ACS Medicinal Chemistry Letters

Letter

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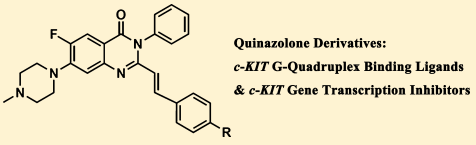
Synthesis and Evaluation of Quinazolone Derivatives as a New Class of *c-KIT* G-Quadruplex Binding Ligands

Xiaoxiao Wang,[†] Chen-Xi Zhou,[†] Jin-Wu Yan, Jin-Qiang Hou, Shuo-Bin Chen, Tian-Miao Ou, Lian-Quan Gu, Zhi-Shu Huang, and Jia-Heng Tan*

School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510006, China

Supporting Information

ABSTRACT: The *c-KIT* G-quadruplex structures are a novel class of attractive targets for the treatment of gastrointestinal stromal tumor (GIST). Herein, a series of new quinazolone derivatives with the expansion of unfused aromatic ring system were designed and synthesized. Subsequent biophysical studies demonstrated that the derivatives with adaptive scaffold could effectively bind to and stabilize *c-KIT* G-quadruplexes with good selectivity against duplex DNA. More importantly, these ligands further inhibited the transcription and expression of *c-KIT* gene and exhibited significant cytotoxicity on the GIST cell line HGC-27. Overall, these quinazolone derivatives represent a new class of promising *c-KIT* G-quadruplex ligands. The experimental results have also reinforced the idea of inhibition of *c-KIT* expression through targeting *c-KIT* G-quadruplex DNA.



KEYWORDS: *c-KIT G-quadruplex, quinazolone derivatives, adaptive scaffold, biophysical study, cellular study*

In the presence of certain cations, guanine-rich single strands can form higher-order structures defined as G-quadruplexes. The primary building block of these structures is the π-stacked G-quartet, in which four guanine bases are connected by a network of eight Hoogsteen hydrogen bonds.[1] G-quadruplex motifs are widely dispersed in the eukaryotic genomes and concentrate in close vicinity to transcriptional start sites.[2,3] A series of G-quadruplex motifs in gene promoter regions have been investigated, including *c-MYC*,[4] *BCL-2*,[5] *VEGF*,[6] *KRAS*, and two motifs in the *c-KIT* promoter (*c-KIT1* and *c-KIT2*).[7−9] The unique G-quadruplex structures of these promoters and their potential biological functions in gene regulation make them attractive targets for drug design.[3]

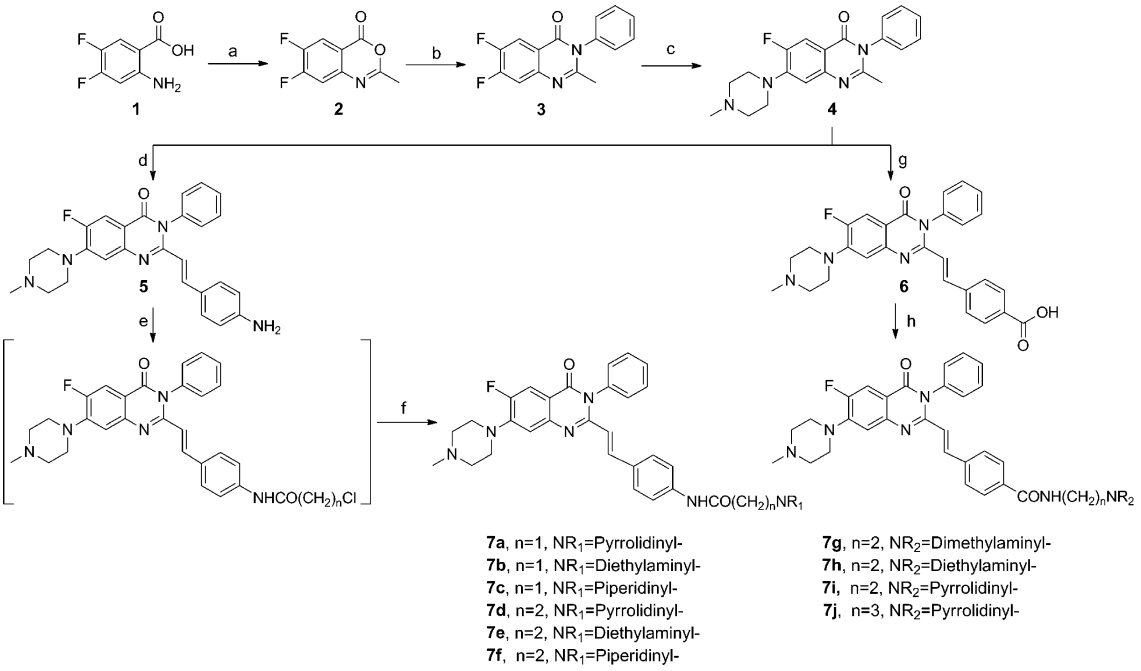
The proto-oncogene *c-KIT*, which encodes a membrane-bound glycoprotein of the family of growth factor receptors with tyrosine kinase activity, constitutes a cell signaling system that can stimulate cell proliferation, differentiation, migration, and survival.[10] The excessive activation of *c-KIT* is considered to be the primary pathogenic event in gastrointestinal stromal tumor (GIST) and the c-KIT protein has become a major molecular target for GIST therapy.[11] As we know, the kinase inhibitor Gleevec (imatinib mesylate) has been approved by the Food and Drug Administration (FDA) for use of the treatment of GIST.[12] However, new patterns of drug resistance owing to mutations in c-KIT protein have reduced its efficacy.[13] Recently it has been shown that treatment of cells with *c-KIT* G-quadruplex binding ligands can reduce their *c-KIT* expression levels thus exhibiting an alternative pathway for the design of small molecules inhibiting GIST cell growth.[14−16] Therefore, development of small molecules as c-*KIT* G-quadruplex ligands has been considered a promising strategy to overcome the c-KIT protein mutation related resistance. Inspiringly, the structure of *c-KIT* G-quadruplex DNA has been determined by the NMR and crystallographic studies.[17,18] Such well-resolved structure exhibits unprecedented conformation as compared with other G-quadruplex DNA and provides an important platform for further structure-based drug design.

Several small molecules including trisubstituted isoalloxazines,[19] naphthalene diimide derivatives,[14] substituted indenoisoquinolines, and benzo[a]phenoxazines have been identified to be *c-KIT* G-quadruplex binding ligands and subsequently inhibit *c-KIT* transcription and expression levels in cellular studies.[15,16] These rigid ligands comprise a planar, aromatic core with the capability of stacking on the G-quartet. Some flexible ligands with unfused aromatic scaffolds, such as bisindole carboxamides and diarylethynyl amides,[20,21] are also found to bind to the *c-KIT* G-quadruplex DNA in vitro. However, the inhibitory effects of unfused aromatic ligands on the *c-KIT* transcription and expression levels have not been explored. Whether they could bind to the G-quadruplex in *c-KIT* oncogene at the transcription level still needs to be clarified. Moreover, further efforts in appropriate drug design are also needed to control the *c-KIT* G-quadruplex selectivity over duplex DNA because ligand interaction with duplex DNA leads to acute toxic and intolerable side effects on normal tissues. Unlike rigid aromatic compounds, unfused aromatic molecules with adaptive structural feature could prevent themselves from intercalating into the duplex DNAs. This may be more promising to achieve the desirable selectivity.[21,22] To further explore these stories and to discover drug-like lead compounds for GIST therapy, we designed a new class of

Received: January 22, 2013
Accepted: August 13, 2013
Published: August 13, 2013

   dx.doi.org/10.1021/ml400271y | *ACS Med. Chem. Lett.* 2013, 4, 909−914

Scheme 1. Synthesis of Quinazolone Derivatives[a]



[a]Reagents and conditions: (a) Ac_2O (reflux); (b) aniline (room temperature); (c) *N*-methylpiperazine, DMF (reflux); (d) 4-nitrobenzaldehyde, glacial acetic acid, NaOAc (reflux); $Na_2S{\cdot}9H_2O$, NaOH, EtOH/H_2O (reflux); (e) 2-chloracetyl chloride/3-chloropropionyl chloride (reflux); (f) R_1NH, NaI, EtOH (reflux); (g) 4-carboxylbenzaldehyde, glacial acetic acid, NaOAc (reflux); (h) R_2NH, BOP, DMF (reflux).

unfused aromatic molecules targeting *c-KIT* G-quadruplex DNA.

Quinazolone and their derivatives are building blocks for approximately 150 naturally alkaloids and have been reported to have wide-ranging biological activities including antibacterial, antidiabetic, anti-inflamatory, antitumor, and several other useful properties.[23−25] Several quinazolone derivatives have been determined to selectively bind and stabilize the G-quadruplex DNA.[25,26] On the basis of this pharmacophore, we designed and synthesized a new class of quinazolone derivatives (**7a**−**7j**) targeting c-*KIT* G-quadruplex DNA. These small molecules contain an expanded aromatic system via the introduction of a benzene ring and a benzylidene group into the quinazolone moiety and two cationic amino side chains (Scheme 1). The unfused aromatic scaffold not only maximizes the stacking interaction of the derivatives with G-quartet but also incorporates features of flexibility that prevent from intercalating into the duplex DNAs. The two cationic amino side chains were attached to the scaffold for improving the G-quadruplex DNA binding potency and selectivity of the derivatives as well as the solubility in aqueous medium. Furthermore, we also interconverted the amido bond of the derivatives to uncover its influence on the *c-KIT* G-quadruplex recognition.

The facile and efficient synthetic route for quinazolone derivatives (QDs) is shown in Scheme 1. Treatment of 2-amino-4,5-difluorobenzoic acid (**1**) in acetic anhydride produced compound **2**, which was stirred at room temperature in aniline to give compound **3**. Regioselective nucleophilic substitution of the fluorine atom of **3** with *N*-methylpiperazine gave compound **4**. The structure of **4** was further confirmed by HMBC and NOESY experiments (Supporting Information). Claisen−Schmidt condensation between **4** and 4-nitrobenzaldehyde yielded the nitro intermediate product. Without further purification, the product was reduced to the corresponding amino **5**, acylation of which was then performed through reaction with the required acid chloride. The target compounds **7a**−**7f** were prepared by substitution of **5** with appropriate secondary amines. However, Claisen−Schmidt condensation between **4** and 4-carboxylbenzaldehyde yielded the compound **6**. The condensation of **6** with various linear aliphatic amines was catalyzed by BOP in DMF to give compounds **7g**−**7j**. The coupling constant across the double bond is ∼16 Hz, suggesting these final compounds adopted *E* configuration (Supporting Information).

To evaluate the stabilizing ability and selectivity of QDs for *c-KIT* G-quadruplex DNA, FRET-melting experiments were carried out. Two dual-labeled *c-KIT* G-quadruplex forming sequences, F-*c-KIT*1-T (5′-*FAM*-d[$AG_3AG_3CGCTG_3AGAG_3$]-*TAMRA*-3′) and F-*c-KIT*2-T (5′-*FAM*-d[$G_3CG_3CGCGAG_3AG_4$]-*TAMRA*-3′), and a self-complementary duplex control F10T (5′-*FAM*-d[TATAGCTATA-HEG-TATAGCTATA]-*TAMRA*-3′) were employed in the experiments. The melting temperatures (ΔT_m) of all tested strands were determined and are listed in Table 1.

As shown in Table 1, the derivatives generally exhibited stabilization effects on the *c-KIT*1 and *c-KIT*2 G-quadruplexes by the enhanced melting temperatures. Compounds **7a**-**7c** with short side chains showed relatively modest quadruplex stabilizing ability (4.2−9.4 °C), while **7d**-**7f** with longer chains apparently increased the melting temperatures (10.7−13.6 °C). This suggested that chain length could be an important factor for G-quadruplex stabilization. On the other hand, it has been shown that the amido bond would enlarge ligand's aromatic plane and/or participate in water-relayed hydrogen bonds with G-quadruplex.[27] This may be important for G-quadruplex stabilization and selectivity. In this study, we interconverted the position of in-chain amido bond to study its influence on G-

dx.doi.org/10.1021/ml400271y | *ACS Med. Chem. Lett.* 2013, 4, 909−914

Table 1. Stabilization Temperatures (ΔT_m) Determined by FRET-Melting Experiment and Equilibrium Dissociation Constant (K_D) Determined by SPR Assay

	FRET ΔT_m (°C)[a]			SPR K_D (M)	
	F-*c-KIT*1-T	F-*c-KIT*2-T	F10T	*c-KIT*1	duplex
7a	6.4 ± 0.4	9.4 ± 0.2	0 ± 0.4	7.84×10^{-7}	*c*
7b	5.5 ± 0.4	7.8 ± 0.2	0 ± 0.4	*b*	*b*
7c	4.2 ± 0.4	7.5 ± 0.2	0.1 ± 0.4	*b*	*b*
7d	11.3 ± 0.3	12.4 ± 0.1	0 ± 0.4	1.88×10^{-6}	*c*
7e	11.1 ± 0.3	13.6 ± 0.1	0 ± 0.5	6.12×10^{-7}	*c*
7f	10.7 ± 0.3	12.6 ± 0.1	0 ± 0.4	9.16×10^{-7}	*c*
7g	15.1 ± 0.3	15.9 ± 0.1	0.5 ± 0.4	1.16×10^{-6}	*c*
7h	13.4 ± 0.3	14.3 ± 0.1	0.4 ± 0.4	1.37×10^{-6}	*c*
7i	12.3 ± 0.3	13.4 ± 0.1	0.4 ± 0.4	15.7×10^{-6}	*c*
7j	15.9 ± 0.3	16.8 ± 0.1	0.4 ± 0.4	1.99×10^{-6}	*c*

[a] $\Delta T_m = T_m$(DNA + ligand) − T_m(DNA). The concentrations of F-*c-KIT*1-T, F-*c-KIT*2-T, and F10T were all 1 μM. The concentrations of the compounds were 5 μM. In the absence of ligand, T_m values of annealed F-*c-KIT*1-T, F-*c-KIT*2-T, and F10T are 56.4, 63.4, and 61.9 °C, respectively. [b] Not determined. [c] No significant binding was found for the addition of up to 20 μM ligand, which might indicate no specific interactions between the ligand and the DNA.

quadruplex stabilization and selectivity. Comparing the melting temperatures of **7g**-**7j** with those of **7d**-**7f**, the compounds with QD-CO-NH- arrangement had stronger quadruplex stabilization by 2−4 °C. Besides, as expected, all the derivatives have excellent selectivity between G-quadruplex and duplex DNA. None of them were found to significantly increase the melting temperature of FRET-tagged duplex DNA F10T. This suggested their poor ability of binding to duplex DNA.

To evaluate the kinetic constants of QDs for *c-KIT* G-quadruplex DNA, surface plasmon resonance (SPR) experiments were carried out. The *c-KIT*1 G-quadruplex DNA and duplex DNA were attached to a streptavidin-coated sensor chip. K_D values were calculated by global fitting of the kinetic data from various concentrations of the derivatives using 1:1 Langmuir binding. As shown in Table 1, K_D values of QDs for *c-KIT*1 G-quadruplex DNA were mostly less than 2 μM, while no obvious binding of the derivatives to duplex DNA was observed. This was consistent with the FRET-melting study. The K_D values of **7b** and **7c** were not determined because they were precipitated after dilution to the working concentration for SPR experiments. The binding of **7a**, **7e** and **7f** to *c-KIT*1 G-quadruplex DNA was slightly stronger (lower K_D values) than that of **7g**-**7j**. For example, compound **7e** (K_D = 0.612 μM) showed a clear preference to bind *c-KIT*1 G-quadruplex compared with **7h** (K_D = 1.37 μM). This indicated that the QD-NH-CO- arrangement performed better in binding interaction than the QD-CO-NH- arrangement. Also, compound **7a** with a short side chain has better binding affinity (K_D = 0.784 μM) than compound **7d** (K_D = 1.88 μM). These data showed some discrepancy with the results from FRET-melting assay. It may be because the affinity indicated by equilibrium binding in SPR studies and stability from T_m measurements in FRET-melting cannot be simply compared.[28]

Furthermore, circular dichroism (CD) spectroscopy was applied to investigate the binding properties of QDs to *c-KIT*1 and *c-KIT*2 G-quadruplex DNA. Compound **7e** with greatest binding affinity and competitive ΔT_m was chosen in this study. To explore the influence of amido bonds on c-*KIT* G-quadruplex recognition, **7h** with an interconverted amido bond was also selected. In the presence of 100 mM K^+, the CD spectrum of *c-KIT*1 without ligands showed a major positive band at 263 nm, a shoulder at about 293 nm, and a negative band near 240 nm. As shown in Figure 1A, upon the addition of

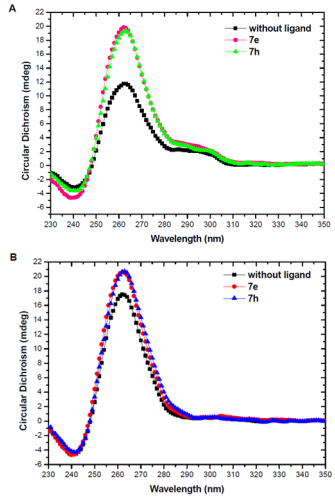


Figure 1. CD spectra of 5 μM *c-KIT*1 (A) and *c-KIT*2 (B) in 10 mM Tris−HCl buffer, pH 7.2, 100 mM KCl, without ligand (■), with 25 μM compound **7e** (●), and 25 μM compound **7h** (▲).

excess compounds **7e** and **7h** to the solution, remarkable enhancement of the bands near 263 nm was observed. Under the same condition, the CD spectrum of *c-KIT*2 without ligands had a major positive band at 263 nm and a negative band near 240 nm. As shown in Figure 1B, the addition of excess **7e** and **7h** to the solution induced a moderate enhancement of the bands near 263 nm. Overall, **7e** and **7h** could induce or enhance the G-quadruplex formation. Meanwhile, the quadruplex topology is retained with the binding of **7e** and **7h**, indicating that none of these ligands would induce major changes in *c-KIT*1 and *c-KIT*2 quadruplex topology.

Beyond the biophysical studies, it is more important to evaluate the cellular effects of these compounds and see whether they could also bind to the *c-KIT* G-quadruplexes in cellular conditions and accordingly reduce the gene transcriptional and expression level. Thus, the short-term cell growth inhibitory activity of the derivatives **7a**−**7j** was first evaluated using MTT assay in HGC-27 cell, a human gastric carcinoma cell line with *c-KIT* overexpression. All the derivatives showed cytotoxicity on the HGC-27 cells, ranging from 2.3 to 21.2 μM (Table 2). The activities of compounds **7a**, **7c**, and **7e** were comparable with that of imatinib.

To further explore the effect of QDs on *c-KIT* promoter activity, luciferase activity assay was employed. Two luciferase constructs were used in the assay. One of them contained a full-length wild promoter of *c-KIT* with native *c-KIT*1 and *c-KIT*2 sequences, while the other had the mutant promoter with mutated *c-KIT*1 and *c-KIT*2 sequences that could not form G-quadruplex structures. All the derivatives (**7a**−**7j**) were included in the screening of the assay with imatinib as control. As shown in Figure 2A, all the derivatives showed obvious inhibitory effects on luciferase activity at the concentration of 2.5 μM for wild promoter construct instead of mutant promoter construct. No significant inhibitory activity was observed in control experiments using imatinib. Compounds **7a**−**7c** with short side chains showed relatively modest

dx.doi.org/10.1021/ml400271y | *ACS Med. Chem. Lett.* 2013, 4, 909−914

Table 2. IC_{50} (μM) Values of Quinazolone Derivatives against HGC-27 Cell Line

MTT IC_{50} (μM)										
7a	7b	7c	7d	7e	7f	7g	7h	7i	7j	imatinib
2.3	18.4	1.3	13.4	6.8	8.2	21.2	8.6	17.2	12.3	3.8

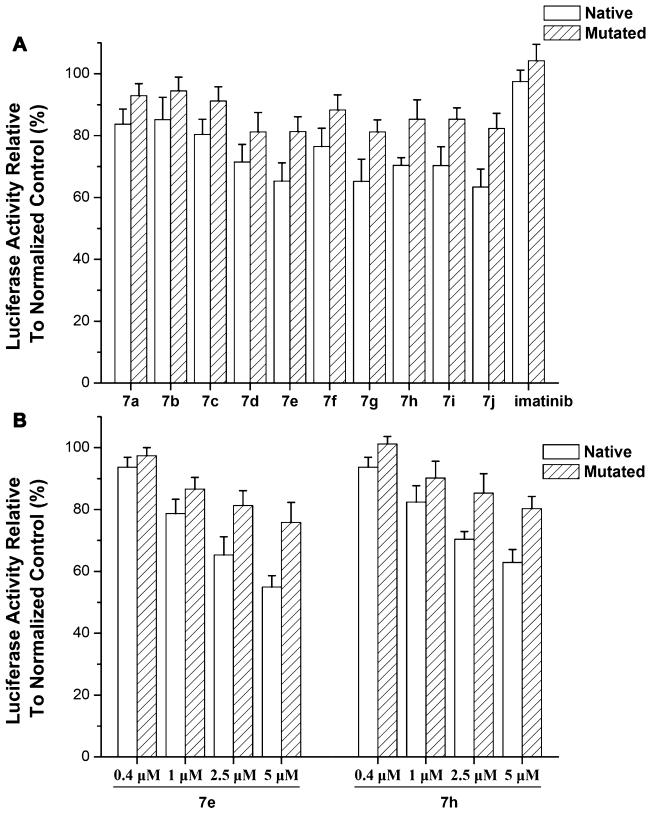


Figure 2. Effects of the derivatives on *c-KIT* promoter activity. MCF-7 cells were transient transfected with plasmid containing full-length wild promoter of *c-KIT* or its mutant and pRL-TK and then treated with different compounds at the concentration of 2.5 μM (A) and compounds **7e** and **7h** at different concentrations (B) for 48 h. The efficiency of transfection was normalized by pRL-TK. Luciferase activity was plotted relative to the untreated group, which was assigned a value of 100%. The error bars represent the standard error from triplicates of three independent experiments.

inhibitory activities as compared with compounds **7d**–**7j** containing longer side chains.

Considering the similar inhibitory activities displayed by **7d**–**7j**, compounds **7e** and **7h** with interconverted amido bonds and best inhibitory effects on luciferase activity for wild promoter construct were chosen for further study. The effects of **7e** and **7h** at different concentrations on *c-KIT* promoter activity are shown in Figure 2B. Obviously, they both could dose-dependently decrease the luciferase activity for wild promoter construct. The addition of **7e** (5 μM) resulted in about 45% reduction of luciferase activity for wild promoter construct, two times stronger than its reduction for mutant promoter construct (22%). At the same concentration, **7h** could also exert inhibitory effects on luciferase activity of wild and mutant construct (37% vs 20%). All these results tell us that QDs could inhibit the activity of *c-KIT* gene through their effective interaction with the promoter G-quadruplex structures, and in parallel with SPR and MTT results, compound **7e** showed stronger inhibitory effects on *c-KIT* promoter activity than its analogue **7h**.

On the basis of results from luciferase activity assay, a study on the effects of compounds **7e** and **7h** on *c-KIT* transcription was carried out using HGC-27 cell line. We evaluated **7e** and **7h** at the final concentration of 1.25, 2.5, and 5 μM through quantitation of mRNA using reverse transcription polymerase chain reaction (RT-PCR). Upon the treatment of **7e** at three different concentrations for 24 h, transcription of *c-KIT* was reduced by 4.9%, 22.8%, and 52.5%, respectively, related to a control gene β-actin (Figure 3). For compound **7h**, 5.7%,

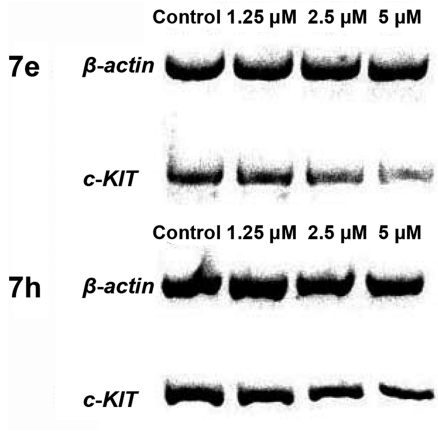


Figure 3. Effects of compound **7e** and **7h** at the concentration of 1.25, 2.5, and 5 μM on *c-KIT* transcription.

10.4%, and 35.7% were observed. Obviously, **7e** and **7h** showed good dose-dependent inhibition on the transcription of *c-KIT* gene in HGC-27 cells. It was also found that **7e**, compared with **7h**, exhibited superior inhibitory effects on *c-KIT* transcription levels in most cases. This is in agreement with the results from SPR, MTT, and luciferase assays. It has been further confirmed that QDs are *c-KIT* G-quadruplex binding ligands act in both biophysical and cellular conditions.

In summary, a novel series of quinazolone derivatives with an unfused aromatic scaffold were designed, synthesized, and evaluated. These small molecules showed promising binding to G-quadruplex DNA in *c-KIT* oncogene instead of duplex DNA. Moreover, the derivatives effectively inhibited the transcription and expression of *c-KIT* gene and exhibited significant cytotoxicity on the GIST cell line HGC-27. All these results provided enlightenment for the design of flexible and adaptive *c-KIT* G-quadruplex ligands. The experimental results have also proved and reinforced the idea of inhibition of *c-KIT* expression through targeting *c-KIT* G-quadruplex DNA.

However, structures of these existing derivatives need to be further modified because their stabilizing ability for *c-KIT* G-quadruplex DNA and inhibitory effects on *c-KIT* transcription levels were apparently less potent than those of reported planar fused aromatic compounds.[14,15] The unfused aromatic ring system seems too feasible and nonplanar to effectively stack on the G-quartet and stabilize the G-quadurplex DNA. In addition,

dx.doi.org/10.1021/ml400271y | ACS Med. Chem. Lett. 2013, 4, 909–914

although there were correlations between biophysical data and cellular results to some extent, it was also noteworthy that several compounds with similar binding affinities for *c-KIT* G-quadruplex showed different cytotoxicity on the HGC-27 cells and that there was no significant correlation between cell proliferation and luciferase inhibition data. It has been reported that the cellular effects of G-quadruplex binding ligands were complex.[3,29] Thus, the cellular effects of these quinazolone derivatives could not be simply explained by *c-KIT* G-quadruplex interactions. Other possible genomic G-quadruplex targets (telomere, *c-MYC*, *BCL-2*, etc.) could be involved in drug action. To overcome the problem of drug selectivity, structural modification was still needed.

Further progress on the development of quinazolone derivatives with high stabilizing ability, binding affinity, and specificity for *c-KIT* G-quadruplex DNA could come from the application of structure-based design methods on the basis of the unprecedented NMR or crystallographic *c-KIT* G-quadruplex structures.[17,18] In order to understand the nature of the ligand−DNA interactions and to help rationalize the experimental results, we did an initial molecular docking study using compounds **7e** and **7h**. The propeller-type *c-KIT* G-quadruplex NMR structure (PDB ID: 2O3M) was applied as a template.[17] The binding free energy for **7e** and **7h** was estimated to be −12.67 and −10.77 kcal·mol^{-1}, respectively (Supporting Information). This trend is consistent with the results of SPR, MTT, luciferase, and RT-PCR assays. The docking utilizing the NMR structure will be used to provide additional information for the better design of more effective ligands targeting c-*KIT* G-quadruplex DNA. For example, more planar aromatic moiety (indole, benzimidazole, etc.) could be introduced instead of 3-phenyl group to control the planarity and aromaticity of the compounds. Other methodologies such as the in situ click chemistry could be employed to explore appropriate side chains of the quinazolone and/or benzylidene moieties.[30] Related investigations on the structural modification of these small molecules are now underway.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures for synthesis and characterization of quinazolone derivatives, biophycial assays, cellular assays, and docking experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(J.-H.T.) Tel: 8620-39943053. E-mail: tanjiah@mail.sysu.edu.cn.

Author Contributions

†X.W. and C.-X.Z. contributed equally to this work.

Funding

This work was supported by the Natural Science Foundation of China (Nos. 91213302, 21272291, and 81001400).

Notes

The authors declare no competing financial interest.

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