence spectra of GW-2974 and SCH-442416. GW-2974 gave a fluorescence band at 410 nm when excited at 243 nm while SCH-442416 gave a broad band centered at 420 nm when excited at 267 nm. The latter band seems to be composed of two overlapping bands at 408 and 423 nm.

Sequential additions of G-quadruplex DNA to GW-2974 resulted in a continuous decrease in the fluorescence intensity at 410 nm. A 65.0% hypochromicity was observed after adding 20 folds of G-quadruplex DNA (Fig. 3a). The band at 484 nm is attributed to Rayleigh scattering, normally shown at double the excitation wavelength. Similarly, additions of G-quadruplex DNA to SCH-442416 continuously decreased the fluorescence emission at 420 nm (Fig. 3b). At 20 folds of added DNA, the band was slightly red shifted and reduced its intensity by 91.4% (hypochromic effect).

Reductions in fluorescence emissions of GW-2974 and SCH-442416 by $\geq 65 - 91\%$, indicated that both compounds bound to human telomeric G-quadruplex DNA through intercalation binding modes [63].

Fluorescence Quenching

Additional evidence on intercalation of GW-2974 and SCH-442416 with telomere G-quadruplex DNA was obtained from fluorescence quenching measurements. Fluorescein labeled telomeric G-quadruplex DNA (5'-FI-AGGG(TTAGGG)3-3') gave a strong fluorescence band at 518 nm when excited at 494 nm. Sequential additions of GW-2974 or SCH-442416 (10^{-4} M) to human telomeric FI-G-quadruplex DNA (2×10^{-6} M) quenched its fluorescence emission at 518 nm by more than 90% (Fig. 4). The extent of quenching depends on the number of molecules bound per DNA molecule.

Decreases of fluorescence emission at 518 nm confirmed intercalations of both compounds in proximity of the fluorescein flag moiety. Since fluorescein molecule is connected to the 5' prime, one may infer that both GW-2974 and SCH-442416 bind between the G-quartets adjacent to the TTA loop cavity.

These results are consistent with previous findings from UV-Vis absorption and fluorescence measurements and confirm intercalation binding modes between each of GW-2974 and SCH-442416 with G-quadruplex DNA.

Circular Dichroism Titration

CD spectroscopy is the tool of choice for studying conformational changes in DNA upon interactions with drugs. Structural conformation of human telomeric G-quadruplex DNA in solution depends on its sequence, length, and environment. It forms antiparallel structure in Na^+ solution whereas in K^+ solution, a hybrid mixed with parallel / antiparallel structure is formed [76–77].

Figure 5 shows the CD spectrum of the twenty-two bases - human telomere G-quadruplex DNA in Tris-KCl buffer pH 7.4. The negative band centered at 235 nm, the positive shoulder at 253 nm and the positive band at 293 nm confirmed the formation of a hybrid parallel-antiparallel structure in K^+ solution [67–68].

Figure 6 shows the effect of GW-2974 and SCH-442416 additions to telomeric G-quadruplex DNA. Gradual decreases in the intensity of the bands at 235, 253 and 293 nm with no change in the spectral shapes or bands' positions indicated no change in the hybrid parallel antiparallel conformation of the telomeric G-quadruplex DNA at the end of titration. Changes in CD spectra of G-quadruplex DNA during titration have been correlated with the binding mode of the ligand involved. A continuous decrease in CD intensity was correlated with intercalation-binding mode while increase was correlated with groove-binding mode [78].

Thus, decreases in CD intensities upon sequential additions of GW-2974 or SCH-442416 could be correlated with intercalation binding modes by π - π stacking between the G-quartets' faces of G-quadruplex (Fig. 6). These results are also consistent with previous results obtained from absorption and fluorescence titrations.

3.3. Binding Stoichiometry

Stoichiometry of GW-2974 and SCH-442416 interactions with human telomere G-quadruplex DNA was estimated using the molar ratio method based on fluorescence measurements. Figure 7 shows the molar ratio curves obtained by adding successive increments of telomeric G-quadruplex (1.33×10^{-4} M) to 2.0 ml of 5×10^{-6} M of GW-2974 or SCH-442416. Plots of fluorescence intensity at 410 and 420 nm versus molar ratio gave the molar stoichiometric ratios of GW-2974 and SCH-442416 to G-quadruplex DNA (Figs. 7a and 7b). A 2:1 molar ratio was obtained. These ratios indicated that two molecules of GW-2974 or SCH-442416 bound per each G-quadruplex molecule. This ratio is consistent with quercetin to human telomeric G-quadruplex DNA molar ratio.

3.4. Binding Affinity

Several modes have been proposed for binding G-quadruplex DNA to ligands. These included intercalative binding between adjacent G-quartets, face π - π stacking on external G-quartets or with the

loops through and a weaker external binding

Decreases in the intensity of the GW-2974 and SCH-442416 fluorescence soret bands upon additions of G-quadruplex favored their intercalations by face π - π stacking in the ratio 2:1 (GW-2974 or SCH-442416 to G-quadruplex DNA) (Fig. 7).

Binding constants of the two compounds to G-quadruplex DNA were estimated using Scatchard plots. Figure 8 shows non-linear downward concave curves of r/C_f versus r . The plots suggested that more than one type of dependent binding sites exist on G-quadruplex molecule. Dependent sites can synergistically or antagonistically affect each other (neighbor exclusion effect). In the former case, the first bound ligand encourages the next binding ligand while in the latter the first bound ligand suppress the next binding [79–80].

Resolving the nonlinear Scatchard plots in Fig. 7 gave two intersecting straight lines each represents one type of binding site. The slopes of these lines gave binding constants of 1.3×10^8 and 1.72×10^6 M⁻¹ for GW-2974 and 1.55×10^7 and 3.74×10^5 M⁻¹ for SCH-442416. The number of binding sites ranged between 0.7–1.27 for GW-2974 and 0.4–1.5 for SCH-442416. (Figs. 8a and 8c, Table 1). These results apparently show a type of dependent binding sites.

Table 1
Binding constants (K) and number of binding sites (n) per G-quadruplex DNA molecule for GW-2974 and SCH-442416.

G-quadruplex complex	K1 (M-1)	K2 (M-1)	n1	n2
Graphical Method				
GW2974-G-quadruplex	1.3x108	1.72x106	0.7	1.27
SCH442416-G-quadruplex	1.55x107	3.74x105	0.4	1.5
Nonlinear Curve Fitting				
GW2974-G-quadruplex	2.65x106	–	1.15	–
SCH442416-G-quadruplex	7.0x105	–	1.88	–

Applying the Scatchard modified equation (5), A non-linear r versus C_f curves were obtained (Figs. 8b and 8d). Nonlinear fitting of the two curves resulted in an average binding constant of 2.65×10^6 and 7.0×10^5 M⁻¹ with number of binding sites of 1.15 and 1.88 for GW-2974 and SCH-442416, respectively (Table 1).

These results reflected high stabilization effects for both compounds on human telomeric G-quadruplex DNA. However, GW-2974 showed higher binding affinities than SCH-442416. The number of binding sites are also consistent with the molar ratio analysis and published values (Section 3.3).

3.5. Melting Temperature

Figure 9 shows the melting temperature curves for G-quadruplex, ct-DNA and their complexes with GW-2974 and SCH-442416, based on CD measurements. Melting temperature (T_m) was calculated as the midway CD signal on the melting curve and represents the temperature at which DNA is half unfolded to its single strands.

The melting curve of human telomere G-quadruplex DNA gave a T_m value of 65.5°C while its complexes with GW-2974 and SCH-442416 gave T_m values of 75.4 and 75.1°C (Figs. 9a and 9b) (Table 2). These results revealed stabilization effect for G-quadruplex DNA by $\Delta T_m = 9.9$ and 9.6°C respectively. These results indicated a higher stabilizing effect for GW2974 compared to SCH-442416. The results are also consistent with the binding constants shown in Table 1. On the other hand, Fig. 9c shows a melting temperature of 88.0 °C for ct-DNA. Complexations with GW-2974 and SCH-442416 stabilized the ct-DNA by ΔT_m of 2.1 and 2.4°C, respectively.

Table 2
Melting temperature of human telomere G-quardruplex DNA and its GW-2974 and SCH-442416 complexes at different [drug]/[DNA] ratios.

Ligand	[Ligand]/[G-quadruplex] molar ratio					
	1:1		3:1		5:1	
	T_m °C	ΔT_m °C	T_m °C	ΔT_m °C	T_m °C	ΔT_m °C
G-quadruplex DNA	65.5	0.0	65.5	0.0	65.5	0.0
GW-2974-G-quadruplex	75.4	9.9	69.7	4.2	69.0	3.5
SCH-442416-G-quadruplex	75.1	9.6	69.3	3.8	68.2	2.7
ct-DNA	88.0	0.0	–	–	–	–
GW-2974-ct-DNA	90.1	2.1	–	–	–	–
SCH-442416-ct-DNA	90.4	2.4	–	–	–	–

These results indicated that GW-2974 and SCH-442416 have ≥ 4 four folds stronger stabilization effects on human telomeric G-quadruplex compared to duplex ct-DNA (4.71 times for GW2974 and 4.00 times for SCH442416). The results also indicated that both compounds have preferential and selective binding to G-quadruplex over duplex ct-DNA.

Figure 9 also shows the effect of GW-2974 and SCH-442416 concentrations on stabilizing human telomeric G-quadruplex DNA. Complexes with molar ratios of 1:1, 3:1 and 5:1 (ligand:G-quadruplex) were measured for their melting curves. Increasing the molar ratio of GW-2974 from 1:1 to 5:1 resulted in decreasing ΔT_m values from 9.9 to 3.5 °C. Similar effect was observed for SCH-442416 where ΔT_m decreased from 9.6 to 2.7 °C upon changing the ratio from 1:1 to 5:1 (Table 2). These results

demonstrated that increasing the ligand's concentrations above 1:1 molar ratio has destabilized the human telomeric G-quadruplex DNA.

These results suggest that the two investigated compounds act as stabilizers for G-quadruplex DNA. They also teach us that they have higher affinity and selectivity towards human telomeric G-quadruplex DNA over duplex ct-DNA [81].

3.6. Selectivity of binding towards G-quadruplex DNA

Binding selectivity of GW-2974 and SCH-442416 towards telomeric G-quadruplex DNA over duplex DNA was estimated using telomeric dsDNA and ct-DNA as interfering species. A 10.0, 50.0 and 100.0 folds of duplex DNA were added to complexes formed between FI-G-quadruplex (10-10 M) and either GW-2974 or SCH-442416. The mixtures were scanned for their fluorescence intensity at 518 nm as described in section 2.4.5.

Figure 10 shows changes in fluorescence intensity of FI-G-quadruplex DNA-GW-2794 upon adding different folds of telomere dsDNA (10a and 10c) and ct-DNA (10b and 10d). Selectivity coefficient was calculated by dividing the change in fluorescence intensity by the intensity of FI-G-quadruplex complex. Coefficients having values of $(1.1-2.2) \times 10^{-2}$ and $(2.2-4.4) \times 10^{-2}$ were obtained. These results indicated preferential binding selectivity for binding GW-2974 and SCH-442416 to bind with human telomere G-quadruplex DNA over duplex dsDNA and ct-DNA.

Thus, the two compounds may selectively bind towards G-quadruplex DNA over dsDNA predominant in human cells. These results are also consistent with the results obtained from melting temperature experiments (section 3.5).

3.7. Molecular Docking

Several crystal structures for human telomere G-quadruplex DNA were obtained from the protein data bank and tried in this work. The crystal structure PDB:2MS6 was found the most suitable since its co-crystallized ligand; quercetin has almost a similar size to that of GW-2974 and SCH-442416 molecules. The presence of two co-crystallized ligand molecules bound to a human telomeric G-quadruplex DNA indicated the presence of two binding sites on the crystal structure of DNA. Therefore, our compounds were docked into these two pockets as per the conditions given in the experimental part.

Table 3 shows the docking scores of GW-2974 and SCH-442416 along with the co-crystallized ligand quercetin. The two compounds gave favorable exothermic binding energies ranged from -6.07 to -8.14 kcal/mol. GW-2974 has the best docking score (-8.14 kcal/mol) which is comparable to quercetin (-9.03 kcal/mol) when docked into the same pocket (Site 1, Table 1). On site 2, GW-2974 and SCH-442416 gave comparable binding scores of -6.17 and -6.37 kcal/mol which are inferior to the score obtained by the co-crystallized ligand: quercetin (-9.27 kcal/mol).

Table 3
Docking scores of SCH-442416 and GW-2974 along with the co-crystallized ligand into the binding site of Telomeric G-quadruplex DNA.

Ligand	Docking score (kcal/mol)	
	Site 1	Site 2
GW2974	-8.14	-6.17
SCH-442416	-6.07	-6.37
The co-crystallized ligand; quercetin	-9.03	-9.72

Figure 11 shows that GW-2974 was able to slide in-between the nitrogen bases surrounding site 1 and make hydrogen bonding with DG20, associated with multiple stacking interactions to DG6 and DG27. The Fig. 11 also shows that SCH-442416 was able to slide in-between the nitrogen bases comprising the binding site 2, making the important interaction; hydrogen bonding with DT16.

To sum up, our docking results are -in line with the experimental data- suggest that GW-2974 and SCH-442416 are favorably and exothermally bind to human telomeric G-quadruplex DNA. The two compounds conveniently bind on the quercetin's binding sites with comparable binding scores.

4. Conclusion

GW-2974 is a highly potent tyrosine kinase receptor that inhibits EGFR and ErbB-2 in tumor cells with selectivity for tumor cells over normal cells. SCH-442416-442416 is a potent adenosine A2a antagonist with high selectivity for human A2a receptors. The anticancer effects of both compounds are documented in literature with several proposed mechanisms.

In this work, human telomere G-quadruplex DNA was evaluated as a molecular target for both compounds underlying their anticancer mechanism. Changes in UV-Vis, fluorescence and CD spectra as well as melting temperature and in silico molecular docking were used to provide evidence for their interactions with G-quadruplex DNA.

Changes in UV-Vis and fluorescence spectra indicated that GW-2974 and SCH-442416 bind to human telomere G-quadruplex DNA in intercalative modes. Fluorescence quenching of FI-G-quadruplex DNA by additions of the two compounds indicated that they bound in the close proximity of the fluorescein flag; close to the TTA loop. Decreases in the CD intensities at 235, 253 and 293 nm indicated intercalation bindings.

Estimations of binding affinities using Scatchard plots, revealed non-linear downwards concave plots. These plots indicated the presence of two types of dependent binding sites. With binding constants of 1.3×10^8 and 1.72×10^6 M⁻¹ for GW-2974 and of 1.55×10^7 and 3.74×10^5 M⁻¹ for SCH-442416. The corresponding number of binding sites were 0.7-1.27 and 0.4-1.5 per G-quadruplex DNA molecule, respectively. Nonlinear curve fitting resulted in average binding constants of 2.65×10^6 and 7.0×10^5 M⁻¹

with binding sites of 1.15 and 1.88 for both compounds, respectively. The molar ratio method indicated that GW-2974 and SCH-442416 form complexes with telomere G-quadruplex DNA in a stoichiometric ratio of 2:1. Melting temperature curves indicated that both compounds stabilized human telomere G-quadruplex by 9.6 and 9.1 °C, respectively. This is more than four times higher than their stabilization effects on duplex ct-DNA, amounted to 2.1 and 2.4 °C, respectively.

Selectivity experiment gave coefficients of $\approx 1.5 \times 10^{-2}$ and 3.0×10^{-2} revealing a preferential binding for both compounds to human telomere G-quadruplex DNA over telomeric duplex DNA and ct-DNA, respectively. This conclusion was also supported by obtaining Δm values that are 4.7 and 4.0 folds higher than their Δm 's for ct-DNA. In silico molecular docking revealed favorable and exothermal binding on two sites of G-quadruplex DNA. Binding scores in agreements with quercetin' binding and our experimental data were obtained.

These results indicated high affinity and selectivity for the two compounds towards G-quadruplex DNA over duplex DNAs. GW-2974 showed higher affinity and melting temperature ΔT_m values compared to SCH-442416. Interaction modes based on intercalative binding between adjacent G-quartets, end-stacking on G-quartet and π - π stacking with ligand molecules have been proposed for interactions of hybrid parallel-antiparallel G-quadruplex DNA with small ligand molecules. Given the quenching of FL-G-quadruplex DNA fluorescence as well as the strong binding affinities and stoichiometry shown by the two compounds, one may infer that binding of GW-2974 and SCH-442416 involved intercalation between adjacent G quartets (π - π^* stacking) and end-stacking on G-quartets. These results suggest the two compounds can act as human telomere G-quadruplex DNA stabilizers. Such stabilization and selective recognition powers could be an underlying mechanism for their anticancer effects.

Declarations

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Data availability

- The following pre-defined DNA sequences were purchased from Alpha DNA, Canada. Purchased DNA sequences were HPLC-purified grade and used without further purification.

1-Human telomeric DNA sequence: 5'-AGGGTTAGGGTTAGGGTTAGGG-3'

2-Human telomeric complementary sequence: 3'-TCCCAATCCCAATCCCAATCCC-5'

3-Fluorescein labeled telomeric sequence: 5'-FI-AGGGTTAGGGTTAGGGTTAGGG-3'

- The crystal structure of the human telomeric G-quadruplex DNA (sequence: 5'AGGGTTAGGGTTAGGGTTAGGG-3') complexed with flavonoid quercetin was used in the molecular docking study. This structure is publicly available in the Protein Data Bank under the code (PDB:2MS6) and can be accessed through; <https://www.rcsb.org/structure/2MS6>

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Scheme

Scheme 1 is available in the Supplemental Files section.

Figures

Figure 1

CD spectrum of 2.36×10^{-6} M telomeric G-quadruplex in Tris-KCl buffer pH 7.4 titrated with ethylene glycol (0 – 200 μ L).

Figure 2

Spectrophotometric titration spectra of GW-2974 (a) and SCH-442416 (b) (5×10^{-5} M) titrated with human telomere G-quadruplex DNA (1.18×10^{-4} M) in Tris-KCl buffer, pH 7.4.

Figure 3

Fluorescence titrations of GW-2974 (3a) and SCH-442416 (3b) (5×10^{-6} M) with human telomeric G-quadruplex DNA (1.33×10^{-4} M). Titrations were done in Tris-KCl buffer, pH 7.4 - 10% ethylene glycol.

Figure 4

Fluorescence quenching of 5'-FI-G-quadruplex DNA (2x10⁻⁶ M) by additions of GW2974 (6a) and SCH442416 (6b) (10⁻⁴ M) in Tris-KCl buffer, pH 7.4. 5'-FI-G-quadruplex DNA was excited at 494 nm and emitted at 518 nm.

Figure 5

CD spectrum of human telomeric G-quadruplex (4x10⁻⁶ M) in Tris-KCl buffer, pH 7.4.

Figure 6

CD titration of human telomere G-quadruplex DNA (4×10^{-6} M) with 5×10^{-6} M of GW2974 (8a) and SCH442416 (8b) in Tris-KCl buffer, pH 7.4.

Figure 7

Stoichiometric ratios of 5×10^{-6} M GW2974 (9a) and SCH442416 (9b) with G-quadruplex DNA (1.33×10^{-4} M) using the molar ratio method based on fluorescence measurements in Tris-KCl buffer, pH 7.4.

Figure 8

Scatchard plots of GW2974 (a-b) and SCH442416 (c-d) ($5 \times 10^{-6} \text{M}$) by adding increments of human telomere G-quadruplex DNA using fluorescence spectroscopy. Tris-KCl buffer, pH 7.4 – 10 % ethylene glycol was used.

Figure 9

Melting temperature curves for human telomere G-quadruplex and its complexes with GW2974 (9a) and SCH442416 (9b) using 1:1, 3:1 and 5:1 (Drug)/[G-quadruplex) molar ratios. Fig. 9c shows the melting temperature curves for ct-DNA and its complexes with GW2974 and SCH442416.

Figure 10

Selectivity of GW2974 and SCH442416 towards G-quadruplex DNA. Fluorescence of GW-2974 and SCH-442416 complexes of Fl-G-quadruplex complex (5×10^{-10} M) were measured in presence of 10.0, 50.0 and 100.0 folds telomere dsDNA (12a & 12c) and ct-DNA (12b & 12d), respectively. Measurements were made in Tris-KCl buffer, pH 7.4 – 10% ethylene glycol.

Figure 11

Predicted binding pose of GW-2974 (pink sticks) and SCH-442416 (blue sticks) docked into the binding site of human telomeric G-quadruplex DNA (green sticks and cartoon), along with their 2D ligand interaction diagrams. Hydrogen bonding and π - π interaction are shown as purple and green dotted lines, respectively

Supplementary Files

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- [SCHEME1.png](#)