ACS Medicinal Chemistry Letters

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Targeting the *c-Kit* Promoter G-quadruplexes with 6-Substituted Indenoisoquinolines

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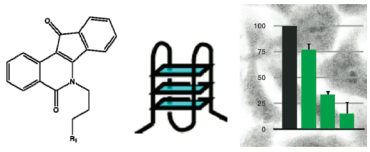
ABSTRACT Herein, we demonstrate the design, synthesis, biophysical properties, and preliminary biological evaluation of 6-substituted indenoisoquinolines as a new class of G-quadruplex stabilizing small molecule ligands. We have synthesized 6-substituted indenoisoquinolines **1a**−**e** in two steps from commercially available starting materials with excellent yields. The G-quadruplex stabilization potential of indenoisoquinolines **1a**−**e** was evaluated by fluorescence resonance energy transfer-melting analysis, which showed that indenoisoquinolines show a high level of stabilization of various G-quadruplex DNA structures. Indenoisoquinolines demonstrated potent inhibition of cell growth in the GIST882 patient-derived gastrointestinal stromal tumor cell line, accompanied by inhibition of both *c-Kit* transcription and KIT oncoprotein levels.

KEYWORDS DNA, quadruplex, c-kit regulation, inhibition, ligand



G-quadruplexes constitute a structural form of DNA that is distinct from the double helix.[1] They are formed from particular guanine-rich nucleic acid sequences in which guanines form planar quartets stabilized using the Watson−Crick and Hoogsteen edges to form hydrogen bonds. Their occurrence has been demonstrated within ciliate telomeres.[2,3] In addition, G-quadruplex sequence motifs ($G_{3+}N_{1-7}G_{3+}N_{1-7}G_{3+}N_{1-7}G_{3+}$) are found at many positions within genomes,[4,5] with a notable increase in density in close vicinity to transcriptional start sites, highly suggestive of a biological function in gene regulation.[5,6] NMR spectroscopy and X-ray crystallographical studies have shown that these guanine-rich sequences can fold into G-quadruplex structures in vitro.[7,8] Their unique structural features and possible biological functions make them attractive targets for drug design.[9,10] Genomic locations that are known to possess quadruplex structures include the immunoglobulin switch region and the promoters of numerous proto-oncogenes, such as *c-MYC*,[11] VEGF,[12] BCL-2,[13] and K-RAS.[14] Two quadruplex-forming motifs have been identified in the *c-Kit* promoter (*c-Kit* 1[15,16] and *c-Kit* 2[17,18]). Here, we present a new class of quadruplex stabilizing compounds that have been used to explore relationships between quadruplexes and their biological functions.

c-Kit is a proto-oncogene that codes for a 145−160 kDa protein belonging to the receptor tyrosine kinase (RTK) family. The KIT protein regulates signal transduction cascades that control cell growth and differentiation.[19] *c-Kit* expression has been shown to control the growth of various cell lines, including gastrointestinal stromal tumors (GIST), hematopoietic cells, small cell lung cancer, and colorectal cancer.[20−23] Gain-of-function mutations in the KIT protein are found in various highly malignant human cancers, especially GIST. Gleevec (imatinib mesylate), a small molecule antagonist originally developed to treat chronic myelogenous leukemia (CML), acts by maintaining the bcr-abl kinase in an inactive conformation. Gleevec is also the mainstay of target therapy in GIST where it targets the KIT kinase,[24] whose oncogenic disregulation is a major factor in the development of this disease. A small molecule that could reduce *c-Kit* expression (i.e., reduce transcription) might be an effective way to treat GIST, with the potential to inhibit KIT-dependent GIST cells with secondary mutations in the KIT protein that confer acquired clinical resistance to Gleevec and other kinase inhibitors.

A large number of G-quadruplex binding and/or stabilizing ligands have been developed, and most have structural features that include a planar aromatic or heteroaromatic surface capable of stacking on the G-tetrads and positively charged substituents to enhance interaction with loops, grooves, and the negatively charged sugar−phosphate backbone of the G-quadruplex DNA.[25] A recent report suggests that monosubstituted quindoline derivatives show high

Received Date: March 31, 2010
Accepted Date: June 24, 2010
Published on Web Date: July 01, 2010

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DOI: 10.1021/ml100062z | *ACS Med. Chem. Lett.* **2010**, *1*, 306–310

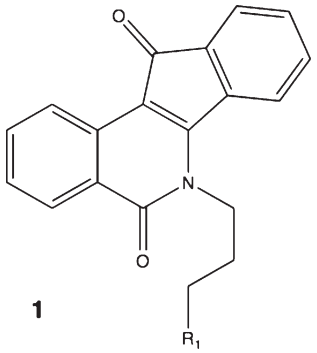


Figure 1. Core structure of indenoisoquinoline (**1**).

stabilization of the *c-MYC* promoter G-quadruplex DNA and down-regulate *c-MYC* gene expression in HepG2 cells at concentrations over 50 μM.[26] We designed a tetracyclic indenoisoquinoline with just a single side chain to target G-quadruplex DNA (**1** in Figure 1). Indenoisoquinoline derivatives have previously shown antiproliferative properties in various human cancer cell lines and have been considered to be topoisomerase 1 inhibitors.[27−29]

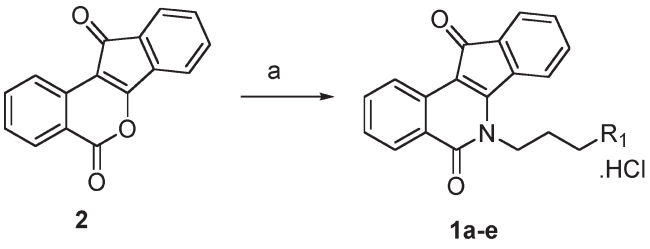
Herein, we present the design, synthesis, and biophysical and primary biological evaluation of 6-substituted indenoisoquinolines as a new class of G-quadruplex stabilizing ligands. We have synthesized indenoisoquinolines **1a**−**e** from commercially available indeno[1,2-*c*]isochromene-5,11-dione (**2**) with excellent yields (Scheme 1) (generally > 90%, see the Supporting Information for details).

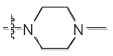
The G-quadruplex stabilization potential of indenoisoquinolines **1a**−**e** was evaluated by a fluorescence resonance energy transfer (FRET)-melting assay, which measures the shift in melting temperature (ΔT_m) of the folded G-quadruplex structures as a function of ligand concentration.[30−32] We have included three native G-quadruplex-forming sequences in this study: the human telomeric DNA quadruplex sequence H-telo 5′-GGG(TTAGGG)$_3$-3′, the two *c-Kit* promoter G-quadruplex sequences of *c-Kit* 2[17] 5′-GGG CGG GCG CGA GGG AGG GG-3′ and *c-Kit* 1[15] 5′-GGG AGG GCG CTG GGA GGA GGG-3′, all of which are dual labeled (5′-FAM and 3′-TAMRA). We also included a dual-labeled duplex DNA as a control (see the Experimental Procedures).

The FRET-melting results revealed that the ligands showed a T_m shift ranging from 3.9 to 25.9 K, at 1 μM ligand for the DNA quadruplexes evaluated. A low level stabilization of control duplex DNA was also observed (Table 1 and Supporting Information). Ligand **1e** showed a different preference for quadruplexes as compared to ligands **1a**−**d**. The weaker G-quadruplex stabilization effect of ligands **1e** and **1d** may be a reflection of the lower pK_a of the protonatable nitrogen that we hypothesize is involved in DNA recognition and mirrors the trend we have seen for other quadruplex ligands.[33,34] We observed some stabilization of ds-DNA, but the ligand concentrations needed to achieve high stabilization of ds-DNA are much higher as compared to the quadruplex targets as shown in Table 1.

A number of indenoisoquinoline derivatives showed high stabilization of the *c-Kit* promoter G-quadruplexes, in particular *c-Kit* 1. We then evaluated the ability of indenoisoquinolines **1a**−**e** to inhibit the growth of the cancer cell lines GIST882 and HT-29, using a 96 h sulforhodamine B (SRB) assay. GIST882 is a primary GIST cancer cell line that was established from a newly diagnosed patient with untreated GIST and contains a homozygous constitutively activating (ligand-independent) mutation, K642E, in the ATP binding pocket region of the KIT protein.[24] GIST882 proliferation is known to be driven by expression of oncogenic *c-Kit*. HT-29 is

Scheme 1. Synthesis of Indenoisoquinolines **1a**−**e**[a]



Ligand	R_1
1a	
1b	
1c	
1d	
1e	

[a]Conditions: (a) (1) R_1R_1N $(CH_2)_3NH_2$, $CHCl_3$, room temperature, 48 h. (2) Ethanolic HCl, room temperature, 1 h.

Table 1. G-Quadruplex Stabilization (ΔT_m) Potential of Indenoisoquinolines **1a**−**e** by FRET-Melting Assay[a]

	concentrations of ligands in μM at half maximal ΔT_m (with ΔT_m at 1 μM ligand in K in parenthesis)			
ligand	G4 DNA of c-KIT 1	G4 DNA of c-KIT 2	G4 DNA of htelo	ds-DNA
1a	0.93 (23.7)	0.72 (14.4)	1.63 (14.4)	9.00 (4.0)
1b	0.95 (20.1)	0.69 (13.0)	1.60 (13.0)	> 10.00 (3.9)
1c	0.59 (25.9)	0.84 (12.2)	0.98 (17.6)	> 10.00 (1.7)
1d	8.90 (8.7)	4.40 (3.9)	> 10.00 (4.8)	> 10.00 (0.6)
1e	> 10.00 (11.7)	0.62 (13.2)	> 10.00 (6.3)	> 10.00 (0.0)

[a]FRET-melting measurements were performed in triplicate with excitation at 483 nm and detection at 533 nm; $\Delta T_m \pm 1.0$ K. The T_m of the quadruplexes in 60 mM K^+ in the absence of ligand are 54.4 °C (c-KIT 1), 72.7C (c-KIT 2), 59.4 (htelo), and 62.7C (ds-DNA).[35] The concentration of DNA was 200 nM (full curves giving ΔT_m values at all ligand concentrations are shown in Supporting Information).

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DOI: 10.1021/ml100062z | *ACS Med. Chem. Lett.* **2010**, *1*, 306–310

Table 2. IC_{50} of Growth Inhibition (in μM) by Indenoisoquinolines **1a**−**e** in *c-kit* Expressing Cancer Cell Lines GIST882 and HT-29 Determined by the SRB Assay[a]

	IC_{50} (μM)	
ligand	GIST882	HT-29
1a	5.0 (±0.52)	0.4 (±0.08)
1b	4.1 (±0.43)	0.3 (±0.034)
1c	13.9 (±1.61)	0.7 (±0.11)
1d	> 50.0	> 50.0
1e	23.0 (±1.28)	2.2 (±0.47)
Gleevec (imatinib)	1.7 (±0.32)	ND[b]

[a] SRB assays were performed in quadruplicate, and the IC_{50} values given are averages of four experiments; the standard deviation is noted in parentheses. [b] Not determined.

a colorectal cancer cell line, which is known to express both c-Kit and its ligand SCF (stem cell factor).[23] Down-regulation of Kit expression by the addition of TGFB was demonstrated to significantly reduce proliferation in vitro, indicating *c-Kit*-dependent proliferation in this cell line. This cell line has also previously been utilized for studies on Kit inhibitors.[20]

IC_{50} values for growth inhibition ranged from 2.7 to 37.2 μM (Table 2). Ligand **1b** showed comparable growth inhibition (IC_{50}) to Gleevec in the GIST882 cell line (Table 2). The IC_{50} values qualitatively correlated with the FRET-melting data of ligands **1a**−**e** (Tables 1 and 2), with the compounds that show high stabilization temperatures exhibiting low IC_{50} values, suggesting a link between the ability to stabilize a G-quadruplex and the growth inhibition properties of the ligands.

A central question regarding G-quadruplex DNA is in which way these structures are part of the regulatory machinery affecting gene expression. A well-studied example is *c-MYC*, in which the alterations in the G-quadruplex structure have been shown to change expression of the gene.[11,36] To determine the effect of **1b** on the expression of *c-Kit*, GIST882 cell mRNA was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). GIST882 cells were treated with varying concentrations of **1b** for 24 h, and the level of *c-Kit* mRNA was measured relative to the expression of two housekeeping genes: β-actin (ACTB) and YWHAZ (see the Experimental Procedures). Figure 2 shows that treatment of GIST882 with **1b** leads to a significant reduction in KIT mRNA in a dose-dependent manner. At 4 μM, levels of KIT mRNA are reduced to 33% of the control, and expression is reduced even further to 11% of the control at 20 μM.

To confirm that the observed reduction in *c-Kit* mRNA levels lead to a reduction of KIT protein, we employed immunoblotting using anti-KIT antibodies. These experiments showed that treatment of GIST882 cells with **1b** resulted in dose-dependent reduction in the level of KIT protein, with virtually undetectable levels of KIT present after 24 h treatment with 20 μM **1b** (At concentrations of ligand of 20 μM and above, the first signs of toxicity of the compounds could be detected. At 20 μM, this was manifest as slightly lower recovery yields of total protein.) (Figure 3).

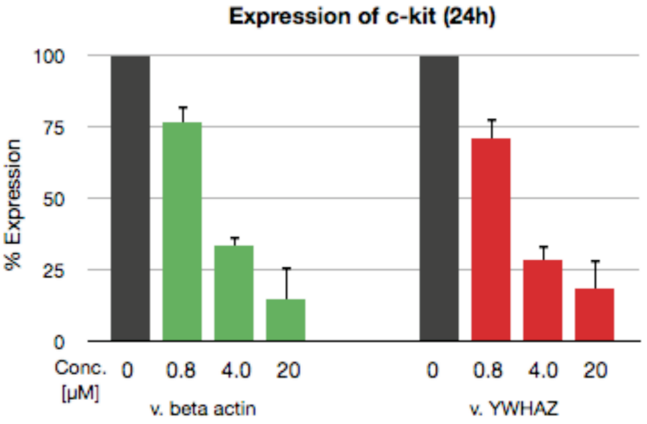

Figure 2. qRT-PCR analysis of GIST882 cells following treatment for 24 h with ligand **1b** at 0, 0.8, 4, and 20 μM. *c-Kit* mRNA was determined by normalizing mRNA expression vs β-actin or YWHAZ. Values are shown as a % of control.

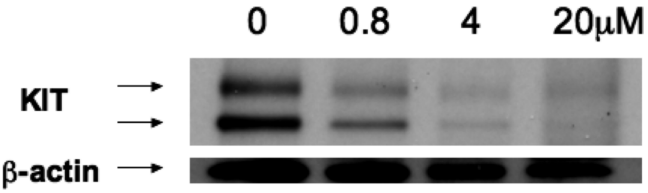

Figure 3. Immunoblot of total KIT protein (showing the glycosylated mature and the unglycosylated immature isoforms) in GIST882 cells following treatment for 24 h with DMSO only (0) and 0.8, 4, and 20 μM **1b**. β-Actin was used as a loading control.

In summary, we have shown that 6-substituted indenoisoquinolines, a new class of G-quadruplex stabilizing ligands, have antiproliferative activity in human cancer cells. In particular, some members of this ligand family showed a high level of stabilization of G-quadruplexes derived from the promotor sequence of *c-Kit*. One such ligand was shown to inhibit the expression of *c-Kit* mRNA and showed a concomitant reduction in KIT protein in the GIST cancer cell line GIST882. This study provides a further example of a quadruplex targeting ligand that can reduce the expression of oncogenes and supports the view that DNA quadruplexes may be attractive targets for small molecule therapeutics.

EXPERIMENTAL PROCEDURES Oligonucleotides were initially dissolved as a 100 μM stock solution in Milli-Q water; further dilutions were carried out in 60 mM potassium cacodylate buffer, pH 7.4, and FRET experiments were carried out with a 200 nM oligonucleotide solution. Oligonucleotides used in these experiments were dual fluorescently labeled. The sequences were as follows: c-kit1 (5′-FAM-GGG AGG GCG CTG GGA GGA GGG-TAMRA-3′); c-kit2 (5′-FAM-GGG CGG GCG CGA GGG AGG GG-TAMRA-3′); h-Telo (5′-FAM-GGG TTA GGG TTA GGG TTA GGG-TAMRA-3′); and ds-DNA (5′-FAM-TAT AGC TAT A-HEG-TAT AGC TAT A-TAMRA-3′), a dual-labeled 20-mer oligonucleotide comprising a self-complementary sequence with a central polyethylene glycol linker able to fold into a hairpin. Dual-labeled DNA was annealed at a concentration of 400 nM by heating at 94 °C for 10 min followed by cooling to room temperature at a rate of 0.1 °C/min. Ninety-six-well plates were prepared by addition of 50 μL of the annealed DNA solution to each

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DOI: 10.1021/ml100062z | *ACS Med. Chem. Lett.* **2010**, *1*, 306–310

well, followed by 50 μL of a solution of the respective molecule at an appropriate concentration. Measurements were made in triplicate with an excitation wavelength of 483 nm and a detection wavelength of 533 nm using a LightCycler 480 System RT-PCR machine (Roche). Final analysis of the data was carried out using OriginPro 7.5 data analysis and graphing software (OriginLab).

GIST-882 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Human colorectal adenocarcinoma cells (HT-29) were maintained in DMEM and MEM supplemented with 10% fetal bovine serum, respectively. Cells were routinely passaged at 70−80% confluence and incubated at 37 °C and 5% CO_2.

Short-term growth inhibition was measured using the SRB assay as described previously.[37] Briefly, cells were seeded at optimal seeding densities into the wells of 96-well plates in appropriate medium and incubated overnight at 37 °C, 5% CO_2, to allow the cells to attach. Subsequently, cells were exposed to freshly made solutions of various ligands at increasing concentrations in the range of 0.1−50 μM in quadruplicate and incubated for a further 96 h. Following this, the cells were fixed with ice cold trichloroacetic acid (TCA) (10%, w/v) for 30 min and stained with 0.4% SRB dissolved in 1% acetic acid for 15 min. All incubations were carried out at room temperature. The IC_{50} value (concentration required to inhibit cell growth by 50%) was determined from the mean absorbance at 540 nm for each drug concentration and expressed as a percentage of the control untreated well absorbance.

For gene expression studies, 1×10^6 cells were seeded in T25 cm^2 flasks and treated with ligand **1b** at 0.8, 4, and 20 μM (IC_{50} value and concentrations five times higher and lower) or DMSO control for 24 h. Cells were harvested with trypsin and centrifuged at 8000*g* for 3 min. Pellets were stored at −80 °C prior to total RNA extraction. RNA was extracted using the Qiagen RNeasy kit according to the manufacturer's instructions. The quantity of the RNA was measured by UV spectrometry. Synthesis of cDNA was performed using 1 μg of total RNA and SuperScript III reverse transcriptase (Invitrogen), with 1 h of elongation time at 55 °C and oligo dT primers. The cDNA was quantified on a Roche Light Cycler 480 real-time PCR machine, using the SYBR Green I Master kit (Roche). The sequences of the *c-Kit* primers used were as follows: 5′-CGTGGAAAAGAGAAAACAGTCA-3′ and 5′-CACCGTGATGCCAGCTATTA-3′.

Housekeeping genes were measured using prevalidated primers for ACTB and YWHAZ, provided by PrimerDesign Ltd. (United Kigndom). The annealing temperature was 64 °C for all primers. The presence of a single amplicon in the RT-PCR reaction was confirmed by melting curve analysis carried out at the end of the amplification cycles. Two independent experiments were carried out, and each of these was performed in triplicate.

For c-kit protein expression analysis, cells were seeded at a density of 1×10^6 T25 cm^2 flasks and incubated with ligand **1b** at 0.8, 4, and 20 μM (IC_{50} value and concentrations five times higher and lower) or DMSO control for 24 h. At the end of treatment, cells were washed with PBS and scraped off from the flasks with a cell scraper. Samples were pelleted and stored at −80 °C for protein extraction. Cells were lysed using RIPA buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, and 1 mM PMSF and supplemented with complete mini-protease inhibitor mix (Roche). Cell lysates were incubated at 4 °C for 1 h and clarified by centrifugation at 13000 rpm for 10 min at 4 °C. The protein concentration was determined by use of the BCA assay (Pierce). Cell lysates containing 10−20 μg of protein were resolved by 8−16% gradient sodium dodecyl sulfate−polyacrylamide gel electrophoresis gel, transferred to a nitrocellulose membrane (Invitrogen), and probed with antibodies for KIT (Dako Cytomation) and β-actin (Cell Signaling technology). Detection was performed with enhanced luminescence reagents (Cell Signaling technology).

SUPPORTING INFORMATION AVAILABLE

General methods and detailed procedures for the synthesis of indenoisoquinolines **1a−e** and FRET melting profiles. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding Sources: We thank Cancer Research UK for program funding to S.B. and S.N., the BBSRC for project funding to S.B., and the National Institutes of Health (Grant 1P50CA127003-03) and grant GI SPORE IP50CA127003-04 for support to J.A.F.

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DOI: 10.1021/ml100062z | *ACS Med. Chem. Lett.* **2010,** *1*, 306–310

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DOI: 10.1021/ml100062z | *ACS Med. Chem. Lett.* **2010,** *1*, 306–310