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Discovery of a Drug-Like G-Quadruplex Binding Ligand by High-Throughput Docking

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There has been considerable interest in the study of G-quadruplex DNA owing to its involvement in the regulation of telomerase activities.[1,2] Human telomeric DNA is composed of a repeating double-stranded [TTAGGG/CCCTAA]$_n$ sequence except in the 3′-terminal region, which consists of a single-stranded tandem [TTAGGG] repeat sequence over several hundred bases.[3–6] In normal somatic cells, approximately 100 bases are lost in each cell division, and after the telomeres have been shortened to a critical threshold, the cell undergoes apoptosis. In cancer cells, telomeric length is maintained by telomerase, and telomerase activity is expressed in $>90\%$ of tumor cell lines, but in relatively few normal cell types.[6] Therefore, the inhibition of telomerase activity by ligand-induced stabilization of G-quadruplexes has become an attractive strategy for developing new anticancer drugs.[1,2,7–10] Planar aromatic molecules with scaffolds that have extended delocalized π-electron systems such as cationic porphyrins,[1,2,7] BRACO-19,[2,9a] 9-anilinoproflavin,[2,7] triazines,[2] pentacyclic acridines,[2] and telomestatin[2,7,9b,10a] are known to bind to and stabilize G-quadruplex DNA, resulting in anti-telomerase activity. This gives rise to telomere shortening and suppression of cell growth, ultimately leading to cell death. Recently, we also demonstrated by molecular modeling studies that quindoline derivatives have the ability to stabilize the G-quadruplex structure in *c-myc*.[10f] However, most reported small-molecule G-quadruplex stabilizers have extended planar structures that result in poor bioavailability.

Virtual screening of chemical databases by molecular docking is one of the most powerful approaches to discover small-molecule inhibitors.[11] The major advantage of virtual screening of drug-like compounds is that chemical diversity is generated without the need for chemical synthesis; confirmed hits identified in a screen could be used to guide further synthesis and quantitative structure–activity relationship analysis. Abagyan and co-workers recently demonstrated the applicability of high-throughput virtual screening of a marketed drug database in the identification of anti-androgen scaffolds.[12] Inspired by this promising result, we extended the scope of identifying G-quadruplex DNA binding ligands through the virtual screening of a drug-like compound database. To develop a high-throughput screening platform for G-quadruplex DNA stabilizing ligands, a computer model was constructed by using the X-ray crystal structure of the intramolecular human telomeric G-quadruplex DNA (PDB code: 1KF1).[13a] It is common to use X-ray crystal structures for virtual screening of novel compounds from large databases because X-ray crystallography generally provides a larger amount of high-quality experimental data than NMR spectroscopy, and thus crystal structures are thought to provide a more accurate depiction. NMR structures are solved in a more biologically relevant environment; however, they provide a dynamic representation of the biomolecule when used as a collection. In the current study, the NMR structure of the intramolecular human telomeric G-quadruplex DNA in K^+ solution (PDB code: 2GKU)[13b] is different from the X-ray crystal structure; the DNA strands are oriented in a $(3+1)$ direction in the NMR structure,[13b,14] whereas the X-ray structure shows an all parallel direction, and as such, studies on the structure of the intramolecular human telomere quadruplex in physiological K^+ solution have raised extreme controversy. Tan and co-workers recently reported the intramolecular human telomere quadruplex to adopt a parallel-stranded conformation in the noncrystalline state in K^+ solution under molecular crowding conditions, as the K^+ crystal structure quadruplex does.[15] We report herein a new drug-like compound identified through in silico screening that is an effective stabilizer of G-quadruplex DNA. This compound also possesses high selectivity for G-quadruplex versus duplex DNA.

Over 100 000 compounds in a drug-like database that passed the Lipinski filters[16] were screened in silico. The continuously flexible ligands were docked to a grid representation of the receptor and assigned a score reflecting the quality of the complex according to the ICM method (Molsoft).[17] The best-scoring molecule in this new class of drug-like hits, 1*H*-pyrazole-3-carboxy-4-methyl-5-phenyl-(1*H*-indol-3-ylmethylene)hydrazide, was evaluated for its ability to stabilize G-quadruplex DNA (Figure 1). To the best of our knowledge, compound **1** has not yet been reported to stabilize G-quadruplex DNA.

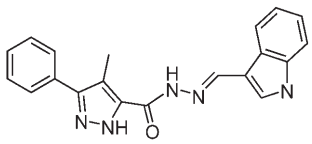

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Figure 1. Structure of 1*H*-pyrazole-3-carboxy-4-methyl-5-phenyl-(1*H*-indol-3-ylmethylene)hydrazide (**1**).

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The ability of compound **1** to stabilize G-quadruplex DNA was studied by using a high-throughput fluorescence resonance energy transfer (FRET) assay.[18] Table 1 shows the effect of various concentrations of compound **1** on the melting temperature (ΔT_m) of two labeled oligomers in potassium cacodylate buffer (60 mM, pH 7.4). F21T represents the human telomeric sequence (5′-*FAM*-d(GGG[TTAGGG]$_3$)-*TAMRA*-3′), whereas F10T (ds) is a labeled hairpin double-helix-forming oligomer (5′-*FAM*-d(TATAGCTATA)-HEG-d(TATAGCTATA)-*TAMRA*-3′) that has an internal hexa(ethylene glycol) (HEG) linker to form a hairpin loop. In the absence of **1**, the DNA melting temperature (T_m) of the F21T quadruplex is 50 °C. However, upon treatment of the F21T quadruplex with **1** at 2 μM, a significant increase in T_m (18 °C) was registered. Similarly, a known G-quadruplex-binding quindoline derivative also induced remarkable changes in T_m under the same conditions. In contrast, addition of the low-scoring molecule 2-[[(4-ethoxyphenyl)methylene]amino]-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carbonitrile to the F21T quadruplex does not increase T_m (Supporting Information). Notably, **1** does not elevate the T_m of the duplex-forming oligomer F10T (ds). The excellent selectivity may be due to the presence of the flexible side chain in **1**, which is able to discriminate between G-quadruplex DNA and duplex DNA.

Table 1. G-quadruplex stabilization temperature in the presence of compound **1**, determined by FRET.

Conditions	Oligonucleotide		
	F21T	F21T	F10T (ds)
Concentration of **1** [μM]:	1.0	2.0	1.0
ΔT_m [°C]:	13.5	17.9	0

To provide insight into the mode of binding, we used circular dichroism (CD) to elucidate the effects of **1** on the folded conformations of the human telomere sequence, hTelo [d(AG$_3$(T$_2$AG$_3$)$_3$)]. In the absence of **1**, the hTelo oligonucleotide (at 5 μM) in K$^+$-containing buffer is present as a mixture of parallel and antiparallel G-quadruplex conformations, which exhibit two absorption maxima at 262 and 292 nm as well as a minimum at 240 nm. When hTelo is folded in the presence of **1** (at 5 μM), a decrease in the antiparallel signal (292 nm) was observed simultaneously with a significant increase in the band at 262 nm, characteristic of a parallel conformation (Supporting Information). It was interesting to note that furan-based cyclic oligopeptides have been reported to induce a similar spectral change under comparable conditions, supporting our hypothesis that both **1** and furan-based cyclic oligopeptides recognize the G-quadruplex through a similar binding mode.[19]

To further evaluate the mode of binding, molecular modeling of the binding of **1** with the X-ray crystal structure (PDB code: 1KF1) of intramolecular G-quadruplex DNA was carried out. The results indicate that **1** strongly binds to the parallel intramolecular G-quadruplex with a binding energy of −38.46 kcal mol^{-1} (Figure 2) and is stacked on the ends of the G-quadruplex at the GT quadruplex terminus, close to the 3′-terminal face of the G-quadruplex. Moreover, the unfavorable binding energies of 28–31 kcal mol^{-1} suggest that the interactions between **1** and G-quadruplex DNA should not be intercalative in nature. The results are given in the Supporting Information.

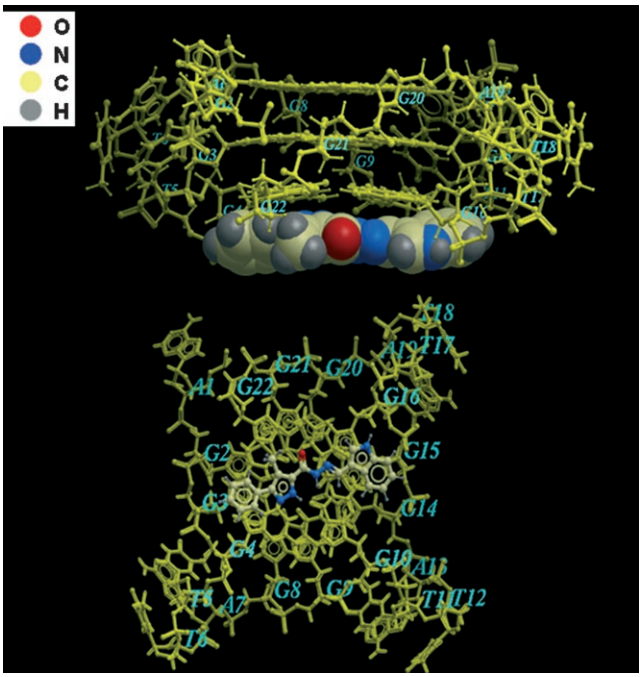

Figure 2. Molecular models showing the interaction of **1** with an intramolecular G-quadruplex.

In summary, by using high-throughput screening in silico we have successfully identified a new highly selective G-quadruplex binding ligand, namely 1*H*-pyrazole-3-carboxy-4-methyl-5-phenyl-(1*H*-indol-3-ylmethylene)hydrazide, from a database of drug-like compounds. Our research group is currently pursuing structural optimization of this class of ligands by in silico high-throughput docking in parallel with organic synthesis.

Experimental Section

Materials. DNA oligomers and their fluorescent conjugates were purchased from Tech Dragon Ltd.: F21T = 5′-*FAM*-d(GGG-[TTAGGG]$_3$)-*TAMRA*-3′, donor fluorophore *FAM* (6-carboxyfluorescein), acceptor fluorophore *TAMRA* (6-carboxytetramethylrhodamine); F10T (ds) = 5′-*FAM*-d(TATAGCTATA)-HEG-d(TATAGCTATA)-*TAMRA*-3′, HEG (hexathyleneglycol). The human telomere sequence was purchased from Genset Singapore Biotechnology Ltd.: hTelo = 5′-AGGG TTAGGG TTAGGG TTAGGG-3′. 1*H*-pyrazole-3-carboxy-4-methyl-5-phenyl-(1*H*-indol-3-ylmethylene)hydrazide and 2-[[(4-ethoxyphenyl)methylene]amino]-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carbonitrile were obtained from Otava chemicals and Interchim Intermediates, respectively. Stock solutions (10 mM) of compound for FRET assays was prepared in DMSO and diluted with deionized water. All stock solutions were kept at −20 °C in the dark between experiments.

Physical measurements. FRET measurements were performed using a BioRad iQ 5 multicolor real-time PCR detection system with excitation at 450–495 nm and detection at 515–545 nm. Fluorescence readings were taken at intervals of 0.5 °C over the range of

30–100 °C, with a constant temperature being maintained for 30 s prior to each reading to ensure a stable value. Circular dichroism spectra were recorded on a Jasco J-810 spectropolarimeter equipped with a Peltier temperature control device.

FRET measurements. DNA was dissolved as a 20 μM stock solution. All dilutions were carried out with potassium cacodylate buffer (50 mM, pH 7.4). The ability of the compounds to stabilize G-quadruplex DNA was investigated with a FRET assay modified for use in high-throughput screening in a 96-well plate format. The labeled oligonucleotide F21T used as the FRET probe was diluted from stock to 400 nM in the potassium cacodylate buffer mentioned above and then annealed by heating at 92 °C for 5 min, followed by slow cooling to room temperature in the heating block. Compound preparations were made from stock concentrations (described above) on the day of use. Final solutions were prepared using DMSO in the initial 1:10 dilution, after which potassium cacodylate buffer (50 mM, pH 7.4) was used in all subsequent steps. The 96-well plates (MJ Research, Waltham, MA, USA) were prepared by portioning 10 μL of the annealed DNA into each well, followed by 10 μL of the compound solutions. Final analysis of the data was carried out by using GraphPad software Prism 3.0. Emission of *FAM* was normalized between 0 and 1, and $T_{1/2}$ was defined as the temperature at which the normalized emission is 0.5. $\Delta T_{1/2}$ values are the mean of two to four experiments.

Circular dichroism. The oligonucleotide hTelo [$d(AG_3(T_2AG_3)_3)$], at a final concentration of 5 μM, was resuspended in a buffer containing Tris·HCl (50 mM, pH 7.4), KCl (100 mM), and compound **1** (5 μM). The sample was heated at 90 °C, then gradually cooled to room temperature, and was incubated at 4 °C for several hours. The CD spectra were recorded on a Jasco J-810 spectropolarimeter at 320–220 nm using 16 scans at 100 nm min^{-1}, a response time of 1 s, and a bandwidth of 1 nm. Cuvettes of 1 mm width with black quartz sides to mask the light beam were used for the measurements. A buffer baseline was collected in the same cuvette and subtracted from the sample spectra. The CD spectra were obtained by taking the average of at least three scans made from 220 to 320 nm. The final spectra were normalized to have zero ellipticity at 320 nm.

High-throughput docking.[17] A drug-like compound database containing 100 000 compounds from ZINC[20] that passed the Lipinski filters was screened in silico. High-throughput docking was performed using the ICM-Pro 3.4-8a program (Molsoft). All continuously flexible ligands were docked to a grid representation of the receptor (PDB code: 1KF1) and assigned a score reflecting the quality of the complex by the ICM method (Molsoft). The binding site was assigned to the whole intramolecular G-quadruplex DNA molecule in the docking procedure. According to the ICM method, the molecular system was described using internal coordinates as variables. Energy calculations were based on the ECEPP/3 force field with a distance-dependent dielectric constant. The biased probability Monte Carlo (BPMC) minimization procedure was used for global energy optimization. The BPMC global energy optimization method consists of the following steps: 1) a random conformational change of the free variables according to a predefined continuous probability distribution, 2) local energy minimization of analytical differentiable terms, 3) calculation of the complete energy including non-differentiable terms such us entropy and solvation energy, and 4) acceptance or rejection of the total energy based on the Metropolis criterion and return to step 1. In the flexible ligand and rigid receptor docking, the receptor was represented by six potential energy maps: electrostatic, hydrogen bond, hydrophobic, and three van der Waals. The flexible ligand in the receptor field was subjected to global optimization so that both the intramolecular ligand energy and the ligand–receptor interaction energy were optimized during the calculation. Each docked compound was assigned a score according to its fit in the receptor that also accounted for desolvation and hydrophobic effects and entropy loss, which occurred on docking. This methodology allows fast and accurate screening of hundreds of thousands of compounds (the average computing time is approximately 5 min per compound per processor). Each ligand from the drug-like compound database was docked three times, and a minimum of the three scores was used. As a reference, molecular docking of the well-known G-quadruplex binder telomestatin showed a score of −30. Thus, a permissive cutoff score was chosen as −28 to take into account limitations stemming from the rigid representation of the receptor and inaccuracies in the scoring function. Ten compounds scoring better than −28 were recorded in the hit list table (Supporting Information).

Molecular modeling. A computer model to study the stacking of **1** and G-quadruplex DNA was performed. Molecular modeling was performed using the ICM-Pro 3.4-8a program (Molsoft). The X-ray crystal structure of the intramolecular G-quadruplex DNA was obtained from the Protein Data Bank (PDB code: 1KF1) and used as the initial model to perform molecular modeling. Hydrogen and missing heavy atoms were added to the receptor structure followed by local minimization using the conjugate gradient algorithm and analytical derivatives in the internal coordinates space. Complex **1** was inserted in the four different positions of the G-tetrad, which correspond to two end-stacking sites and two intercalation sites, and the complex was allowed to minimize. Conformations were sampled according to a Metropolis criterion with $T=$ 600 K followed by up to 2000 steps of conjugate gradient minimization after each stochastic move. The binding energy calculation implemented in ICM included an electrostatic term for Coulombic interactions and partial charge desolvation, a hydrophobic term, and an entropy term for loss of torsional entropy upon binding. A constant term for loss of translational/rotational entropy and change in entropy from variations in the concentration of free molecules was also included. The molecular modeling was performed to find the most favorable orientation. The resulting **1**–G-quadruplex DNA complex trajectories were energy minimized, and the interaction energies were computed.

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Keywords: DNA · G-quadruplexes · high-throughput docking · ligands · selectivity

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