ACCEPTED ARTICLE: This manuscript is the version that was accepted after peer review and appeared as an Accepted Article online on June 4, 2019, prior to editing, proofing, and formal publication of the final Version of Record (VoR). Readers should obtain the VoR from the journal website to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

Combining 1,3-ditriazolyl-benzene and quinoline to discover a new G-quadruplex interactive small molecule active against cancer stem-like cells

Eduarda Mendes,[a] Enrico Cadoni,[a] Filipa Carneiro,[a] Marta B. Afonso,[a] Hugo Brito,[a] João Lavrado,[a] Daniel J. V. A. dos Santos,[a] Jorge B. Vítor,[a,b] Stephen Neidle,[c] Cecília M. P. Rodrigues,[a] Alexandra Paulo [a]*

[a] Dr. E. Mendes, E. Cadoni, F. Carneiro, Dr. M. B. Afonso, H. Brito, Dr. J. Lavrado, Dr. D.J.V.A. dos Santos, Dr. J. B. Vítor, Prof. C.M.P. Rodrigues, Prof. A. Paulo
Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649 003 Lisbon, Portugal
E-mail: mapaulo@ff.ulisboa.pt

[b] Dr. J. B. Vítor
Department of Biochemistry & Human Biology, Faculty of Pharmacy, Universidade de Lisboa, 1649-003 Lisbon, Portugal

[c] Prof. S. Neidle
School of Pharmacy, UCL, London WC1N 1AX, UK

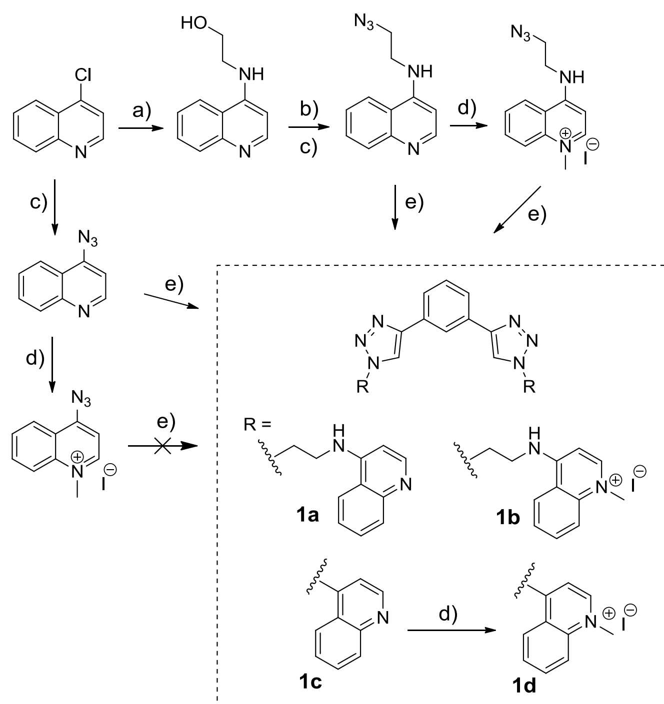
Supporting information for this article is given via a link at the end of the document.

Abstract: Quadruplex nucleic acids are promising targets for cancer therapy. In this study we used a fragment-based approach to create new flexible G-quadruplex (G4) DNA interactive small molecules with good calculated oral drug-like properties, based on quinoline and triazole heterocycles. G4 melting temperature and Polymerase Chain Reaction-stop assays showed that two of these compounds are selective G4 ligands, since they were able to induce and stabilize G4s in a dose- and DNA sequence-dependent manner. Molecular docking studies have suggested plausible quadruplex binding to both the G-quartet and groove, with the quinoline module playing the major role. Compounds were screened for cytotoxicity against four cancer cell lines, where 4,4'-(4,4'-(1,3-phenylene)bis(1H-1,2,3-triazole-4,1-diyl))bis(1-methylquinolin-1-ium) (**1d**) showed the greater activity. Importantly, dose-response curves show that **1d** is cytotoxic in the human colon cancer HT-29 cell line enriched in cancer stem-like cells, a subpopulation of cells implicated in chemo-resistance. Overall, this study identified a new small molecule as a promising lead for the development of drugs targeting G4 in cancer stem cells.

DNA binding compounds have been widely used in cancer therapy, but their general toxicity due to lack of selectivity demands new approaches. An emerging and promising new approach to develop more selective anticancer drugs, has four-stranded G-quadruplex (G4) DNA structures formed by certain guanine rich-sequences, as targets.[1][2][3] The basic unit of a G4 is the G-quartet, a square-planar arrangement of four guanine bases linked with hydrogen bonds. The G-quartets can be linked by intervening loop sequences so that they can stack on top of each other to form a G4, which is additionally stabilized in solution by monovalent cations coordinated with the O6 atoms of guanines.[1] There is now good evidence that G4s can be formed in cells[4][5] where they have important regulatory roles such as in telomere maintenance,[6] in DNA replication, in epigenetic responses and in control of gene expression.[1][7][8] Moreover, the enrichment of these structures in promoter regions of, in particular, proto-oncogenes has been revealed by informatics analysis of the human genome[9] and supported by antibody-based G4 chromatin immunoprecipitation and high-throughput sequencing.[10] The discovery that in many cases they negatively control transcription, has led to the exploitation of these nucleic acid structures as potential targets for selective anticancer drugs.[1][3] Strikingly, it has been shown that G4-stabilizing ligands might differentially target human cancer stem cells,[11][12] a subpopulation of cancer cells implied in tumor formation, metastases and recurrence due to their long-lasting properties and resistance to chemotherapy.[13] Many G4-interactive small molecules with anti-proliferative activity have been described to date but only two of them have reached clinical trials,[14] possibly in part because the rational design of many efficient and selective G4-binders has led to molecules with poor or only moderate pharmacokinetic properties. Differences between nucleic acid sequences forming G4s can create diverse G4 topologies that may have major influence on G4-ligand interactions. The design of flexible non-fused polycyclic G4-interactive small molecules that may also target grooves and loops of G4s, has emerged as one way to achieve selectivity and also to lead to more drug-like compounds.[15][16][17] The 1,3-di(1*H*-1,2,3-triazol-4-yl)benzene system was previously shown by biophysical and molecular modeling studies to provide an efficient central module, able to interact effectively with the 3'-end G-quartet of the human telomeric parallel-stranded G4 structure.[18][19] Also, quinolines have been shown to be important modules in the design of several potent G4 ligands.[15][20]

In order to further explore triazole and quinoline rings in the design of G4-interactive small molecules with potential anticancer activity, we have designed compounds **1a-d** with predicted good oral drug-like properties (Table S1). Synthesis of compounds **1a-c** was readily achieved by microwave-assisted Cu(I)-catalysed Huisgen cycloaddition reaction between 1,3-diethynylbenzene and the appropriate azide as depicted in Scheme 1 and described in the Supporting Information. Interestingly, methylation of the 4-azidoquinoline nitrogen atom precludes the cycloaddition reaction, even in the presence of bathophenanthroline disulphonic acid which was previously described as a 'click catalyst'.[21] Instead, compound **1d** was obtained by direct methylation of **1c**. The ability of compounds **1a-d** to interact with different G4 DNA structures and a duplex DNA sequence was evaluated by a Fluorescence Resonance Energy Transfer (FRET)-melting assay. The DNA sequences used, their respective folding topologies and melting temperatures at the assay conditions, are presented in Table S2.



Scheme 1. Synthesis of compounds **1a-d**. *Conditions*: a) Ethanolamine, TEA, reflux, 24h; b) Methanesulphonyl chloride, pyridine, 0°C, 8h; c) NaN_3, DMF, 110 °C, 2-3h; d) MeI (10 eq.), ACN, r.t.; e) 1,3-diethynylbenzene, $CuSO_4.5H_2O$, Sodium L-ascorbate, $^tBuOH:H_2O$ (1:1), MW 110 ˚C, 30 min.

The results shown in Figure 1A reveal that **1a-d** are superior stabilizers of G4 structures compared to duplex DNA (Tloop), but no significant differences were observed between the melting temperatures of DNA:ligand complexes with different G4-sequences and folds (parallel or hybrid). Surprisingly, **1c** showed no capacity to stabilize G4s structures whereas compounds **1b** and **1d** showed very similar ability to interact and stabilize G4s DNA, possibly because their binding mode to G4 is governed by a common chemical feature, the quinolinium cation. Figure 1B shows the results of a FRET melting competition assay, which indicates that **1d** is more selective for G4 structures than **1b.** In the presence of 50-fold higher molar concentration of double strand DNA, the melting temperature of the G4:**1d** complex decreases by only 10%.

To further assess the capacity of compounds to induce and stabilize DNA G4 structures we performed a Polymerase Chain Reaction (PCR)-stop assay using two different G4-forming sequences. The 27 nucleotides sequence present in the wild-type promoter region of the *c-MYC* oncogene (Pu27, Figure 2A) is able to form three different G4s, the more stable loop-isomer involving G-runs 2-3-4-5 and the less stable loop isomers 1-2-3-4 and 1-2-4-5;[22][23] whereas substitution of two guanines in G-run 3 (Pu27mut, Figure 2B) abrogates the formation of two of the G4s. Figure 2 illustrates that compounds **1b** and **1d** can inhibit DNA hybridization at G-run 5 and consequent formation of the PCR product in a dose- and sequence-dependent manner, clearly suggesting selective activity by induction of G4 formation.

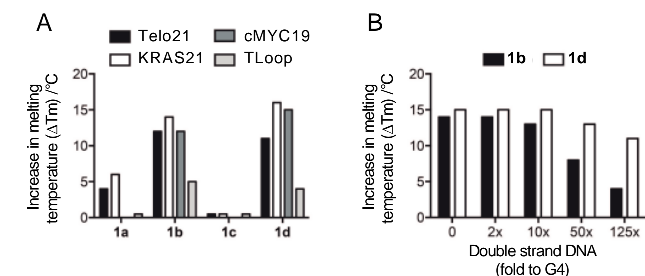


Figure 1. G4 stabilizing efficiency and selectivity of compounds measured by a FRET melting assay**.** (A) Melting temperature variations (ΔTm) of labeled G4s present in promoters of *k-RAS* (KRAS21) and *c-MYC* (cMYC19), human telomere (Telo21) and hairpin loop sequence (T-loop) at 0.2 μM, stabilized by compounds (5 μM). ΔTm values are averages from two independent experiments each in triplicate; std errors < 0.25 °C. (B) FRET melting competition assay data for **1b** and **1d** in complex with KRAS21 G4s, challenged with increasing concentrations of non-labeled 26mer dsDNA competitor.

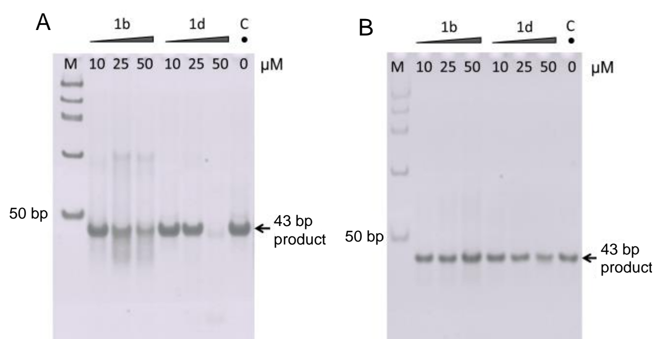


Figure 2. PCR-Stop effect of compounds on two DNA sequences**.** Polyacrylamide gels stained with ethidium bromide showing the effect of compounds on polymerization of DNA constructs (A) containing the *c-MYC* promoter wild type sequence (Pu27) or (B) with a mutated *c-MYC* promoter sequence (Pu27mut). Legend: G-runs are shown in red, mutated bases in blue and the hybridization region in grey; C – control of PCR product in the absence of G4 ligand (0 μM); M – PCR molecular weight marker.

The binding mode to G4s was investigated by molecular docking using a well-studied G4, the human telomere G4 structure (PDB: 143D) and a previously validated protocol.[24] The score results are presented in Table S5 and have the same trend as the FRET results, i.e. the methylated compounds **1b** and **1d** form more stable complexes and compound **1c** is the poorest performer. The top-ranked docking poses have a quinoline ring sitting on top of the G-quartet formed by nucleotides G1, G9, G21, G13, and below T11 with the rest of the molecule occupying the groove, starting at G1 and ending at G3 (Figures 3 and S1B). Although this stacking, to be effective, tends to put the quinoline rings all in the same plane, they occupy nevertheless different relative positions which impact on the conformation of the molecule and change the possibilities for suitable interactions. This is clearly seen in the number of direct interactions found by the MOE software, with a 0.5 kcal mol^{-1}

ACCEPTED ARTICLE: This manuscript is the version that was accepted after peer review and appeared as an Accepted Article online on June 4, 2019, prior to editing, proofing, and formal publication of the final Version of Record (VoR). Readers should obtain the VoR from the journal website to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

cutoff interaction (Figure S2). While molecule **1a** interacts with G8 through a hydrogen bond with the triazole nitrogen atom at position 2, **1b** makes π-π interactions both with G1 and T11 and arene-H with G1 and G2, while T11 also functions as a side-chain acceptor. On the other hand, while molecule **1c** only makes π-π interactions with G1 and T11, **1d** makes these same interactions together with an extra arene-H interaction with G2. The results also show that the reduced partial charge on the methylated nitrogen atom, as in **1b** and **1d**, plays an important role by allowing a tighter interaction with the quadruplex. It is interesting to note that the central ditriazolyl-benzene moiety is not responsible for defining the relative docking position of the molecule as in previously ditriazolyl-benzene derivatives,[19] but instead this role is played by the quinoline rings at both ends of the molecules.

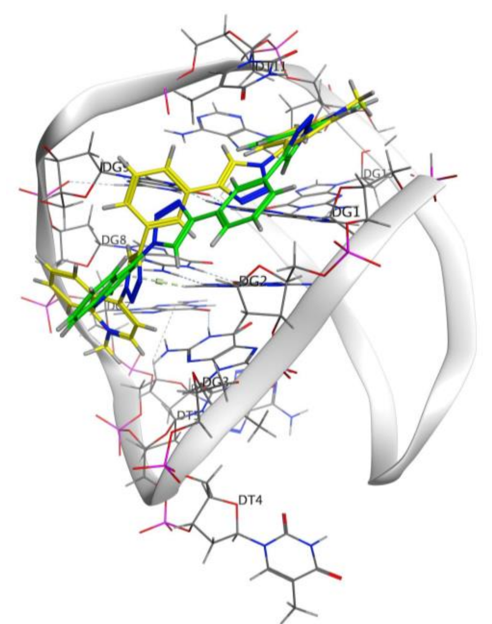


Figure 3. Top-ranked docking poses for compounds **1b** (carbon atoms in green) and **1d** (carbon atoms in yellow). Interactions are represented by dotted lines and only bases closer than 4.5 Å to the molecules are represented.

Compounds **1b** and **1d** were first screened for their cell growth inhibitory activity in a panel of cancer cell lines. The results (Figure 4A) show a consistent potency trend of **1d** > **1b**, which is particularly clear for human colon cancer HT-29 and HCT116 cell lines. A dose-response curve confirmed the activity of **1d** for HT-29 cancer cells cultured in adherent conditions (Figure 4B). It has been proposed that cancer stem cells play a key role in colon cancer initiation, growth, metastasis and therapy resistance.[13] To assess the inhibitory potency of **1d** in colon cancer stem-like cells, a dose-response curve was calculated using human colon cancer HT-29-derived tumor spheres.[25] Our results showed that **1d** also targets HT-29 cells enriched in cancer stem-like cells, with a IC_{50} value in the low micromolar range (IC_{50} 10.63 μM; 95% CI 7.39 – 20.08) (Figure 4B). Conventional chemotherapeutic regimens are effective in reducing tumor mass, but often fail to eliminate stem-like cancer cells, leading to eventual tumor relapse. Thus, the discovery of drug-like small molecules active against cancer stem cells could be used in controlling tumor progression, metastatic spread and disease recurrence.[13]

In summary, we have used a fragment-based approach to design a family of new flexible G4 ligands with good calculated oral drug-like properties (Table S1).[26] Studies of interaction with different DNA sequences indicate that compounds **1b** and **1d** are able to induce and selectively stabilize G4 DNA structures in a dose-dependent manner and preliminary molecular docking studies suggest that these molecules target both the G-quartet and the groove, with the quinoline rings having a major role in the interaction with G4s. Importantly, compound **1d** showed short-term anti-proliferative activity, particularly against colon cancer cells, and good activity against colon cancer stem-like cells.

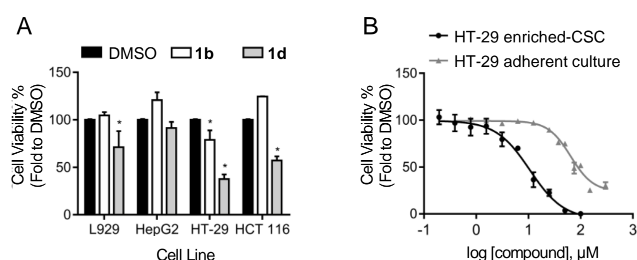


Figure 4. Evaluation of the cytotoxic effect of compounds **1b** and **1d**. (A) Human cancer cell lines HCT116, HT29, HepG2 and murine fibrosarcoma cell line L929 were cultured in adherent conditions and incubated with compounds at 50 μM for 72 h. (B) Dose-response curves for compound **1d** incubated with HT29 cancer cells cultured in adherent conditions for 72 h and with HT-29-derived tumor spheres (HT-29 enriched-CSC) for 7 days. Results are expressed as mean percentage ± SEM from at least 3 independent experiments and normalized to vehicle control (DMSO). $^{*}p < 0.05$ from vehicle control.

The fact that **1d** binds to more than one G4 type suggests that it can target multiple G4s. This would confer therapeutic advantage in complex human cancers, where multiple cancer genes and pathways need to be down-regulated in order to achieve high levels of anticancer potency.[27] The relative importance of G4 nucleic acids in the control of cancer stem cells proliferation compared with cancer non-stem cells has been poorly studied. A G4 has been implicated as a positive regulator of octamer-binding protein 4 (Oct4) expression, a transcription factor associated with pluripotent properties of embryonic and cancer stem cells.[28] Furthermore, a few studies have suggested that cancer stem cells may be more susceptible to loss of telomerase function, either by transcriptional inhibition of *TERT* and *c-MYC*,[29] or by induction of G4 formation at telomeres.[11][12] Overall, compound **1d** is a G4-stabilizing ligand based on quinoline and triazole heterocycles, which shows activity against colon cancer stem-like cells and good predicted oral drug-like properties. As such, this compound could be a promising lead for the development of novel drugs targeting long-lasting and chemo-resistant cancer stem cells.

Experimental Section

Detailed experimental procedures, including compounds characterization data and additional docking studies results can be found in the supporting information file.

ACCEPTED ARTICLE: This manuscript is the version that was accepted after peer review and appeared as an Accepted Article online on June 4, 2019, prior to editing, proofing, and formal publication of the final Version of Record (VoR). Readers should obtain the VoR from the journal website to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

Acknowledgements

This work has received funding from from European Structural & Investment Funds through the COMPETE Program – Programa Operacional de Lisboa, under the Program grant LISBOA-01-0145-FEDER-016405 and from National Funds through FCT, under the Program grant SAICTPAC/0019/2015. J. Vitor's lab has been financed by New England Biolabs, Inc. (USA).

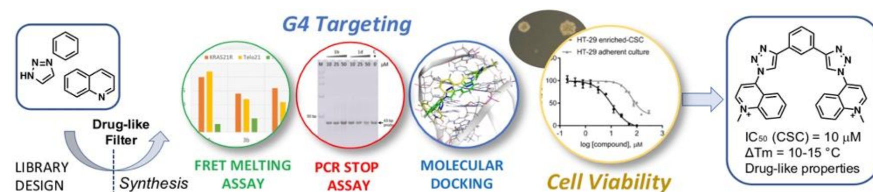
Keywords: DNA • quadruplex • heterocycle • cancer stem cell • drug design

References:

[1] A. Paulo, C. C. Castillo, S. Neidle, in *Compr. Med. Chem. III* (Eds.: S. Chakckalamannil, D.P. Rotella, S.S. Ward), Oxford: Elsevier, **2017**, pp. 308–340.
[2] R. Hänsel-Hertsch, M. Di Antonio, S. Balasubramanian, *Nat. Rev. Mol. Cell Biol.* **2017**, DOI 10.1038/nrm.2017.3.
[3] A. P. Francisco, A. Paulo, *Curr. Med. Chem.* **2017**, *24*, 4873–4904.
[4] G. Biffi, D. Tannahill, J. McCafferty, S. Balasubramanian, *Nat. Chem.* **2013**, *5*, 182–186.
[5] A. Henderson, Y. Wu, Y. C. Huang, E. A. Chavez, J. Platt, F. B. Johnson, R. M. Brosh, D. Sen, P. M. Lansdorp, *Nucleic Acids Res.* **2014**, *42*, 860–869.
[6] M. Ghosh, M. Singh, *Nucleic Acids Res.* **2018**, *46*, 10246–10261.
[7] N. Maizels, L. T. Gray, *PLoS Genet.* **2013**, *9*, DOI 10.1371/journal.pgen.1003468.
[8] B. Herdy, C. Mayer, D. Varshney, G. Marsico, P. Murat, C. Taylor, C. D'Santos, D. Tannahill, S. Balasubramanian, *Nucleic Acids Res.* **2018**, *46*, 11592–11604.
[9] J. L. Huppert, S. Balasubramanian, *Nucleic Acids Res.* **2007**, *35*, 406–413.
[10] R. Hänsel-hertsch, D. Beraldi, S. V Lensing, G. Marsico, K. Zyner, A. Parry, M. Di Antonio, J. Pike, H. Kimura, M. Narita, et al., *Nat. Genet.* **2016**, *48*, 1267–1272.
[11] P. Phatak, J. C. Cookson, F. Dai, V. Smith, R. B. Gartenhaus, M. F. G. Stevens, A. M. Burger, *Br. J. Cancer* **2007**, *96*, 1223.
[12] T. Nakamura, S. Okabe, H. Yoshida, K. Iida, Y. Ma, S. Sasaki, T. Yamori, K. Shin-Ya, I. Nakano, K. Nagasawa, et al., *Sci. Rep.* **2017**, *7*, 1–11.
[13] A. Zeuner, M. Todaro, G. Stassi, R. De Maria, *Cell Stem Cell* **2014**, *15*, 692–705.
[14] H. Xu, M. Di Antonio, S. McKinney, V. Mathew, B. Ho, N. J. O'Neil, N. Dos Santos, J. Silvester, V. Wei, J. Garcia, et al., *Nat. Commun.* **2017**, *8*, 14432.
[15] A. R. Duarte, E. Cadoni, A. S. Ressurreição, R. Moreira, A. Paulo, *ChemMedChem* **2018**, *13*, 869–893.
[16] J. Amato, A. Pagano, D. Capasso, S. Di Gaetano, M. Giustiniano, E. Novellino, A. Randazzo, B. Pagano, *ChemMedChem* **2018**, *13*, 406–410.
[17] D. Musumeci, J. Amato, P. Zizza, C. Platella, S. Cosconati, C. Cingolani, A. Biroccio, E. Novellino, A. Randazzo, B. Pagano, *Biochim. Biophys. Acta - Gen. Subj.* **2017**, *1861*, 1341–1352.
[18] A. D. Moorhouse, S. Haider, M. Gunaratnam, D. Munnur, S. Neidle, J. E. Moses, *Mol. Biosyst.* **2008**, *4*, 629–642.
[19] A. D. Moorhouse, A. M. Santos, M. Gunaratnam, M. Moore, S. Neidle, J. E. Moses, *J. Am. Chem. Soc.* **2006**, *128*, 15972–15973.
[20] S. Müller, K. Laxmi-Reddy, P. V Jena, B. Baptiste, Z. Dong, F. Godde, T. Ha, R. Rodriguez, S. Balasubramanian, I. Huc, *Chembiochem* **2014**, *15*, 2563–2570.
[21] C. M. Lombardo, I. S. Martínez, S. Haider, V. Gabelica, E. De Pauw, J. E. Moses, S. Neidle, *Chem. Commun.* **2010**, *46*, 9116–9118.
[22] T. A. Phan, Y. S. Modi, D. J. Patel, *J. Am. Chem. Soc.* **2004**, *126*, 8710–8716.
[23] R. I. Mathad, E. Hatzakis, J. Dai, D. Yang, *Nucleic Acids Res.* **2011**, *39*, 9023–9033.
[24] J. Lavrado, S. A. Ohnmacht, I. Correia, S. Pisco, M. Gunaratnam, R. Moreira, S. Neidle, J. V. A. Santos, A. Paulo, *ChemMedChem* **2015**, *10*, 836–849.
[25] D. M. Pereira, S. E. Gomes, P. M. Borralho, C. M. P. Rodrigues, *Cell Death Discov.* **2019**, *5*, 1–13.
[26] D. F. Veber, S. R. Johnson, H. Cheng, B. R. Smith, K. W. Ward, K. D. Kopple, *J. Med. Chem.* **2002**, *45*, 2615–2623.
[27] C. Marchetti, K. G. Zyner, S. A. Ohnmacht, M. Robson, S. M. Haider, J. P. Morton, G. Marsico, T. Vo, S. Laughlin-Toth, A. A. Ahmed, et al., *J. Med. Chem.* **2018**, *61*, 2500–2517.
[28] D. Renčiuk, J. Ryneš, I. Kejnovská, S. Foldynová-Trantírková, M. Andäng, L. Trantírek, M. Vorlíčková, *Biochim. Biophys. Acta - Gene Regul. Mech.* **2017**, *1860*, 175–183.
[29] J. Chappell, S. Dalton, M. F. Roussel, G. W. Robinson, P. Gallant, *Cold Spring Harb. Perspect. Med.* **2013**, *3*, DOI 10.1101/cshperspect.a014381.

ACCEPTED ARTICLE: This manuscript is the version that was accepted after peer review and appeared as an Accepted Article online on June 4, 2019, prior to editing, proofing, and formal publication of the final Version of Record (VoR). Readers should obtain the VoR from the journal website to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

Entry for the Table of Contents



Targeting cancer stem-cells with G4 ligands. Conventional chemotherapeutic regimens are effective in reducing tumor mass, but often fail to eliminate cancer stem cells (CSC) leading to tumor relapse. Combining heterocycles we discovered a new G-quadruplex (G4)-interactive small molecule with predicted oral drug-like properties and good activity against colon CSC.