In Silico Identification of Piperidinyl-amine Derivatives as Novel Dual Binders of Oncogene *c-myc*/*c-Kit* G-quadruplexes

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Supporting Information

ABSTRACT: In the last years, it has been shown that the DNA secondary structure known as G-quadruplex is also involved in the regulation of oncogenes transcription, such as *c-myc*, *c-Kit*, *KRAS*, *Bcl-2*, *VEGF*, and *PDGF*. DNA G-quadruplexes, formed in the promoter region of these proto-oncogenes, are considered alternative anticancer targets since their stabilization causes a reduction of the related oncoprotein overexpression. In this study, a structure-based virtual screening toward the experimental DNA G-quadruplex structures of *c-myc* and *c-Kit* was performed by using Glide for the docking analysis of a commercial library of approximately 693 000 compounds. The best hits were submitted to thermodynamic and biophysical studies, highlighting the effective stabilization of both G-quadruplex oncogene promoter structures for three *N*-(4-piperidinylmethyl)amine derivatives, thus proposed as a new class of dual G-quadruplex binders.

KEYWORDS: G-quadruplex, *c-myc*, *c-Kit*, drug design, virtual screening

G-quadruplexes (G4s) are noncanonical high-order secondary DNA structures that occur in guanine-rich sequences, especially in human genome.¹ They are characterized by the planar arrangement of four guanines interacting via Hoogsteen hydrogen bonding and further stabilized by a monovalent cation coordinated by the O6 lone pairs.² The G4 forming sequences are located in essential regions of eukaryotic chromosomes, underlining their crucial role in many important biological processes. Recently, Biffi et al. quantitatively visualized DNA G4 structures in human cells through a specific antibody, confirming them as a valid target for the rational drug development.³ Although chemically distinct, RNA can also fold *in vitro* into G4 structures, and they were observed within the cytoplasm of human cells using a specific antibody.⁴ Chen et al. even developed a molecular probe for the unique visualization of the G4 structure formed by the G-rich sequence within the 5'-UTR of NRAS mRNA.⁵ Moreover, recently the application of a new red-emitting fluorescent probe allowed to track the dynamic folding and unfolding of RNA G4 in live cells.⁶

The G4 expression in gene promoter regions is explained as a transcriptional repression of oncogenes, thus representing emerging therapeutic targets in oncology. Indeed, many G4 gene promoters have physicochemical properties and structural characteristics that make them druggable.⁷ Thus, these structures have been studied for different proto-oncogenes that include *c-myc*,⁸ *VEGF*,⁹ *bcl-2*,¹⁰ *KRAS*,¹¹ and *c-Kit*.^{12,13}

Several studies showed the down-regulation of the expression of these genes in cell based systems when G4 binders have been used.^{14,15} Among all oncogene promoter G4s, *c-myc* is the most investigated one since its abnormal overexpression is frequently observed in some tumors, including breast, colon, and cervix carcinomas, as well as small-cell lung cancer, osteosarcomas, glioblastomas, and myeloid leukemias.¹⁶ Experimental studies, carried out by circular dichroism (CD) and nuclear magnetic resonance (NMR), revealed the presence of a single G4 isomer with a parallel-stranded structure containing three lateral loops¹⁷ in the nuclear hypersensitivity element III₁ (NHE III₁), which is located upstream of the P1 promoter of *c-myc*.^{18,19} Also *c-Kit* proto-oncogene is widely studied since it codes for a membrane-bound glycoprotein of the family of growth factor receptors with tyrosine kinase activity involved in cell survival, proliferation, and differentiation. Therefore, it is implicated in several human cancers, including gastro-intestinal cancer (GIST), where the dysregulation of *c-Kit* expression is the main causative event at the base of this disease.^{20,21} In the promoter region of this gene, two quadruplex-forming sequences have been identified, namely, *c-Kit1* and *c-Kit2*.^{12,13} Both in NMR and crystallo-

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graphic models, the *c-kit1* quadruplex shows an identical parallel topology in which one non-G-tract guanine unusually participates in the core of stacked G-quartets.²²

The use of small molecules able to stabilize these G4 structures can regulate related protein expression and thus inhibit the growth of a wide range of cancer cells.¹⁴ Interestingly, it has been shown that the overexpression of both *c-myc* and *c-Kit* occurs in the stroma of phyllodes tumors, predominantly those of a sarcomatous type.²³ Therefore, compounds interacting concurrently on *c-Kit* and *c-myc* G4 would be desirable in these types of tumors. Even if ligands with dual action are known in literature, none of them has made it through the drug discovery pipeline because of their poor drug-like properties or their poor selectivity profile, or both. This is strongly encouraging research toward the identification of new G4 binding agents as potential anticancer drugs. In particular, for this purpose, computational approaches have been widely and successfully used,^{24–26} resulting also capable to elucidate ligand binding mechanisms and to provide the molecular information useful for drug design studies.^{27,28}

Consequently, in this work, in order to identify promising G4 stabilizers able to act on both *c-myc* and *c-Kit* promoters, we performed a high-throughput *in silico* screening of a commercial database by means of a structure-based approach. Regarding the *c-myc* G4 sequence, we selected the two NMR models with the following PDB codes: 1XAV and 2L7V. The first is formed by 20 conformations of a monomeric parallel-stranded quadruplex,²⁹ while the latter is formed by 10 different conformations³⁰ and is complexed with the quindoline ligand in a stoichiometry ratio of 2:1. As concerns the *c-Kit* sequence, two X-ray models (PDB codes: 4WO2 and 4WO3) and an NMR structure (PDB code: 2O3M) have been taken into account. The X-ray models are dimers, with resolutions of 1.82 and 2.73 Å, respectively.^{31,32} On the contrary, the NMR structure is a monomer characterized by 11 different conformations.³³ All the above-described experimental structures were prepared and refined. Then, in order to validate our protocol and to identify, among X-ray, NMR and their minimized conformations the structure to be used in our Virtual Screening (VS) protocol, enrichment studies were performed by applying all Glide accuracy levels (HTVS, SP, and XP) to the *consensus* library built, respectively, for *c-myc* and *c-Kit*. Globally, 40 active compounds (1–40) were collected and selected from the literature on the basis of their activity against *c-myc*^{34–36} (1–20) and *c-Kit* G4s^{37–39} (21–40) (Supporting Information Figures S1–S2). Thus, two combinatorial libraries of 1249 compounds were generated by means of the DUD-E, starting from active molecules, and were therefore employed as decoy sets.⁴⁰

With the aim to select the NMR conformation to be used for the docking analysis toward both G4 promoter structures, we considered the receiver operating characteristic (ROC) and the robust initial enhancement (RIE) (Supporting Information Tables S1–S2). In particular, for *c-Kit* sequence, the best model resulted in the sixth NMR conformation of 2O3M, with a ROC value equal to 0.94 and a RIE equal to 12.31; by contrast, for *c-myc* structure, the fifth conformation of the NMR model 2L7V was chosen since it showed ROC and RIE values of 0.95 and 12.45, respectively (Supporting Information Figure S3).

For our study, we adopted a multiconformational database from the Asinex vendor including comprehensively ~693 thousands of compounds. The physiological relevant ionization state (pH = 7.4) was assigned to all compounds, and the stereochemistry of chiral centers was determined. First, such

database was reduced in size by applying several physical and chemical filters. The popular empirical Lipinski's rule of five⁴¹ was applied to remove those molecules without drug-like properties. Hence, the filtered molecules were characterized for their ADME profile by means of QikProp,^{42,43} and only those with appropriate pharmacokinetic properties were selected. Finally, all compounds with toxic functional groups were removed from the database. The resulting 462 045 compounds were submitted to ensemble docking simulations and evaluated on the basis of their G-Score value.

Taking into account only those compounds with a G-Score within 2 kcal/mol from that of the best ranked hits (ranging from –12.54 to –10.54 kcal/mol for *c-myc* and from –11.39 to –9.39 kcal/mol for *c-Kit*, respectively), we obtained 442 and 634 molecules for *c-myc* and *c-Kit* G4s, respectively. The 76 shared hits in complex with both receptors were further submitted to full energy minimization, with the aim to calculate their binding energies and to investigate their thermodynamic behavior. In order to select the best promising hits with dual activity,⁴⁴ we used the same protocol for three known compounds, reported in literature as dual binders (41–43) (Supporting Information Figure S4). Therefore, their average $\Delta\Delta E$ value of 6.60 ± 2.00 kcal/mol was used as a cutoff, globally leading to 36 hits characterized by a good theoretical dual activity (Supporting Information Table S3).

Finally, after a careful visual inspection analysis of the best poses and the evaluation of their commercial availability, 10 hits (44–53) were purchased and submitted to biophysical assays (Supporting Information Table S4 and Figure S5). Structurally, all the hits present a similar scaffold with a small planar portion and a positively charged side chain, confirming the essential role of these moieties in G4 recognition. Moreover, all selected hits fulfill the ranges of drug properties for 90% (Supporting Information Table S5). This is a very important issue since none of the G4 binders is currently approved for marketing due to their nonoptimal pharmacokinetics. In fact, although several ligands have shown good antiproliferative and anticancer efficacy at preclinical level, only quarfloxin entered in the phase I/II of clinical trials (<http://clinicaltrials.gov/>).

Unfortunately, among the 10 selected compounds, four were not sufficiently soluble in water to be proficiently tested and thus were not included in the experimental evaluation.

To assess the potential of the most soluble compounds to interact with the G4 sequences in solution, we screened them by using a fluorescence melting assay. For this purpose the melting profiles of appropriately labeled oligonucleotides corresponding to the *c-myc* and *c-Kit* sequences used along the virtual screening were acquired in the presence/absence of increasing concentrations of the identified binders. For comparison, the same analysis was performed with a duplex DNA unable to fold into G4. Data are summarized in Table 1. Out of the six tested candidates, three compounds (i.e., derivatives 45, 46, and 47) stabilized both G4 structures. Conversely, only derivative 47 was able to stabilize also the double helix, although to a reduced extent in comparison to the G4 arrangement.

On *c-Kit* G4, 45, 46, and 47 behave as loops binders, in particular interacting with the long five-nucleotide lateral stem loop (Figure 1A,C,E). It is important to underline that the most pronounced difference between the crystal structures and the NMR models is just associated with the large cleft between the stem-loop (G18, A19, and G20) and the adjacent G-quartet. The net effect of this difference is that the large cleft is narrower in all crystal structures with respect to NMR conformations,

Table 1. Increase in Melting Temperature of Selected DNA Sequences Induced by 20 μ M of Tested Ligands^a

hit	ΔT_m [°C]		
	dsDNA ^b	<i>c-Kit</i> ^c	<i>c-myc</i> ^d
45	n.o.	3.2	5.4
46	n.o.	2.3	1.3
47	1.5	3.0	2.0
49	n.o.	0.3	0.1
51	n.o.	0.1	0.6
53	n.o.	1.1	0.4

^an.o. indicates no observed shift of the thermal transition. Error was ± 0.2 °C. ^b T_m dsDNA = 61.8 °C. ^c T_m *c-Kit* = 58.2 °C. ^d T_m *c-myc* = 70.4 °C.

Figure 1. Best docked poses of 45 (A,D), 46 (B,E), and 47 (C,F) against *c-myc* and *c-Kit* G4 sequences, respectively. The G4s DNA of *c-myc* and *c-Kit* are shown as orange and dark-cyan cartoons, respectively. The best 45, 46, and 47 hits are represented as green, violet, and pink carbon sticks, respectively. Coordinating K⁺ ions are reported as orange spheres, while hydrogen bond interactions are indicated as dashed yellow lines.

almost creating a distinct binding site.⁴⁵ Such an observation could rationalize the best ROC and RIE data obtained for NMR structures in our enrichment studies. On the contrary, as regards the complexes of *c-myc*, compounds 45 and 46 appeared to prefer the 3'-end position by acting in part as grooves binders (Figure 1A,B), while 47 was found to interact, by means of two stacking contacts, with the guanines exposed to the solvent at the 5'-end position (Figure 1C).

Specifically, from the qualitative analysis of the contacts established by the best biophysical hits 45 and 46 on *c-myc* (Figure 2A,B), it is evident that both of them are involved in the

Figure 2. Contacts analysis of the best hits 45, 46, and 47 toward (A) *c-myc* and (B) *c-Kit* G4 sequences.

same number of hydrogen bond (Hbond), salt bridge, and cation- π interactions. This is in line with the very similar binding orientation adopted by 45 and 46, due to their low structural differences.

Nevertheless, the presence of the 3-ethoxy portion in 45 induced a conformational reorganization so that the 4-chlorobenzyl portion was able to make two π - π stacking interactions with G13 residue and could explain 45's slight preference against *c-myc* with respect to 46. By contrast, hit 47, characterized by less flexibility of the phenyl-2-furyl portion, interacted with the 3' end of *c-myc*, clearly reducing the number of Hbonds and salt bridges (Figure 2A) and thus justifying the low increase of ΔT_m . As regards the contact analysis toward the *c-Kit* G4, all the best hits preferably bound in the stem-loop formed by G18, A19, and G20 and the adjacent G-quartet. In particular, 45 and 46 assumed a very similar binding accommodation, with the positively charged 4-piperidinylmethyl-amine group oriented toward the phosphate backbone of C9, G21, G8, A19, and G4 residues wherewith they established the same number of salt bridges (Figure 1D,E). However, it is worth to mention that, due to the steric hindrance of the 3-ethoxy portion, the positively charged nitrogen atom of the piperidine in 45 was driven to the negatively charged phosphate backbone. Conversely, 47 assumed a different binding mode since the 4-piperidinylmethyl-amine group was involved in salt bridge interactions with A19 and G4 nucleobases (Figure 1F).

This proposed binding mode well fits with the modest stabilization of the target sequences above-described. To validate it, we monitored by CD titrations the ligand binding mode of 45, 46, and 47 to *c-myc* and *c-Kit* sequences in solution (Supporting Information Figure S6). Upon addition of the ligands to the folded G4, only a modest decrement of the positive band centered at 265 nm was recorded. This reflects lack of extensive G4 rearrangement upon ligand binding as predicted by the molecular dynamics simulations (MDs) (see Supporting Information Section "Molecular Dynamics").

HGC27

HMC1.2

Figure 3. Dose–response and IC_{50} values derived for tested derivative 45, 46, and 47 in HGC27 and HMC1.2 after 72 h treatment.

Nevertheless, this process reached saturation at high ligand/DNA molar ratio, which points out to a modest affinity for the target. SPR titrations performed with the three ligands confirmed it. Compound 47 extensively binds to the reference blank cell, thus preventing proper data acquisition. Conversely, a concentration-dependent increment of the RU at the steady state was recorded by flashing 45 and 46 on chip surfaces functionalized with the target sequences (Supporting Information Figure S8). However, the signals were quite low and a complete isotherm was derived only for the 45-*c-Kit* system. These data are comparable with the parallel analysis performed using the CD data and provided a $K_d = 18 \pm 3 \mu M$. This moderate value is actually in line with the expected behavior for a new hit.

To fully define the profile of these derivatives as potential candidates for the regulation of *c-Kit/c-myc* expression, an evaluation of their behavior at the cellular level was clearly required. In most publications focused on *c-Kit/c-myc* and G4s, the HMC 1.2 and HGC27 cell lines are widely used to check the level of *c-Kit/c-myc* expression upon treatment with G4 binders,^{46–48} thus justifying the choice to test our candidate G4-ligands on these cells. Cell cytotoxicity was evaluated by the Alamar Blue test after a 72 h cell treatment. All three selected hits showed dose-dependent cytotoxic effects in both tested cell lines. Dose–response curves, the relative IC_{50} values, and the corresponding linear regression coefficients (R^2), for each G4-ligand, are reported in Figure 3. Interestingly, 45, 46, and 47 showed comparable IC_{50} values that were conserved among the two cell lines. Likely, the new hits need to be further optimized in terms of target recognition to better exploit their potential activity on the target sequences.

In conclusion, in this work a structure-based virtual screening of a commercial database was performed toward *c-myc* and *c-Kit* G4 structures. Both theoretical and biophysical experiments put in evidence compounds 45, 46, and 47 as moderate stabilizers of both oncogenic promoter G4s. The innovative aspect of our work is related to the identification of a new chemical scaffold if

compared to that of the already known G4 binders. Moreover, all the selected hits showed good theoretical pharmacokinetics profiles with respect to the used filters. Despite the moderate efficiency in the recognition of the selected targets, the most interesting compounds could be further optimized in future studies with the aim to design new lead compounds and build an *in silico* structure-based focused library.

It can be concluded that the applied workflow was successful in retrieving new promising drug-like scaffolds, different with respect to the known G4 binders and worthy to further investigation. In particular, starting from 45, future lead optimization and SAR studies will be carried out in order to enhance its binding affinity and G4 stabilization.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.8b00275.

Detailed experimental procedures for molecular modeling and biophysical and biological assays. Molecular dynamics simulations. Tables S1–S5 and Figures S1–S7 (PDF)

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Author Contributions

[‡]These authors contributed equally. R.R. and S.A. conceived and designed the research; R.R., F.M., and C.T. performed *in silico* experiments; F.M., R.R., G.C., and F.O. analyzed the data; F.O. contributed with analysis tools; S.D.R., G.N., and C.S. performed biophysical and biological assays; A.A., C.S., and S.A. wrote the paper. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

G4s, G-quadruplexes; CD, circular dichroism; NMR, nuclear magnetic resonance; NHE III₁, nuclear hypersensitivity element III₁; GIST, gastrointestinal cancer; ROC, receiver operating characteristic; RIE, robust initial enhancement; Hbonds, hydrogen bonds; SAR, structure–activity relationship

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