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Design, synthesis and evaluation of 4,5-di-substituted acridone ligands with high G-quadruplex affinity and selectivity, together with low toxicity to normal cells

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ABSTRACT

A series of 4,5-di-substituted acridones have been designed and synthesized. Several compounds show high affinity for telomeric G-quadruplex DNA in classical and competition FRET assays, together with low duplex DNA affinity, although they do not show activity in a telomerase assay or evidence of telomere shortening. They have low toxicity against a panel of cancer cell lines and a normal human fibroblast line, and produce potent senescence-based long-term growth arrest in the MCF7 and A549 cancer cell lines.

Telomeres are highly specialized DNA–protein structures at the end of eukaryotic chromosomes.[1] Telomeric DNA erosion in each replication cycle due to the end-replication problem[2] occurs in normal somatic cells, so that eventually telomeric DNA reaches a critically short length, the Hayflick limit, and cells enter the replicative senescence state.[3,4] The senescent phenotype must be overcome for cells to reach the immortalized state that characterizes cancer,[5] in most cases by activation of the reverse transcriptase enzyme telomerase,[6] which is expressed in 85–90% of cancer cells yet is not significantly expressed in somatic tissue.[7] Telomerase maintains telomeric DNA length and its inhibition induces cell senescence and is thus a target for therapeutic intervention.[8,9]

One approach to selectively inhibit telomere maintenance in cancer cells is to target the telomerase substrate, the short sequence of single-stranded DNA at the extreme 3′ end of telomeric DNA. A wide range of small-molecule ligands have been found to stabilize the G-quadruplex (G4) DNA conformation that the single-stranded 3′ end of telomeric DNA can adopt. G4 formation can displace bound hPOT1 protein at the single-stranded end, which is involved in the regulation of telomerase activity,[10] and may dissociate telomerase from its telomere capping function.[11]

The majority of G4-binding ligands[12] have a planar aromatic chromophore that complements the G-quartet surface of a human telomeric DNA quadruplex structure.[13] Crystal structures are now available for human telomeric G4 DNAs and their ligand complexes with a tri-substituted acridine[14] and a tetra-substituted naphthalenediimide compound,[15] and can be used in structure-based drug design studies.

Several series of 2,6, 2,7 and 3,6-di-substituted acridone compounds have been reported previously as G4 ligands and show patterns of activity that are similar to their di-substituted acridine counterparts,[16] with short-term toxicities in the low μM range but with only low levels of discrimination between cancer and normal cell lines. In this work we explore further the potential of acridone-based compounds as G4 ligands and selective telomere targeting agents through the synthesis and biological evaluation of a novel series of 4,5-di-substituted acridone derivatives. We postulated that the incorporation of aromatic side chains to the acridone core in the 4 and 5 positions, conjugated by the use of amide bonds, would confer enhanced planarity on the molecule, hence improving π–π stacking interactions with a G-quartet. Compounds were designed with these considerations in mind (Fig. 1), and also contain several features that were anticipated to contribute to the hypothesized improvements in G4 affinity, namely: (i) the NH groups attached to the acridone would play a role in substituent planarity by participating in a pair of bifurcated hydrogen bonds to the carbonyl groups of the 4- and 5-substituted amides, which themselves would be conjugated with the acridone moiety, and (ii) alkyl chains bearing a tertiary amine, a common feature of G4 ligands.[17,18]

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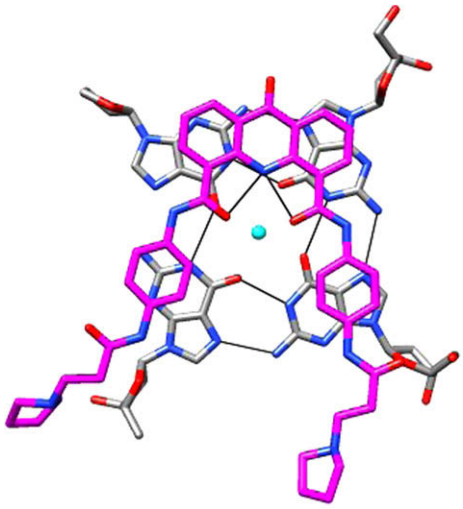


Figure 1. Qualitative molecular model of compound **9** stacked onto a terminal G-quartet at the 3′ face of the human intramolecular G4 structure.[13–15]

Compounds **9–27** were chosen as synthetic targets, (Table 1), in order to examine in particular diversity in side-chain substituents and side-chain length, as well as substitution pattern in the benzene ring and presence or absence of the carbonyl group. The synthesis of the acridone core and the side chains was conducted independently and converged in the last and key step, amide bond formation between the 4,5-dicarboxyl acridone **1** (or the 4-carboxyl acridone **2** for the mono-substituted compound **27**) and the side chains (Scheme 1).

Compound **1** was synthesized using the reported method.[19] Although the overall yield was low (16% for 3 steps), in our hands this method proved to be more time efficient than an alternative literature method.[20] Compound **2** was synthesized by modification of the reported method[19] using the Jourdan–Ullmann reaction with 2-bromobenzoic acid and anthranilic acid followed by ring closure with concentrated H_2SO_4.

The general synthesis pathway for the anilinic side chains is shown in Scheme 2.[21]

The synthesis of the side chain leading to compound **16** involved reaction of 4-nitroaniline with succinic anhydride, followed by amide formation with pyrrolidine and reduction of the nitro group using ammonium formate (Scheme 3). Synthesis of the side chain leading to compound **25** involved synthesis of compound **6**[22] followed by amide formation and reduction of the nitro group using ammonium formate (Scheme 3).

For the coupling reactions an approach using PyBOP was found to be the most efficient for this particular reaction. Several solvent systems (DMF/DMA/DCM/MeCN mixtures) were trialed and a 3:1 DMF:MeCN mixture gave the best results. Up to 4 equiv of the corresponding amines were used to increase conversion, as initial reactions using fewer equivalents gave unwanted mono-coupled byproducts.

Compounds **9–27** were successfully synthesized with this methodology.[23] Isolation involved precipitation of the product from the reaction mixture by EtOAc addition, and purified by repeated precipitation of the product from a DMF solution with EtOAc and preparative HPLC when required (when the purity was lower than 95%). Typically the purity obtained after reprecipitation was >90% and the yield was >50%.

G4 DNA binding of compounds **9–27** and a reference compound (BRACO-19: **BR-19**) were assessed with a FRET (Fluorescence Resonance Electron Transfer) assay using a human telomeric G4 DNA sequence.[24] The results confirm and extend the assumptions that had been made when designing the library (Table 1). The benzene rings in the side chains had been postulated as important for G4 interaction. This is supported by the low increase in melting temperature (ΔT_m) for the compound with nonaromatic side chains **26** (ΔT_m(1 μM) = 7 °C). Compound **25**, which has *N*-methyl pyrrole substituting for benzene, shows only limited stabilization (ΔT_m(1 μM) = 15 °C) possibly due to a less favourable out-of plane conformation as a result of the methyl groups. Compounds **9**, **11**, **12**, **13**, **17**, **19**, **20** and **24** all showed similar high levels of stabilization ability for the telomeric G4 sequence with ΔT_m(1 μM) values between 28 and 34 °C. These are comparable to the 32 °C (ΔT_m(1 μM)) for the tri-substituted acridine compound **BR-19**.

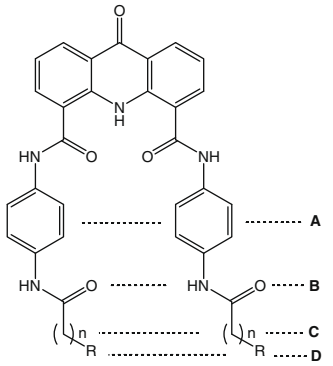
All these acridones have side chains terminating in a basic tertiary amine, which is protonated at pH 7. Comparison with the less basic morpholine analogues **10** and **18** (ΔT_m(1 μM) of 19 and 20 °C, respectively) and especially with the neutral amide analogue **16** (ΔT_m(1 μM) = 0 °C) highlights the importance of the positively charged groups. Compound **14** with a secondary cyclohexyl amine (ΔT_m(1 μM) = 24.5 °C) and **15** with an imidazole group (ΔT_m(1 μM) = 15 °C) were less effective than those with aliphatic tertiary amines. Interestingly, the length of the side chains is critical for their potency. Compounds **9**, **11**, **12**, **13**, **17**, **19**, **20** and **24** all have the same number of side-chain atoms between the acridone and the tertiary amines. Comparing these values with the obtained for the shorter side-chain analogue **21** (ΔT_m(1 μM) = 25 °C) and those with longer side chains **22** (ΔT_m(1 μM) = 15 °C) and **23** (ΔT_m(1 μM) = 22 °C) shows that the optimum length is $n = 2$ (Table 1), in accord with indications from preliminary molecular modelling. The similarity of the ΔT_m(1 μM) values between compounds **9** and **17**, **10** and **18**, **11** and **19** and **12** and **20** shows that the presence or absence of the carbonyl group in the side chains does not affect G4 stabilization. The amide bond is a potential site for metabolism so this finding has relevance for further development of these compounds. Mono-substitution of the acridone core (compound **27**) results in reduced G4 stabilization (ΔT_m(1 μM) = 11 °C).

Duplex-binding experiments show that the compounds as a class have high selectivity with those having the largest G4 ΔT_m(1 μM) values having duplex DNA ΔT_m(1 μM) values of 1.5–3 °C. At 10 μM ligand concentration they showed low duplex DNA stabilization with the exception of the meta-substituted compound **24** (ΔT_m(10 μM) = 30 °C). The shape of this compound may be more adaptable to one of the grooves of duplex DNA. Competition experiments with calf thymus DNA (Fig. 2 and Supplementary data) show that G4 ΔT_m values are little changed in the presence of >400 excess of duplex DNA, confirming that this family of 4,5-acridones have unusually high selectivity for G4 DNAs. Their selectivity for individual G4s, for example, those found in promoter regions, remains to be established. The reference compound BRACO-19 (**BR-19**) showed significant duplex DNA affinity in the FRET assay (ΔT_m(1 μM) = 6 °C), in contrast to the behavior of the 4,5-acridones (Fig. 2).

The short-term (72 h) toxicity of the 4,5-acridones was evaluated using the SRB assay.[25] None of the 4,5-acridones examined (Table 1) show significant toxicity in the somatic fibroblast cell line IMR90, apart from the abnormal behavior shown by compound **27** which is active in all three cell lines and also has low ΔT_m activity. This suggests the possibility of a therapeutic window for cancer versus somatic cells. Several compounds are selectively toxic to the two cancer cell lines examined, A549 (lung) and MCF7 (breast). The latter was overall the most sensitive cell line with compounds **9**, **15**, **16**, **22**, **23**, **25** and **27** having IC_{50} values lower than 25 μM. The A549 line was overall less sensitive although compounds **9**, **11**, **15**, **22**, **25** and **27** have IC_{50} values <25 μM.

Overall there is not a high correlation between G4 affinity and short-term toxicity. Two compounds with the highest ΔT_m values in the G4 FRET assay, **9** and **11** (34 and 32 °C, respectively) were

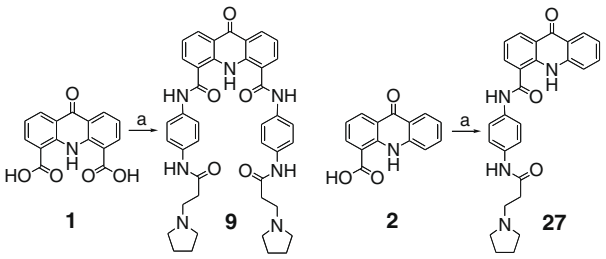
Table 1
The 4,5-acridones synthesized together with details of their G4 and duplex DNA melting behaviour and cell growth inhibition in two cancer cell lines (MCF7 and A549) and a human fibroblast cell line (IMR90)



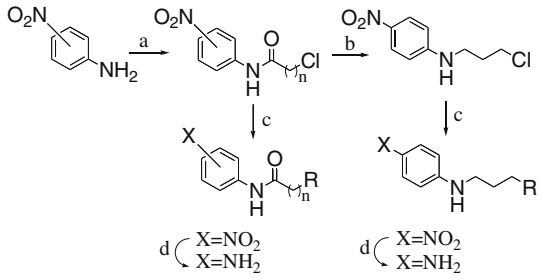
Compound	A group at 1st sector	B Presence of carbonyl group	C linker length (n)	D end group (R)	G4 ΔT_m (°C)	Duplex ΔT_m (°C)	MCF7 IC_{50} (μM)	A549 IC_{50} (μM)	IMR90 IC_{50} (μM)
9	*p*-Ph[a]	Yes	2	Pyrrolidine	34	2.5	23	16	>100
10	*p*-Ph	Yes	2	Morpholine	19	0	>100	>100	>100
11	*p*-Ph	Yes	2	Dimethylamine	32	2.5	55	15	>100
12	*p*-Ph	Yes	2	*N*-methylpiperazine	31	1.5	99	>100	>100
13	*p*-Ph	Yes	2	Piperidine	31	1.5	62	>100	>100
14	*p*-Ph	Yes	2	Cyclohexaneamine	24.5	0	28	31	>100
15	*p*-Ph	Yes	2	Imidazole	15	0.5	17	19	>100
16	*p*-Ph	Yes	2	C=O-pyrrolidine	0	0	21	27	>100
17	*p*-Ph	No	3	Pyrrolidine	33	2.5	60	66	>100
18	*p*-Ph	No	3	Morpholine	20	0	>100	>100	>100
19	*p*-Ph	No	3	Dimethylamine	31	2.5	53	>100	>100
20	*p*-Ph	No	3	*N*-Methylpiperazine	28	1.5	68	>100	>100
21	*p*-Ph	Yes	1	Pyrrolidine	25.5	0.5	72	>100	>100
22	*p*-Ph	Yes	3	Pyrrolidine	15	0	15	8	>100
23	*p*-Ph	Yes	4	Pyrrolidine	22.5	0.75	2	43	>100
24	*m*-Ph[b]	Yes	2	Pyrrolidine	29	3	84	>100	>100
25	*N*-MePy[c]	n.a.	3	Pyrrolidine	15	0	19	21	>100
26	*N*-DMPDA[d]	n.a.	n.a.	n.a.	7	1	40	62	>100
27[e]	*p*-Ph	Yes	2	Pyrrolidine	11	0.5	9	16	7.5
BR-19	n.a.	n.a.	n.a.	n.a.	32	6	2.5	2.4	26

The figure above shows the general structure of the final compounds. The table includes thermal stabilization (ΔT_m) data for each compound, with a human telomeric G4 and a duplex DNA, both determined by FRET methods.[24] The three columns on the right give short-term (IC_{50}) data in μM for 96 hr exposures with the MCF7 and A549 cancer cell lines, and the human fibroblast normal cell line IMR90. Esds for ΔT_m are ±0.5°, and for IC_{50} are ±2 μM.
n.a. = not applicable.

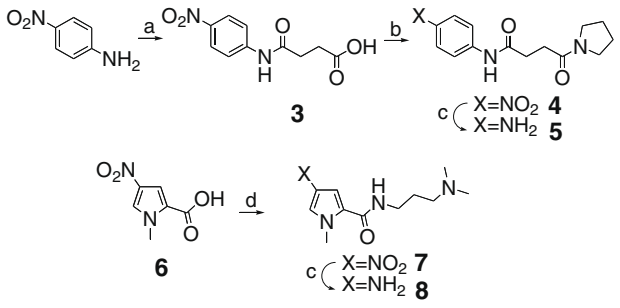
[a] *para*-Phenyl.
[b] *meta*-Phenyl.
[c] 4-Amino-1-methyl-2-carboxamide-1*H*-pyrrole.
[d] *N*,*N*-Dimethylpropane-1,3-diamine.
[e] Mono-substituted analogue.



Scheme 1. Synthetic route for final compounds **9** and **27**. Reagents and conditions: (a) side chain *N*-(4-aminophenyl)-3-(pyrrolidin-1-yl)propanamide, PyBOP, DMF/acetonitrile, rt, 24 h.



Scheme 2. General synthetic route for side chains. Please refer to Supplementary data for specific structures. Reagents and conditions: (a) chloroacyl chloride, Δ; (b) $Me_2S.BH_3$, THF; (c) amine R, neat/THF; (d) ammonium formate, Pd/C, ethanol, Δ.



Scheme 3. Synthetic route for side chains **5** and **8**. Reagents and conditions: (a) succinic anhydride, toluene, reflux, 1 h; (b) pyrrolidine, HOBt, EDCI, DMF, rt, 16 h; (c) ammonium formate, Pd/C, ethanol, 120 °C, 4 min; (d) 3-(pyrrolidin-1-yl)propan-1-amine, HOBt, EDCI, acetonitrile, rt, 16 h.

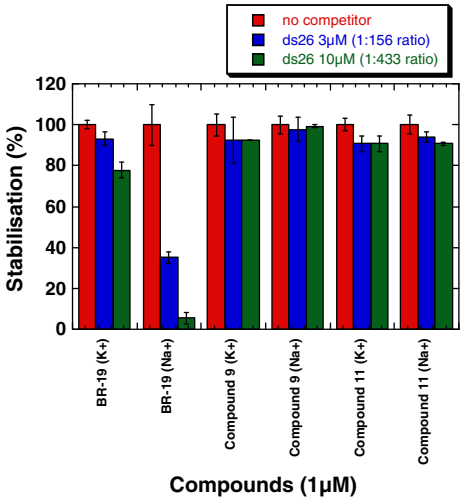


Figure 2. FRET data[25] on BRACO-19 and compounds **9**, **11** in competition with varying ratios of duplex DNA in excess, showing the high G4 selectivity of the acridones.

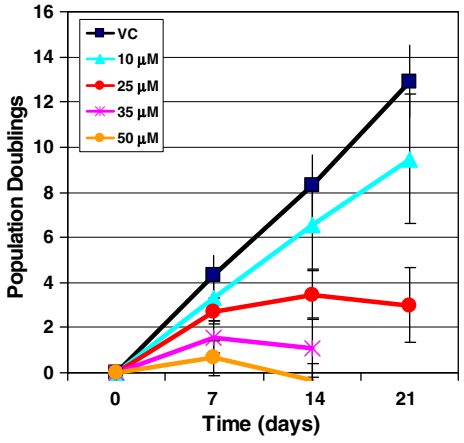


Figure 3. Effects of long-term exposure to compound **24** on proliferation in the MCF7 cell line. **24** was added initially to cells, then three times weekly (on days 2, 4 and 6). At each re-treatment the medium was discarded and the cells washed with PBS. Fresh media and compound solution were then added. VC represents the control experiment in the absence of ligand.

amongst the most active in the SRB assay with the two cancer cell lines used here. The morpholine compounds **10** and **18** had more modest ΔT_m capabilities and showed no cellular toxicity <100 μM, although uptake problems cannot be discounted. Several compounds showed high cancer cell toxicity while being poor G4 ligands and others stabilized the G4 strongly but showed high IC_{50} values.

Anti-proliferative effects were further examined by long-term incubation of selected compounds in the cancer cell lines MCF7 and A459 at sub-toxic doses. For compound **24** (IC_{50} = 84 μM) and the MCF7 cell line, a concentration of 10 μM showed an effect on cell proliferation (Fig. 3); after five weeks cells receiving this treatment had gone through 17.5 pd while the control cells underwent 22.5 pd. Experiments with concentrations >25 μM had to be discontinued after three weeks or less; cell growth was profoundly inhibited after seven days. Similar behavior was observed for A549 cell (see Supplementary data).

The targeting of telomeres with G4 ligands can result in cells with dysfunctional telomeres[26] and the onset of the senescent phenotype.[27] To gain an insight into the mechanism of action of one of these ligands (compound **24**), the number of senescent cells at different stages during the long-term experiments in the MCF7 and A549 cell lines was quantified.[28] The former after 25 μM of compound **24** treatment had 25% of cells undergoing senescence after three weeks; lower concentrations caused no senescence. Compound **24** also caused senescence in A549 cells in a concentration-dependent manner (Fig. 4). However it is not clear from this data alone whether these relatively low levels of senescence are sufficient to explain the inhibition of cell proliferation observed in the long-term studies.

Inhibition of telomerase or telomere uncapping can result in telomere attrition. Thus potentially, G4 ligands can cause telomere shortening by either of these mechanisms and such results have been reported for several ligands.[11] Telomere length measurements were undertaken on DNA extracted from cells following long-term exposure to compounds **11** and **24** but no telomere shortening was detected in cells treated for up to six weeks (results not shown). Measurements of telomerase inhibitory activity using

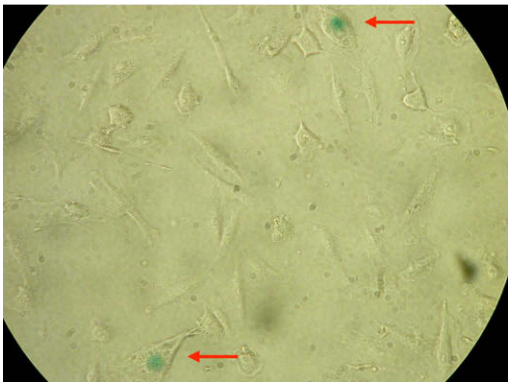


Figure 4. Micrograph of A549 cells after treatment with compound **24** and after staining for senescence. Red arrows indicate cells with the characteristic blue colour associated to senescent cells.

both the TRAP-LIG[29] and direct telomerase assays[30] did not show any activity for any of the 4,5-acridones.[31]

Inhibition of cell proliferation in long-term culture experiments, together with evidence of cellular senescence, is suggestive of a telomere-targeted mechanism of cancer cell toxicity, which is supported by the high G4 affinity of some (but not all) of the 4,5-acridone compounds. The lack of telomerase inhibition and of telomere shortening, demonstrates that the catalytic function of telomerase is not involved, although telomerase uncapping from the 3′ end of telomeres may be a contributor to the observed cancer cell selectivity. We cannot also discount other mechanisms of action, including the involvement of promoter G4s since these compounds are not specific for telomeric G4s.[31] The properties of these 4,5-acridones are in striking contrast with a recently- reported series of 4,5-di-substituted acridines (with amidoalkylamino side chains).[32] These show low G4 stabilization ability, with ΔT_m(1 μM) values in the range 0–3 °C, and with some compounds having potent in vitro telomerase inhibitory activity.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.07.033.

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21. Acylation of either 3- or 4-nitro aniline used the correspondent chloroacyl chloride as a solvent under mild heating for 8–16 h. For the substitution reaction the chloro compounds were treated with the different amines as solvent, except for dimethylamine when a 2 M solution in THF was used, with mild heating for 24 h. The nitro compounds were reduced with ammonium formate and Pd/C in ethanol in a microwave with very short reaction times (2–3 min) at 120 °C to give the targeted anilines. When necessary, an extra step was performed for carbonyl group reduction with a $Me_2S{\cdot}BH_3$ complex in THF, prior to the reaction with the amines.
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23. Synthesis and analytical data for compound **9**. Information on other compounds is provided in the Supplementary data.
N^4,N^5-Bis(4-(3-(pyrrolidin-1-yl)propanamido) phenyl)-9,10-dihydro-9-oxoacridine-4,5-dicarboxamide (**9**): A solution of **1** (20 mg, 0.071 mmol), PyBOP (3 equiv, 0.213 mmol, 111 mg) and *N*-(4-aminophenyl)-3-(pyrrolidin-1-yl)propanamide (4 equiv, 1.818 mmol, 69 mg) in a mixture of anhydrous DMF (3 ml) and acetonitrile (1 ml) under N_2 atmosphere was stirred at rt for 24 h. EtOAc (30 ml) was added and the yellow solid formed was filtered, washed with EtOAc (3 × 5 ml) and ether (3 × 5 ml). Yield: 42.8 mg, 84.5%. Analytical data: decomp. at 250 °C; IR μ (cm^{-1}) 3283, 1644, 1610, 1510, 1433, 1406, 1311, 1238, 1022, 832; ^{1}H NMR (DMSO-d_6) δ: 1.89 (m, 8H), 2.79 (t, 4H, J = 6.9 Hz), 3.10 (m, 8H), 3.33 (m, 4H), 7.46 (t, 2H, J = 7.7 Hz), 7.61 (d, 4H, J = 9.1 Hz), 7.67 (d, 4H, J = 9.1 Hz), 8.34 (dd, 2H, J = 7.5, 1.4 Hz), 8.50 (dd, 2H, J = 8.1, 1.3 Hz), 10.29 (s, 2H), 10.62 (s, 2H), 13.26 (s, 1H); ^{13}C NMR (DMSO-d_6) δ: 23.02 (4 × CH_2), 35.52 (2 × CH_2), 51.28 (2 × CH_2), 53.28 (2 × CH_2), 119.19 (4 × CH), 120.58 (2 × C), 120.63 (2 × C), 121.06 (2 × C), 121.29 (4 × CH), 129.78 (2 × CH), 133.66 (2 × CH), 134.05 (2 × CH), 135.47 (2 × C), 139.25 (2 × C), 165.56 (2 × C═O), 169.60 (2 × C═O), 176.19 (C═O); HRMS (ESI+) calcd $C_{41}H_{44}N_7O_5$ $[M+H]^+$ 714.3398. Found: 714.3395.
24. The FRET DNA melting assay was performed as described previously (Schultes, C. M.; Guyen, B.; Cuesta, J.; Neidle, S. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4347). The full protocol is given in the Supplementary data. The tagged DNA sequences used were: 5′-*FAM*-d(GGG[TTAGGG]$_3$)-*TAMRA*-3′ for the G4 and 5′-*FAM*-dTATAGCTATA-*HEG*-TATAGCTATA-*TAMRA*-3′ (*HEG* linker: [(–CH_2–CH_2–O–)$_6$]) for the duplex experiment.
25. See Supplementary data for more information.
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28. 1 × 10^5 cells per well were cultivated in 2 ml of medium plus compound in 96-well plates. After 24 h the cells were stained for senescence with a β-galactosidase kit (Cell Signalling Technology). Blue stained senescent cells were quantified by microscopy.
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