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Discovery of new G-quadruplex binding chemotypes

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We report here on the discovery and preliminary evaluation of a novel non-macrocyclic low molecular weight quadruplex-stabilising chemotype. The lead compounds, based on a furan core, show high G-quadruplex stabilisation and selectivity as well as potent in vitro antiproliferative activity.

Quadruplexes (G4s) are higher-order nucleic acid arrangements involving a core of π–π stacked guanine-quartets (G-quartets) rather than the Watson-Crick base pairs of double-helical nucleic acids.1 G4-forming sequences are widely prevalent in eukaryotic telomeric sequences as well as being over-represented in other genomes,2 notably promoter and 5’-UTR sequences of genes involved in cellular proliferation.3 The recent demonstration of the presence of G4s in human cells4 has added credence to the concept that G4s can be targets for therapeutic intervention, at the single gene or polygene levels.5 Appropriate small molecules can serve to stabilise G4s and the resulting complexes can then act as impediments to telomere maintenance, transcription or translation, depending on the nature of the quadruplex target site.6 These effects have been shown in several target genes of relevance to human cancer such as c-MYC5 and c-KIT.5

A large number of small molecule chemotypes have been reported as G4-binding ligands.6 The overwhelming majority are heteroaromatic with large flat surfaces, designed to complement the surface characteristics of a terminal G-quartet in a typical quadruplex structure. A second class of ligand is represented by the cyclic polyoxazole natural product telomestatin.7 A number of cyclic and acyclic analogues have been reported, some of which show potent biological activity.10 The acyclic compounds tend to be characterised by a crescent shape. For example, pyridostatin11 and several series of phenyl- and pyridyl-bis-oxazoles12,13 all selectively target G4s (Figure 1). A more general requirement of most G4-binding ligands is the possession of side-chains terminating in cationic charge.6

Few G4-binding small molecules have proceeded to in vivo evaluation in models of human cancer, and to date only one compound, Quarfloxin, has been evaluated in clinical trials.15 The perceived lack of drug-like characteristics in many G4-binding compounds may have hindered progress to the clinic. We report here on a study to discover novel ligands with MWs <400 Da that could be suitable starting-points for future drug discovery efforts.

Figure 1. Structures of various non-polycyclic G-quadruplex ligands

Thirty-eight representative members of a large chemical library from the anti-parasitic drug discovery programme at Georgia State University16,17 (several hundred compounds), with highly diverse scaffolds and functional groups, were screened using a high-throughput 96-well FRET (Fluorescence Resonance Energy Transfer) assay.18 G4 stabilisation was initially evaluated using dual-labelled F21T (human telomeric 21-mer) and c-KIT2 (a tyrosine kinase oncogene) G4s, as well as a duplex DNA sequence (T-loop). The ten most active compounds were subsequently screened against an expanded panel of fluorescently-labelled promoter G4-forming sequences, with HSP90A, HSP90B (heat shock protein 90 promoter sequences),12 k-RAS21 (in the promoter of the k-RAS oncogene)19

Figure 2. Structures of the lead compounds 1-6 identified in this study, together with a control compound 7.

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and AR, a G4 recently identified in the promoter of the androgen receptor (involved in prostate cancer development).29

Six acyclic furan- and thiophene-based compounds (Figure 2: 1-6), representing two distinctive chemotypes were identified with high (>15 °C) \( \Delta T_m \) values. A competition assay using unlabelled calf thymus duplex DNA and compounds 1-6 examined the ability of these compounds to differentiate between duplex and the F21T G4 DNA at high duplex:G4 ratios. The compounds were also examined in a 96 hr short-term sulforhodamine B (SRB) assay, to determine their ability to inhibit cancer cell growth (Tables 1, 2). All six compounds showed potent G4 stabilising abilities, as judged by the large changes in \( \Delta T_m \) values for the selected G4s. In particular the bis-phenyl- mono-furan compounds 1 and 5 had especially high \( \Delta T_m \) values, broadly comparable to those for established high-affinity G4-binding compounds such as tetrabridged naphthalene diimides.21

The tri-furan compound 2 is consistently more effective in stabilising the G4s than the tetrafuran compound 6. Compound 2 and 6, representatives of a tetrafuran second chemotype, were less selective at high duplex ratios and were inactive in the SRB assay, possibly because of aqueous solubility and cellular uptake issues. Switching from a furan (1, 5) to a thiophene (4), does slightly affect G4 stabilisation and selectivity vs duplex DNA, though not in vitro potency, which at least in the cell lines examined, is comparable to that of compounds 1 and 5.

### Table 1

| Cmpnd | Mol Wt | F21T k-CT2 | HSP-90A | HSP-90B | k-RAS 2/1R | AR | T-loop | G4C:CT 1:1 | G4C:CT 1:10 | G4C:CT 1:100 | G4C:CT 1:300 |
|-------|--------|------------|---------|---------|-------------|---|--------|----------|-----------|-------------|-------------|
| 1     | 384.5  | 22.3       | 16.8    | 27.7    | 23.7        | 16.0 | 15.4   | 100      | 100       | 100         | 100         |
| 2     | 364.4  | 20.4       | 16.3    | 26.0    | 22.0        | 15.1 | 13.8   | <2       | 100       | 100         | 28.0        |
| 3     | 330.5  | 17.6       | 17.8    | 21.2    | 21.5        | 11.9 | 10.2   | <2       | 100       | 100         | 65.9        |
| 4     | 372.5  | 18.0       | 13.9    | 25.8    | 19.1        | 18.8 | 11.8   | <2       | 100       | 100         | 77.7        |
| 5     | 412.5  | 22.6       | 18.5    | 26.6    | 22.7        | 13.4 | 10.1   | <2       | 100       | 100         | 53.1        |
| 6     | 350.3  | 18.6       | n/a     | 20.6    | 16.2        | 17.0 | 15.1   | <2       | 100       | 100         | 35.4        |
| 7     | 302.5  | 14.4       | 12.2    | 19.0    | 17.5        | 16.7 | 9.9    | 3.4      | 100       | 100         | 24.5        |
| 8     | 830.6  | 26.6       | 22.0    | 33.1    | 28.6        | n/a  | 15.9   | 4.9      | 100       | 100         | 27.2        |

Overall, F21T and the two HSP90 G4s have been most stabilised by compounds 1-6. Comparison with the behaviour of a tetra-substituted naphthalenediimide compound previously examined by us22, shows that 1-6 exhibit only moderate \( \Delta T_m \) values with the AR G4, which are generally lower than with other G4s, Compounds 1-6 produced slightly reduced but still significant stabilisation with the c-Kit2 and k-RAS2 G4s, suggesting that these compounds have the ability to act simultaneously on multiple G4 targets (G4-polytargeting). The stabilisation of a duplex DNA sequence (T-loop) was not significantly affected by any of the compounds at the biologically relevant concentration employed here (1 \( \mu \)M). \( < \text{SD} > \pm 0.5 ^\circ \text{C} \), from triplicate measurements. n/a: indicates unsuccessful curve fitting to the melting data. Compound 7, a negative control, is a para analogue of the mono-furan compounds. Compound 8 is a tetra-substituted naphthalene diimide derivative21, used here as a G4 control.

Circular dichroism (CD) was employed to qualitatively evaluate the binding mode of the lead compounds and to examine induced structural transitions in the telomeric G4. The CD spectra (Figure 3 and Supplementary Information) show that compounds 1 and 3-6 produce very small induced CD signals in a human telomeric quadruplex sequence. Such weak induced CD signals are characteristic of quadruplex end-stacking compounds and the small differences in the CD signal patterns for different compounds indicate minor differences in the stacking geometries of the ligands at the terminal G-quartets.

Figure 3. Circular dichroism (CD) spectra for compounds 1 and 5 at differing ligand:G4 ratios. F22T is a 22-mer analogue of the F21T sequence.
crucial for shape similarity to telomestatin. The para substitution in the control compound 7 enables it to effectively bind to the minor groove of duplex DNA, which is not possible for any of the six compounds arising from the screen.

Several of the compounds showed low µM anti-proliferative activity (Table 2) in a cancer cell line panel (A549 (lung cancer), MCF7 (breast cancer), RCC4 and 786-O (renal cancer), Panc1 and Mia-PaCa2 (pancreatic cancer), ALT (transformed lung fibroblast cells characterised by Alternative Maintenance of Telomeres) and WI38 (non-transformed lung fibroblast cells)).

### Table 2. IC50 values of compounds 1-8 determined by a 96 hr SRB assay (see the Supplementary Information for further details). *(<Esd>) ± 0.3 µM.*

| Compounds | A549 | MCF7 | ALT | Mia-PaCa2 | Panc1 | RCC4 | 786-O | WI38 |
|-----------|------|------|-----|-----------|------|------|-------|------|
| 1         | 0.3  | 0.9  | 1.0 | 0.3       | 0.5  | 4.9  | 3.6   | 1.3  |
| 2         | >25  | >25  | >25 | >25       | >25  | >25  | >25   | >25  |
| 3         | 0.8  | 0.9  | 6.6 | 2.0       | 1.7  | >25  | >25   | >25  |
| 4         | 0.4  | 1.4  | 1.7 | 0.7       | 1.0  | 3.3  | 5.6   | 2.0  |
| 5         | 0.5  | 1.2  | 1.4 | 0.5       | 1.0  | 3.9  | 4.8   | 2.0  |
| 6         | 4.2  | 15.6 | 18.8| 7.0       | >25  | >25  | 12.8  | >25  |
| 7         | 3.7  | 11.7 | >25 | 8.9       | 10.9 | >25  | >25   | >25  |
| 8         | 0.019| 0.070| 0.063| 0.011     | 0.003| 0.560| 0.320 | 0.230|

The two poly-furan compounds 2 and 6 have low anti-proliferative activity, even though both have G4-stabilising ability comparable to the other four compounds in the group. This may be due to cell uptake and nuclear localisation problems as well as limited aqueous solubility; the lack of observed precipitation during the SRB assay supports the former suggestions. Compounds from the mono-furan and mono-thiophene series on the other hand show limited aqueous solubility; the lack of observed precipitation during the FRET assay against a panel of G4s with a duplex control sequence, we report here that screening putative ligands using a HTS-FRET assay against a panel of G4s with a duplex control sequence, has resulted in the discovery of meta-substituted bisphenylmonofurans as a novel G4 stabilizing chemotype. A similar chemotype, with a urea group replacing the furan ring, has been reported as having high G4 affinity. These compounds are structurally-simple, conformationally flexible and chemically readily accessible with M Wts <400 Da. They have G4 stabilisation ability comparable to those previously observed with polycyclic heteroaromatic compounds1 (cf compound 8 (4,9-bis-(3-(4-methyl-piperazin-1-yl)propyl)amino)2,7-bis-(3-morpholinopropionyl) benzox-[inn] [3,8] phenanthroline-1,3,6,8(2H,7H)-tetraene) in Table 1, but with low duplex DNA affinity. They inhibit cancer cell growth at low µM/high nM levels, suggesting that these or related compounds may have potential as drug-like poly-quadruplex targeting agents.

### Notes and references

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