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PAPER

Unraveling the relationship between structure and stabilization of triarylpyridines as G-quadruplex binding ligands†

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A series of novel 2,4,6-triarylpyridines have been synthesized and their interactions with intramolecular G-quadruplexes have been measured by Förster Resonance Energy Transfer (FRET) melting and Fluorescent Intercalator Displacement (FID) assays. A few of these compounds exhibit stabilization of G4-DNA that is comparable to other benchmark G4-DNA ligands with fair to excellent G4-DNA *vs.* duplex selectivity and significant cytotoxicity towards HeLa cells. The nature of the 4-aryl substituents along with side chain length governs the G4-DNA stabilization ability of the compounds. In addition, we demonstrate that there is a strong correlation between the ability of the compounds to stabilize the same G4-DNA sequence in K^+ and Na^+ conditions and a strong correlation between the ability of the compounds to stabilize different G4-DNA sequences in K^+ or Na^+ buffer.

Introduction

G-quadruplex DNA (G4-DNA) is a highly dynamic and polymorphic four stranded DNA structure that can form from certain guanine-rich (G-rich) sequences such as those occurring at the 3′-terminus of human telomeric DNA and in the promoter region of certain oncogenes.[1–3] Small molecules which stabilize the G-quadruplex structure can lead to the arrest of proliferation of cancer cells, making G4-DNA an attractive target for selective anti-cancer therapy and drug development.[3–7] Most quadruplex binding ligands interact with G4-DNA by π-stacking with the terminal G-quartet making them relatively insensitive to the specific loops and grooves that differ between quadruplex types.[8] A novel trend in G-quadruplex ligand design is emerging based on enhancing G-quadruplex recognition by the introduction of additional structural elements that target the loops and grooves of G4-DNA.[9–12] These secondary interactions enable the possibility of increased affinity for G4-DNA and better G4-DNA *vs.* duplex selectivity.[1]

Triarylpyridines and terpyridines are a class of compounds that provide structural flexibility in their framework to target the loops and grooves of G4-DNA. These have already been reported to have a good affinity for these structures.[13–16] Since the triarylpyridine central ring nitrogen atom is not protonated at physiological pH we postulate that the additional mobility of the triarylpyridine chromophore in this interaction site may allow the protonated side chains to maximize interactions with the quadruplex. It is also noteworthy that the three bonds connecting the aryl rings to the pyridine core deviate from planarity.[17] Consequentially, we envisage that this additional geometric freedom may further enhance the ability of the side chains to optimize interactions with the quadruplex loops and grooves. Herein, we have synthesized a novel series of functionalized 2,4,6-triarylpyridines and examined the effects of structural features such as the 4-aryl substituents on the central pyridine ring, amidoalkylamino side chain length and terminal amino substituents on the ability of these compounds to stabilize G4-DNA and on their selectivity for G4 over duplex DNA. In terms of success at stabilizing G4-DNA our observations show that there exists three categories within this series of compounds: 1) Compounds which exhibit high stabilization of G4-DNA that are comparable or superior in magnitude to the best G4-DNA ligands but with medium specificity for quadruplex over duplex DNA; 2) Compounds which show mid range stabilization but have high specificity for quadruplex over duplex DNA; and 3) Compounds which exhibit low stabilization of G4-DNA. Furthermore, we observed that the majority of compounds which display high to mid range stabilization of G4-DNA exhibit significant cytotoxicity towards HeLa cells. In addition, we demonstrate that

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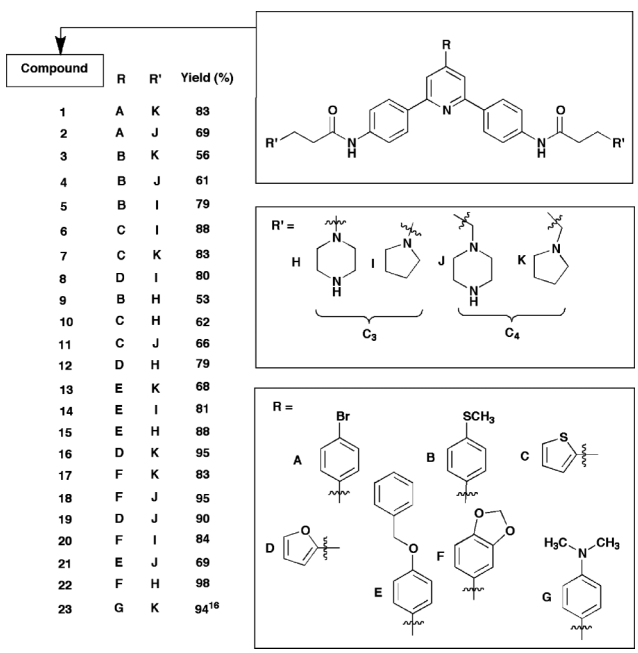
there is a strong correlation between the ability of the compounds to stabilize the same sequence in K^+ and Na^+ buffer and the ability of the compounds to stabilize different sequences in K^+ or Na^+ buffer.

Results and discussion

Synthesis

We previously reported the 'one-pot' synthesis of the triarylpyridine core, 4-aryl-2,6-bis(4-aminophenyl)pyridine, for compounds **1–12**, **16** and **19** (Table 1).[17] This 'one-pot' procedure was also used to synthesize the triarylpyridine core for compounds **13–15**, **17–18** and **20–22** (see Supporting Information for synthetic procedure†). The core platform was then acylated with 3-chloropropionyl chloride or 4-chlorobutyryl chloride followed by aminolysis with pyrrolidine or piperazine to afford the target compounds, **1–22**, in high yields (Table 1) (see Supporting Information for synthetic procedures†). In addition, we previously reported the synthesis of compound **23** using a microfluidic platform.[16] We varied the 4-aryl substituents on the central pyridine ring (R) from five-membered to six-membered rings with various substituents to study the effect of ring size and substituent effects on the ability of these compounds to stabilize G4-DNA (Table 1). We limited the amidoalkylamino side chain length to propyl (C_3) and butyl (C_4) as structure–activity studies[18] on the acridine series showed that increasing the chain length of the 3,6-substituents of BRACO-19 results in a significant loss of affinity for the human telomeric (HTelo) quadruplex (in K^+ conditions) (Table 1). Furthermore, the terminal amino groups used for our study are pyrrolidine (R′ = "I" or "K") and piperazine (R′ = "H" or "J"), as structure–activity studies[18] of the 3,6-disubstituted acridines showed that more bulky terminal amino groups such as piperidine, azepane and azocane result in loss of affinity for the antiparallel HTelo quadruplex (in Na^+ buffer) (Table 1). Amine substituents provide active sites for hydrogen bonding and electrostatic interactions with the sugar–phosphate backbone and the loops and grooves of G4-DNA, hence piperazine was chosen as one of the terminal amino groups in an attempt to compensate for the increase in ring size with the presence of an extra amino group which could engage in hydrogen bonding and electrostatic interactions to improve binding and stabilizing properties.

Table 1 2,4,6-Triarylpyridines synthesized and tested for G-quadruplex stabilization and specificity



FRET-melting

The interaction of each compound with G4-DNA was investigated using Förster Resonance Energy Transfer (FRET) melting (Table 2).[19] The assay is based on the thermal denaturation of fluorescently tagged quadruplex-forming oligonucleotides that mimic the human telomeric overhang, HTelo: 5′-FAM-d($AG_3T_2AG_3T_2AG_3T_2AG_3T$)-TAMRA-3′ (F23T) and 5′-FAM-d($G_3T_2AG_3T_2AG_3T_2AG_3$)-TAMRA-3′ (F21T), and tagged quadruplex-forming oligonucleotides that mimic one of the quadruplex forming sequences within the c-kit promoter and the K-ras promoter respectively: 5′-FAM-d($G_3CG_3CGCGAG_3AG_4$)-TAMRA-3′ (FCkit2T) and 5′-FAM- d($AG_3CG_2TGTG_3A_2GAG_3A_2GAG_5AG_2CAG$)-TAMRA-3′ (FKrasT) (donor fluorophore FAM is 6-carboxy-fluorescein and acceptor fluorophore TAMRA is 6-carboxy-tetramethyl-rhodamine), in 100 mM NaCl 10 mM lithium cacodylate buffer (Na^+) or 10 mM KCl supplemented with 90 mM LiCl (K^+). Though F21T is the more common sequence used for HTelo, we have also used F23T for comparison as it has been reported that placing a fluorophore (usually fluorescein) immediately adjacent to a guanine quenches fluorescence.[20] Ligand-induced change in melting temperature of the quadruplexes (ΔT_m), were obtained to determine the G4-DNA stabilization ability of the ligands (Table 2) (see Supporting Information for ΔT_m values in Na^+†).

Compounds **1** and **3** which contain the C_4-pyrrolidine side chain (R′ = "K") (Table 1) and compound **2** which contains the C_4-piperazine side chain (R′ = "J") (Table 1) exhibited the highest stabilizations for all G4-DNA sequences studied (Table 2). It is also interesting to note that these compounds contain either the *p*-bromophenyl (R = "A") or *p*-thiomethylphenyl (R = "B") 4-aryl substituents on the central pyridine ring (Table 1). These stabilizations are significantly higher than the previously reported stabilizations for a series of triarylpyridines with the c-kit2 and HTelo quadruplexes in 60 mM K^+ buffer.[15] Indeed, these values are comparable or superior in magnitude to the best G4-DNA ligands reported so far such as telomestatin, BRACO-19 and 360A.[21–23] The decrease in chain length by one CH_2 unit for compound **3** (C_4-pyrrolidine) to compound **5** (C_3-pyrrolidine) and for compound **4** (C_4-piperazine) to compound **9** (C_3-piperazine) significantly reduces the potencies with ΔT_m values ≤10 °C (Table 2). Changing the 4-aryl substituent of compounds **1–3** from *p*-bromophenyl and *p*-thiomethylphenyl to either the five-membered thiophene or furan substituents (compounds **7**, **11**, **16** and **19**), the bulky benzyl ether substituent (compounds **13** and **21**), piperonal substituent (compounds **17** and **18**) or *p*-dimethylaminophenyl substituent (compound **23**) also results in a significant loss of G4-DNA stabilization (Table 2). Hence, for high stabilization, a C_4 chain length is preferred over a C_3 chain length, and a *p*-bromophenyl or *p*-thiomethylphenyl 4-aryl substituent on the central pyridine

Table 2 Change in quadruplex melting temperatures (ΔT_m) in the presence of triarylpyridines **1–23** (1 μM) determined using a FRET assay, carried out with G4 oligos (0.2 μM) in 10 mM K^+ buffer (pH 7.2) and specificity (S) determined from a ds26 FRET competition assay

	F21T[e]		F23T[e]		FKrasT[e]		FCkit2T[e]	
Compound	ΔT_m (°C)	S[a]	ΔT_m (°C)	S[a]	ΔT_m (°C)	S[a]	ΔT_m (°C)	S[a]
1	23.4	1.0	23.9	0.59	26.5	0.71	> 30[c]	0.56[d]
2	30.5	0.41	32.6	0.36	32.8	0.43	> 30[c]	0.45[d]
3	> 40[c]	0.65[d]	> 40[c]	0.56[d]	41.6	0.3	> 30[c]	0.76[d]
4	13.9	0.65	15	0.54	18.5	0.82	11.3	0.93
5	9.6	1.0	8.1	1.0	11.1	0.98	5.4	—[b]
6	12.2	0.95	10	1.0	10	0.67	9.4	1.0
7	10.4	0.99	9.1	1.0	10.8	0.92	8.5	—[b]
8	10.7	1.0	8.4	1.0	10.9	0.89	11.2	1.0
9	7.8	—[b]	6.5	—[b]	8.0	—[b]	4.2	—[b]
10	7.7	—[b]	5.3	—[b]	7.5	—[b]	4.7	—[b]
11	8.5	—[b]	6.8	—[b]	9.0	—[b]	4.2	—[b]
12	6.7	—[b]	6.0	—[b]	8.3	—[b]	6.6	—[b]
13	8.7	—[b]	8.7	—[b]	11.6	1.0	3.2	—[b]
14	6.2	—[b]	3.6	—[b]	5.2	—[b]	1.6	—[b]
15	3.3	—[b]	3	—[b]	3.1	—[b]	2.3	—[b]
16	12.6	0.93	10.7	1.0	11.3	1.0	11.7	0.92
17	10.5	0.91	10.5	1.0	11.4	0.98	8.3	0.98
18	7.1	—[b]	5.6	—[b]	7.7	—[b]	5.5	—[b]
19	7.2	—[b]	6.2	—[b]	6.6	—[b]	5.4	—[b]
20	10.1	0.92	9	1.0	11.7	1.0	5.2	—[b]
21	4	—[b]	3	—[b]	4.7	—[b]	1.4	—[b]
22	6.9	—[b]	6.3	—[b]	5.4	—[b]	3.5	—[b]
23	5.9	—[b]	6.0	—[b]	7.1	—[b]	3.6	—[b]

[a] S is the ratio of ligand-induced ΔT_m of G4-DNA in the presence of ds26 (10 μM) competitor and in the absence of competitor. [b] Not determined – selectivity experiments were performed with compounds exhibiting ΔT_m >10 °C. [c] Stabilization was >30 °C for FCkit2T and >40 °C for F21T and F23T and hence absolute ΔT_m could not be determined at 1 μM ligand. [d] S was calculated assuming a ΔT_m of 40 °C for F21T and F23T and 30 °C for FCkit2T. [e] Melting temperature (T_m) of oligos in the absence of compound: F21T (54.7 °C ± 0.4), F23T (52 °C±0.2), FKrasT (46 °C ± 0.7) and FCkit2T (64.8 °C ± 0.6).

ring is preferred. With regard to the terminal amino groups, there does not appear to be any significant change in ΔT_m for the majority of compounds when the terminal amino group is changed from pyrrolidine to piperazine and *vice versa* while keeping the side chain length constant. The only exceptions are compounds **3** and **4**, where changing the C_4-pyrrolidine of compound **3** to C_4-piperazine of compound **4** results in a significant loss of stabilization for all quadruplex targets (Table 2).

For compounds **6–8** and **10–23** which contain either the thiophene, furan, benzylether, piperonal and *p*-dimethylaminophenyl 4-aryl substituents on the central pyridine ring, the decrease in chain length from C_4 to C_3 does not result in a significant change in ΔT_m and the majority of these compounds displayed ΔT_m of ≤12.0 °C (Table 2). Within this category, compounds containing the pyrrolidine terminal amino group and the furan (compounds **8** and **16**), thiophene (compounds **6** and **7**), or piperonal (compounds **17** and **20**) 4-aryl substituent on the central pyridine ring exhibited mid range stabilizations for G4-DNA.

There is an excellent correlation between the ability of the compounds to stabilize the same sequence in K^+ and Na^+ buffer, hence compounds that stabilize each G4-DNA sequence well in K^+ buffer tend to also stabilize it well in Na^+ buffer (see Supporting Information†). There also is a good correlation between the ability of the compounds to stabilize different G4-DNA sequences in the same buffer conditions (see Supporting Information†). A correlation plot of F21T in K^+ *vs.* FKrasT in K^+ is shown in Fig. 1. This graph demonstrates that the stabilizations for these two quadruplexes are highly correlated (R = 0.987).

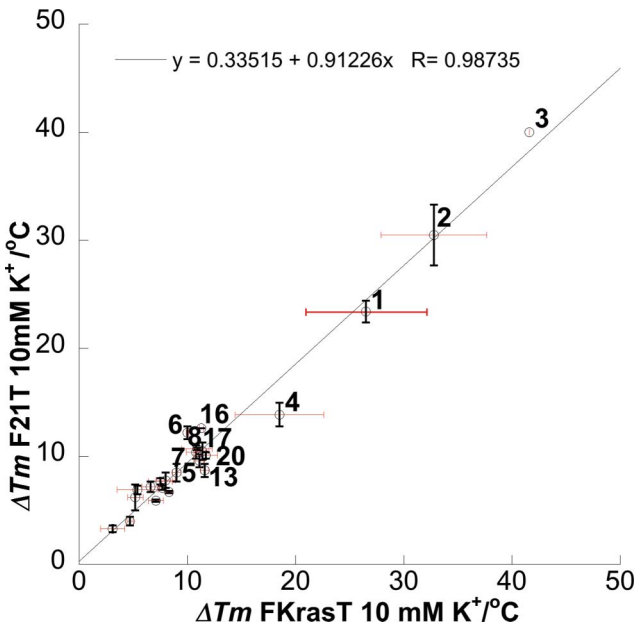


Fig. 1 Correlation plot of ligand induced change in melting temperatures (ΔT_m) for F21T in K^+ *vs.* FKrasT in K^+.

FRET competition with ds26

A FRET competition assay, containing a large excess of duplex DNA, ds26 (15 equiv. (3 μM) and 50 equiv. (10 μM) to G4-DNA),

a self-complementary 26-base-long oligonucleotide of sequence 5′-d-$CA_2TCG_2ATCGA_2T_2CGATC_2GAT_2G$-3′, was conducted on compounds which displayed a high to mid range stabilization of G4-DNA ($\Delta T_m \geq 10$ °C) in K^+ and Na^+ to determine the specificity (*S*) of the ligands for G4-DNA over duplex (Table 2) (see Supporting Information for specificity in Na^+†). *S* is the ratio of ligand-induced ΔT_m of G4-DNA in the presence of ds26 (10 μM) competitor and in the absence of competitor. The duplex was added in excess in order to assess weak binding to double stranded DNA.

The highest stabilizing compounds, **1–4**, generally exhibited relatively low *S* in Na^+ and K^+ (Table 2) (see Supporting Information for specificity in Na^+†). However, there were exceptions with compounds **1** which exhibited a *S* of 1.0 with F21T in K^+ (ΔT_m = 23.4 °C ± 1.0) and **4** which exhibited a *S* of 0.82 with FKrasT in K^+ (ΔT_m = 18.5 °C ± 4.1) and 0.93 with FCkit2T in K^+ (ΔT_m = 11.3 °C ± 2.4). Hence though compounds **1–4** display a high stabilization for G4-DNA their somewhat limited specificity for G4-DNA over duplex DNA questions their efficacy.

Amongst compounds **6–8**, **13**, **16–17** and **20**, the majority of these compounds exhibited a specificity of >0.9 for G4-DNA over duplex DNA, with the exception of compound **6** for FKrasT in K^+ (Table 2) (see Supporting Information for specificity in Na^+†). Although the G4-DNA stabilization of these compounds is not as high as compounds **1–4** their specificity for G4-DNA over duplex DNA combined with their mid range G4-DNA stabilization make them promising G4-DNA ligands. It is also interesting to note that the specificity of these compounds for F21T significantly decreased from >0.9 in K^+ to between 0.45 and 0.77 in Na^+ (see Supporting information†).

FRET competition with TG_5T

A FRET competition assay, containing a large excess of d-$[TG_5T]_4$ (3 μM and 10 μM), a 7-base-long oligonucleotide forming a tetramolecular G-quadruplex, was conducted on compounds **1–4** in the presence of F21T, FCkit2T and FKrasT in K^+ and Na^+ (Table 3) (see Supporting Information for Na^+†). For ligands with a significant affinity for G4-DNA, we expect TG_5T to act as a specific competitor, lowering the stabilization.

For F21T and FKrasT in K^+ and Na^+ there was a decrease in stabilization in the presence of TG_5T competitor for all compounds. For FCkit2T in K^+ and Na^+ there was a decrease in stabilization in the presence of TG_5T competitor for all compounds, except compound **3** in K^+ and compound **4** in Na^+. Hence with the exception of compound **3** with FCkit2T in K^+ and compound **4** in Na^+, the observed decrease in ΔT_m in the presence of TG_5T competitor confirms the high G4-DNA affinity of these compounds. The fact that TG_5T is not a competitor in all cases may look surprising; however, we recently found[24] that this "minimal" quadruplex, with four identical grooves and no loops, may not be an optimal target for all G4 ligands.

Table 4 Concentration of compounds **1–3** required to obtain a ΔT_m of 20 °C determined from a dose response FRET assay, carried out with G4 oligos (0.2 μM) in 10 mM K^+ buffer (pH 7.2)

[Compound] required for ΔT_m of 20 °C (μM)				
Compound	F21T	F23T	FKrasT	FCkit2T
1	0.96	0.93	0.95	0.75
2	0.71	0.70	0.86	0.74
3	0.45	0.42	0.52	0.6

Dose response

For the highest stabilizing compounds, **1–3**, the concentration required to obtain a ΔT_m of 20 °C[18] was obtained from FRET dose response curves in K^+ buffer with F21T, F23T, FCkit2T and FKrasT (Table 4). The most efficient compound in terms of G4-DNA stabilization is compound **3**, which contains the C_4-pyrrolidine side chain and a *p*-thiomethylphenyl 4-aryl substituent on the central pyridine ring. Replacing the *p*-thiomethylphenyl 4-aryl substituent on the central pyridine ring, compound **3**, with the *p*-bromophenyl, compound **1**, results in a 2.0 fold increase in the concentration required to obtain a ΔT_m of 20 °C for F21T, F23T and FKrasT.

FID

A Fluorescent Intercalator Displacement (FID) assay[25] was performed with compounds **1–4**, **15** and **16** in the presence of K^+ with i) an unlabelled quadruplex-forming oligonucleotide that mimics the human telomeric overhang: d($AG_3T_2AG_3T_2AG_3T_2AG_3$) (22AG), ii) quadruplex-forming oligonucleotides that mimic one of the quadruplex forming sequences within the c-kit promoter and the K-ras promoter respectively: d($G_3CG_3CGCGAG_3AG_4$) (Ckit2), d($AG_3CG_2TGTG_3A_2GAG_3A_2GAG_5AG_2CAG$) (Kras) and duplex ds26, to establish affinity of these compounds for the above oligos as a complementary method to FRET melting (see Supplementary Information†). This assay is based on the loss of fluorescence of a DNA-bound intercalator, thiazole orange (TO), upon displacement by a DNA-binding molecule.[25] The displacement ability of a given ligand was monitored by the decrease of TO fluorescence at 516 nm upon selective excitation at

Table 3 Change in quadruplex melting temperatures (ΔT_m) in the presence of triarylpyridines **1–4** (1 μM) determined using a TG_5T FRET competition assay, carried out with G4 oligos (0.2 μM) and TG_5T G4 competitor (3 μM and 10 μM) in 10 mM K^+ buffer (pH 7.2)

	F21T ΔT_m			FKrasT ΔT_m			FCkit2T ΔT_m		
	TG_5T			TG_5T			TG_5T		
Compound	0 μM	3 μM	10 μM	0 μM	3 μM	10 μM	0 μM	3 μM	10 μM
1	20.2	18.3	12.4	23.5	18.1	15.6	> 30	10.3	5.8
2	28.5	24.3	13.0	30.0	22.6	19.7	> 30	13.7	7.7
3	> 40	24.1	17.2	38.3	23.0	19.5	> 30	> 30	> 30
4	12.3	10.4	6.6	16.2	15.1	12.8	8.2	5.7	1.7

492 nm. There was a decrease in TO fluorescence for 22Ag, ckit2 and Kras in the presence of compounds **1–4** and **16** but not for compound **15** (see Supporting Information†). This is in agreement with the results obtained in the FRET-melting assay, where compounds **1–4** and **16** exhibited moderate to high stabilization of G4-DNA and compound **15** displayed poor stabilization of all three oligos by FRET melting (ΔT_m <4 °C) (Table 2). With respect to interaction with ds26 in the FID assay, there was only a slight decrease in TO fluorescence for compound **1** (10 equiv.) and no decrease in TO fluorescence for compound **3** suggesting that these compounds have good and excellent selectivity for G4-DNA over duplex DNA. For compounds **2**, **4** and **16** there was a significant decrease in TO fluorescence with ds26 at 10 equiv. of compounds. This suggests that they have a lower G4-DNA over duplex DNA specificity compared to compounds **1** and **3**.

Cytotoxicity

The relationship between cytotoxicity and G4-DNA stabilization is not straightforward, nevertheless, some rules could be deduced (Fig. 2 and Supporting Information†). Cytotoxicity studies of the compounds in HeLa cells showed that amongst the compounds which displayed the highest stabilization for G4-DNA, **1–3**, there was also high cytotoxicity observed, with IC_{50} values of 1.7 ± 0.6 μM, 5.5 ± 2.2 μM and 2.4 ± 1.4 μM respectively. Compounds with IC_{50} values below 2 μM include compounds **20** (1.4 ± 0.5 μM), **16** (1.4 ± 0.8 μM), **14** (1.4 ± 0.2 μM) and **1** (1.7 ± 0.6 μM), with the exception of compound **14**, these compounds displayed high to mid range stabilization of G4-DNA.

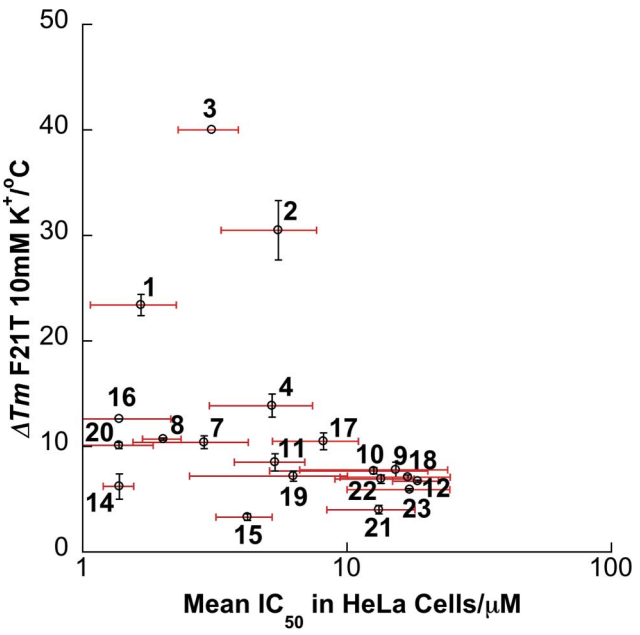


Fig. 2 Correlation plot of change in melting temperature *vs.* IC_{50} in HeLa cells for F21T in K^+.

Conclusions

In conclusion, we have synthesized a series of novel 2,4,6-triarylpyridines, some of which display G4-DNA stabilization comparable to that reported for other benchmark quadruplex ligands. For these high stabilizing compounds a *p*-bromophenyl or *p*-thiomethylphenyl 4-aryl substituent on the central pyridine ring along with a C_4 side chain is preferred, and reducing the chain length by one CH_2 unit, or replacing the *p*-bromophenyl or *p*-thiomethylphenyl 4-aryl substituent, results in a significant loss of G4-DNA stabilization. This suggests that the nature of the 4-aryl substituent on the central pyridine ring along with side chain length governs the ability of the compounds to stabilize G4-DNA. However, despite their high G4-DNA stabilization, their somewhat limited specificity for G4-DNA over duplex DNA raises questions about their efficacy.

Furthermore, we observed that compounds containing the pyrrolidine terminal amino group and the furan, thiophene or piperonal 4-aryl substituent on the central pyridine ring exhibited mid range stabilization for G4-DNA with excellent specificity for G4-DNA over duplex DNA. This combination of good G4-DNA stabilization and excellent G4-DNA specificity make them promising G4-DNA ligands.

We also demonstrate that there is an excellent correlation between the ability of the compounds to stabilize the same G4-DNA sequence in K^+ and Na^+ buffer, also in addition there is a good correlation between the ability of the compounds to stabilize different G4-DNA sequences in the same buffer conditions. Additionally, we reported a simple correlation between cytotoxicity and G4-DNA stabilization, with the majority of good G4-DNA stabilizers displaying significant cytotoxicity. Obviously, a number of other factors may complicate these cellular effects, such as cellular uptake and distribution. Experiments are under way to investigate in depth the biological impact of these molecules.

Experimental

Synthetic chemistry

Synthesis of compounds containing C_3-pyrrolidine and C_4-pyrrolidine side chains. After acylation of the triarylpyridine core with 3-chloropropionyl chloride or 4-chlorobutyryl chloride (see Supporting Information†) the resulting product (54.2 mg - 58.8 mg) was combined with pyrrolidine (130 equiv.) and left stirring at room temperature overnight (~16 h). The reaction mixture was placed in an ice-bath and ice-cold saturated bicarbonate solution was added to give a brown precipitate. The mixture was centrifuged and the solution was decanted, the brown precipitate was washed with ice-cold saturated bicarbonate solution (× 3). The precipitate was dissolved in methanol to precipitate excess salt, centrifuged and the methanol solution was decanted and evaporated to give the crude product, which was then purified by HPLC as oil and freeze dried for 48 h. HPLC (gradient: starting from acetonitrile (0.2% TFA) : water (0.2% TFA) 30 : 70 to 70% acetonitrile (0.2% TFA) over 20 min), 3.00 ml min^{-1} flow rate, with varying run times.

Compound 1. Yellow oil, 83%. HPLC run time 20 min, R_t = 12.057 min. δ_H (500 MHz, MeOD) 8.21 (d, J = 8.71 Hz, 4H), 7.99 (s, 2H), 7.81 (d, J = 8.59 Hz, 2H), 7.75 (d, J = 8.71 Hz, 4H), 7.71 (d, J = 8.59 Hz, 2H), 3.71 (m, 4H, pyr), 3.30 (m, 4H), 3.12 (m, 4H, pyr), 2.62 (t, J = 6.64 Hz, 4H), 2.18 (m, 4H, pyr), 2.11 (m, 4H), 2.04 (m, 4H, pyr). δ_C (125 MHz, MeOD) 173.99, 161.43, 158.26, 146.72, 141.01, 136.05, 133.30, 130.07,128.65, 124.32, 120.97, 116.84, 56.88, 54.96, 35.98, 25.71, 24.19. HRMS

(FAB) for $C_{39}H_{44}N_5O_2Br$ ([M]$^+$): calcd: 693.2678: found: 696.2766 (100%), 694.2731 (98%). Found C, 67.60; H, 6.22; N, 10.17. Calcd C, 67.43; H, 6.38; N, 10.08%.

Compound 3. Yellow oil, 56%. HPLC run time 20 min, R_t = 10.406 min. δ_H (500 MHz, MeOD) 8.15 (d, J = 8.71 Hz, 4H), 8.05 (s, 2H), 7.87 (d, J = 8.48 Hz, 2H), 7.79 (d, J = 8.71 Hz, 4H), 7.44 (d, J = 8.48 Hz, 2H), 3.71 (m, 4H, pyr), 3.30 (m, 4H), 3.12 (m, 4H, pyr), 2.62 (t, J = 6.87 Hz, 4H), 2.56 (s, 3H, SCH3), 2.18 (m, 4H, pyr), 2.11 (m, 4H), 2.05 (m, 4H, pyr). δ_C (125 MHz, MeOD) 173.88, 161.41, 158.00, 150.64, 140.87, 136.16, 129.24, 128.61, 128.45, 127.45, 120.91, 116.59, 56.86, 54.94, 35.97, 25.67, 24.19, 15.28. HRMS (FAB) for $C_{40}H_{47}N_5O_2S$ ([M]$^+$): calcd: 661.345: found: 662.3536 (100%). Found C, 72.57; H, 7.05; N, 10.78. Calcd C, 72.58; H, 7.16; N, 10.58%.

Compound 5. Yellow oil, 79%. HPLC run time 15 min, R_t = 10.162 min. δ_H (500 MHz, MeOD) 8.12 (d, J = 8.82 Hz, 4H), 8.10 (s, 2H), 7.90 (d, J = 8.59 Hz, 2H), 7.83 (d, J = 8.82 Hz, 4H), 7.44 (d, J = 8.59 Hz, 2H), 3.72 (m, 4H, pyr), 3.58 (t, J = 6.64 Hz, 4H), 3.16 (m, 4H, pyr), 2.97 (t, J = 6.64 Hz, 4H), 2.55 (s, 3H, SCH3), 2.18 (m, 4H, pyr), 2.06 (m, 4H, pyr). δ_C (125 MHz, MeOD) 172.60, 158.07, 150.74, 141.84, 140.82, 136.27, 136.09, 128.66, 128.49, 127.50, 120.92, 116.67, 54.81, 52.88, 36.86, 24.33, 15.28. HRMS (FAB) for $C_{38}H_{43}N_5O_2S$ ([M]$^+$): calcd: 633.3137: found: 634.3171 (100%). Found C, 72.14; H, 6.74; N, 11.19. Calcd C, 72.01; H, 6.84; N, 11.05%.

Compound 6. Yellow oil, 88%. HPLC run time 15 min, R_t = 8.191 min. δ_H (500 MHz, MeOD) 8.14 (d, J = 8.82 Hz, 4H), 8.03 (s, 2H), 7.93 (dd, J = 3.67 Hz, 1.03 Hz, 1H), 7.82 (d, J = 8.82 Hz, 4H), 7.68 (dd, J = 5.04 Hz, 1.03 Hz, 1H), 7.25 (dd, J = 5.04 Hz, 3.67 Hz, 1H), 3.73 (m, 4H, pyr), 3.58 (t, J = 6.53 Hz, 4H), 3.18 (m, 4H, pyr), 2.96 (t, 6.53 Hz, 4H), 2.19 (m, 4H, pyr), 2.06 (m, 4H, pyr). δ_C (125 MHz, MeOD) 172.70, 161.49, 158.43, 142.75, 141.01, 136.08, 129.64, 128.64, 128.31, 127.12, 121.00, 115.38, 54.83, 52.90, 36.87, 24.32. HRMS (FAB) for $C_{35}H_{39}N_5O_2S$ ([M]$^+$): calcd: 593.2824: found: 594.2919 (100%). Found C, 70.86; H, 6.53; N, 11.90. Calcd C, 70.80; H, 6.62; N, 11.79%.

Compound 7. Yellow oil, 83%. HPLC run time 15 min, R_t = 8.301 min. δ_H (500 MHz, MeOD) 8.13 (d, J = 8.82 Hz, 4H), 8.02 (s, 2H), 7.92 (dd, J = 3.67 Hz, 1.03 Hz, 1H), 7.79 (d, J = 8.71 Hz, 4H), 7.68 (dd, J = 5.15 Hz, 1.03 Hz, 1H), 7.25 (dd, J = 5.15 Hz, 3.67 Hz, 1H), 3.71 (m, 4H, pyr), 3.30 (m, 4H), 3.12 (m, 4H, pyr), 2.62 (t, 6.87 Hz, 4H), 2.18 (m, 4H, pyr), 2.11 (m, 4H), 2.04 (m, 4H, pyr). δ_C (125 MHz, MeOD) 174.01, 161.68, 158.45, 142.74, 141.00, 135.50, 129.64, 128.62, 128.31, 127.11, 120.99, 115.36, 56.88, 54.97, 35.98, 25.72, 24.20. HRMS (FAB) for $C_{37}H_{43}N_5O_2S$ ([M]$^+$): calcd: 621.3137: found: 622.3189 (100%). Found C, 71.52; H, 7.04; N, 11.38. Calcd C, 71.47; H, 6.97; N, 11.26%.

Compound 8. Yellow oil, 80%. HPLC run time 15 min, R_t = 7.102 min. δ_H (500 MHz, MeOD) 8.14 (s, 2H), 8.08 (d, J = 8.82 Hz, 4H), 7.85 (d, J = 8.71 Hz, 4H), 7.86 (m, 1H, furan), 7.51 (dd, J = 3.67 Hz, 0.46 Hz, 1H), 6.73 (dd, J = 3.55 Hz, 1.72 Hz, 1H), 3.73 (m, 4H, pyr), 3.58 (t, J = 6.53 Hz, 4H), 3.17 (m, 4H, pyr), 2.97 (t, 6.64 Hz, 4H), 2.18 (m, 4H, pyr), 2.06 (m, 4H, pyr). δ_C (125 MHz, MeOD) 170.12, 161.79, 156.51, 151.73, 147.51, 142.14, 132.30, 129.74, 121.03, 116.50, 115.42, 114.19, 55.51, 52.15, 32.98, 24.03. HRMS (FAB) for $C_{35}H_{39}N_5O_3$ ([M]$^+$): calcd: 577.3053: found: 578.3129 (100%). Found C, 72.93; H, 6.67; N, 12.16. Calcd C, 72.76; H, 6.80; N, 12.12%.

Compound 13. Yellow oil, 68%. HPLC run time 26 min, R_t = 12.989 min. δ_H (500 MHz, MeOD) 8.16 (d, J = 8.71 Hz, 4H), 8.00 (s, 2H), 7.88 (d, J = 8.59 Hz, 2H), 7.77 (d, J = 8.71 Hz, 4H), 7.47 (d, J = 7.22 Hz, 2H), 7.39 (t, J = 7.56 Hz, 2H), 7.33 (d, J = 7.56 Hz, 1H), 7.19 (d, J = 8.82 Hz, 2H), 5.19 (s, 2H), 3.71 (m, 4H, pyr), 3.30 (m, 4H), 3.12 (m, 4H, pyr), 2.62 (t, J = 6.76 Hz, 4H), 2.18 (m, 4H, pyr), 2.11 (m, 4H), 2.05 (m, 4H, pyr). δ_C (125 MHz, MeOD) 173.88, 161.44, 157.70, 150.35, 138.43, 136.30, 131.88, 130.34, 129.53, 129.43, 128.92, 128.66, 128.58, 120.93, 116.47, 115.88, 70.97, 56.84, 54.93, 35.97, 25.66, 24.17. HRMS (FAB) for $C_{46}H_{51}N_5O_3$ ([M]$^+$): calcd: 721.3992: found: 722.4105 (100%). Found C, 76.72; H, 7.06; N, 9.87. Calcd C, 76.53; H, 7.12; N, 9.70%.

Compound 14. Yellow oil, 81%. HPLC run time 26 min, R_t = 12.962 min. δ_H (500 MHz, MeOD) 8.17 (d, J = 8.71 Hz, 4H), 7.99 (s, 2H), 7.86 (d, J = 8.82 Hz, 2H), 7.79 (d, J = 8.59 Hz, 4H), 7.47 (d, J = 7.22 Hz, 2H), 7.39 (t, J = 7.22 Hz, 2H), 7.33 (d, J = 7.56 Hz, 1H), 7.18 (d, J = 8.82 Hz, 2H), 5.19 (s, 2H), 3.72 (m, 4H, pyr), 3.58 (t, J = 6.42 Hz, 4H), 3.18 (m, 4H, pyr), 2.96 (t, J = 6.42 Hz, 4H), 2.19 (m, 4H, pyr), 2.06 (m, 4H, pyr). δ_C (125 MHz, MeOD) 172.58, 161.14, 158.01, 150.99, 140.81, 138.46, 136.38, 130.39, 129.55, 129.43, 128.93, 128.69, 128.59, 120.93, 116.67, 115.90, 70.99, 54.79, 52.86, 36.86, 24.31. HRMS (FAB) for $C_{44}H_{47}N_5O_3$ ([M]$^+$): calcd: 693.3679: found: 694.3781 (100%). Found C, 76.31; H, 6.73; N, 10.20. Calcd C, 76.16; H, 6.83; N, 10.09%.

Compound 16. Yellow oil, 95%. HPLC run time 15 min, R_t = 7.707 min. δ_H (500 MHz, MeOD) 8.18 (s, 2H), 8.07 (d, J = 8.82 Hz, 4H), 7.89 (d, J = 1.37 Hz, 1H), 7.85 (d, J = 8.82 Hz, 4H), 7.56 (d, J = 3.44 Hz, 1H), 6.76 (dd, J = 3.44 Hz, 1.72 Hz, 1H), 3.71 (m, 4H, pyr), 3.30 (m, 4H), 3.12 (m, 4H, pyr), 2.62 (t, J = 6.87 Hz, 4H), 2.19 (m, 4H, pyr), 2.11 (m, 4H), 2.04 (m, 4H, pyr). δ_C (125 MHz, MeOD) 173.97, 161.46, 158.20, 153.05, 145.27, 141.01, 136.09, 128.56, 120.96, 116.16, 113.15, 110.29, 56.87, 54.96, 35.97, 25.70, 24.19. HRMS (FAB) for $C_{37}H_{43}N_5O_3$ ([M]$^+$): calcd: 605.3366: found: 606.3432 (100%). Found C, 73.57; H, 6.97; N, 11.64. Calcd C, 73.36; H, 7.15; N, 11.56%.

Compound 17. Yellow oil, 83%. HPLC run time 18 min, R_t = 8.999 min. δ_H (500 MHz, MeOD) 8.19 (s, 2H), 8.06 (d, J = 8.71 Hz, 4H), 7.88 (d, J = 8.71 Hz, 4H), 7.63 (dd, J = 8.13 Hz, 2.06 Hz, 1H), 7.59 (d, 1.37 Hz, 1H), 7.07 (d, J = 8.25 Hz, 1H), 6.11 (s, 2H), 3.70 (m, 4H, pyr), 3.30 (m, 4H), 3.23 (m, 4H, pyr), 2.62 (t, J = 6.76 Hz, 4H), 2.18 (m, 4H, pyr), 2.11 (m, 4H), 2.05 (m, 4H, pyr). δ_C (125 MHz, MeOD) 173.77, 161.40, 157.88, 150.96, 149.86, 140.90, 136.13, 133.78, 128.62, 122.26, 120.89, 116.73, 109.71, 108.31, 102.83, 56.80, 54.92, 35.90, 25.56, 24.18. HRMS (FAB) for $C_{40}H_{45}N_5O_4$ ([M]$^+$): calcd: 659.3472: found: 660.3549 (100%). Found C, 72.67; H, 6.78; N, 10.71. Calcd C, 72.81; H, 6.87; N, 10.61%.

Compound 20. Yellow oil, 84%. HPLC run time 15 min, R_t = 9.018 min. δ_H (500 MHz, MeOD) 8.19 (d, J = 8.71 Hz, 4H), 7.92 (s, 2H), 7.77 (d, J = 8.71 Hz, 4H), 7.39 (m, 2H), 6.99 (d, J = 8.59 Hz, 1H), 6.05 (s, 2H), 3.72 (m, 4H, pyr), 3.58 (t, J = 6.64 Hz, 4H), 3.20 (m, 4H, pyr), 2.95 (t, J = 6.53 Hz, 4H), 2.18 (m, 4H, pyr), 2.07 (m,

4H, pyr). δ_C (125 MHz, MeOD) 172.51, 161.40, 157.82, 150.90, 149.84, 140.79, 136.16, 133.79, 128.63, 122.23, 120.87, 116.72, 109.71, 108.32, 102.84, 54.76, 52.83, 36.85, 24.31. HRMS (FAB) for $C_{38}H_{41}N_5O_4$ ([M]$^+$): calcd: 631.3159: found: 632.3228 (100%). Found C, 72.43; H, 6.38; N, 11.15. Calcd C, 72.24; H, 6.54; N, 11.09%.

Synthesis of compounds containing C_3-piperazine and C_4-piperazine side chains. After acylation of the triarylpyridine core with 3-chloropropionyl chloride or 4-chlorobutyryl chloride (see Supporting Information†) the resulting product (46 mg–59.2 mg) was combined with piperazine (130 equiv.). The solids were then heated with a heat gun until melted and left stirring at 110 °C overnight (~16 h). The reaction mixture was cooled to room temperature and placed in an ice-bath. Ice-cold saturated bicarbonate solution was added to give a brown precipitate. The mixture was centrifuged and the solution was decanted, the brown precipitate was washed with ice-cold saturated bicarbonate solution (× 3). The precipitate was dissolved in methanol to precipitate excess salt, centrifuged and the methanol solution was decanted and evaporated to give the crude product which was then purified by HPLC as oil and freeze dried for 48 h. HPLC (gradient: starting from acetonitrile (0.2% TFA) : water (0.2% TFA) 30 : 70 to 70% acetonitrile (0.2% TFA) over 20 min), 3.00 ml min^{-1} flow rate, with varying run times.

Compound 2. Yellow oil, 69%. HPLC run time 30 min, R_t = 7.913 min. δ_H (500 MHz, MeOD) 8.19 (d, J = 8.82 Hz, 4H), 8.03 (s, 2H), 7.83 (d, J = 8.59 Hz, 2H), 7.78 (d, J = 8.82 Hz, 4H), 7.72 (d, J = 8.59 Hz, 2H), 3.43 (m, 8H, pip), 3.26 (m, 8H, pip), 3.04 (t, J = 7.22 Hz, 4H), 2.60 (t, J = 6.76 Hz, 4H), 2.07 (m, 4H). δ_C (125 MHz, MeOD) 174.12, 161.43, 158.20, 150.38, 141.04, 136.00, 133.29, 130.05, 128.65, 124.30, 120.93, 116.81, 59.53, 54.82, 46.11, 35.93, 23.33. HRMS (FAB) for $C_{39}H_{46}BrN_7O_2$ ([M]$^+$): calcd: 723.2896: found: 724.2982 (100%). Found C, 64.72; H, 6.48; N, 13.58. Calcd C, 64.63; H, 6.40; N, 13.53%.

Compound 4. Yellow oil, 61%. HPLC run time 20 min, R_t = 7.066 min. δ_H (500 MHz, MeOD) 8.17 (d, J = 8.71 Hz, 4H), 8.05 (s, 2H), 7.87 (d, J = 8.48 Hz, 2H), 7.80 (d, J = 8.71 Hz, 4H), 7.45 (d, J = 8.36 Hz, 2H), 3.35 (m, 8H, pip), 3.11 (m, 8H, pip), 2.90 (t, J = 6.87 Hz, 4H), 2.57 (t, J = 6.87 Hz, 4H), 2.56 (s, 3H, SCH3), 2.03 (m, 4H). δ_C (125 MHz, MeOD) 174.09, 161.43, 158.10, 150.50, 140.97, 136.16, 131.77, 128.65, 128.49, 127.50, 120.91, 116.64, 59.41, 54.40, 46.37, 35.92, 23.34, 15.28. HRMS (FAB) for $C_{40}H_{49}N_7O_2S$ ([M]$^+$): calcd: 691.3668: found: 692.3745 (100%). Found C, 69.24; H, 7.02; N, 14.28. Calcd C, 69.43; H, 7.14; N, 14.17%.

Compound 9. Yellow oil, 53%. HPLC run time 15 min, R_t = 7.015 min. δ_H (500 MHz, MeOD) 8.14 (d, J = 8.82 Hz, 4H), 8.10 (s, 2H), 7.90 (d, J = 8.48 Hz, 2H), 7.81 (d, J = 8.71 Hz, 4H), 7.45 (d, J = 8.48 Hz, 2H), 3.37 (m, 8H, pip), 3.13 (t, J = 6.76 Hz, 4H), 3.08 (m, 8H, pip), 2.78 (t, J = 6.76 Hz, 4H), 2.56 (s, 3H, SCH3). δ_C (125 MHz, MeOD) 172.81, 164.24, 163.15, 161.39, 158.06, 140.89, 136.23, 128.67, 128.53, 127.53, 120.92, 116.63, 55.58, 52.78, 47.55, 34.69, 15.29. HRMS (FAB) for $C_{38}H_{45}N_7O_2S$ ([M]$^+$): calcd: 663.3355: found: 664.3449 (100%). Found C, 68.90; H, 6.75; N, 14.83. Calcd C, 68.75; H, 6.83; N, 14.77%.

Compound 10. Yellow oil, 62%. HPLC run time 15 min, R_t = 5.736 min. δ_H (500 MHz, MeOD) 8.10 (d, J = 8.82 Hz, 4H), 8.08 (s, 2H), 8.01 (dd, J = 3.78 Hz, 1.03 Hz, 1H), 7.83 (d, J = 8.71 Hz, 4H), 7.75 (dd, J = 5.04 Hz, 1.03 Hz, 1H), 7.28 (dd, J = 5.04 Hz, 3.67 Hz, 1H), 3.46 (m, 8H, pip), 2.88 (t, J = 6.64 Hz, 4H). δ_C (125 MHz, MeOD) 172.87, 161.38, 158.25, 142.71, 141.00, 135.95, 129.66, 128.61, 128.31, 127.12, 120.94, 115.25, 55.62, 54.48, 46.27, 34.65. HRMS (FAB) for $C_{35}H_{41}N_7O_2S$ ([M]$^+$): calcd: 623.3042: found: 624.3128 (100%). Found C, 67.28; H, 6.77; N, 15.80. Calcd C, 67.39; H, 6.62; N, 15.72%.

Compound 11. Yellow oil, 66%. HPLC run time 15 min, R_t = 5.677 min. δ_H (500 MHz, MeOD) 8.11 (d, J = 8.71 Hz, 4H), 8.07 (s, 2H), 8.00 (d, J = 3.78 Hz, 1H), 7.82 (d, J = 8.71 Hz, 4H), 7.74 (d, J = 4.70 Hz, 1H), 7.28 (dd, J = 4.93 Hz, 3.78 Hz, 1H), 3.51 (m, 8H, pip), 3.44 (m, 8H, pip), 3.18 (t, J = 7.33 Hz, 4H), 2.63 (t, J = 6.64 Hz, 4H), 2.11 (m, 4H). δ_C (125 MHz, MeOD) 174.14, 161.39, 158.30, 142.72, 141.12, 135.88, 129.64, 128.58, 128.28, 127.09, 120.95, 115.24, 59.49, 54.68, 46.06, 35.94, 23.34. HRMS (FAB) for $C_{37}H_{45}N_7O_2S$ ([M]$^+$): calcd: 651.3355: found: 652.3454 (100%). Found C, 68.35; H, 6.89; N, 15.10. Calcd C, 68.17; H, 6.96; N, 15.04%.

Compound 12. Yellow oil, 79%. HPLC run time 15 min, R_t = 5.758 min. δ_H (500 MHz, MeOD) 8.12 (s, 2H), 8.10 (d, J = 8.71 Hz, 4H), 7.84 (m, 1H), 7.82 (d, J = 8.71 Hz, 4H), 7.47 (d, J = 3.55 Hz, 1H), 6.72 (dd, J = 3.55 Hz, 1.83 Hz, 1H), 3.42 (m, 8H, pip), 3.23 (t, J = 6.64 Hz, 4H), 3.20 (m, 8H, pip), 2.83 (t, J = 6.64 Hz, 4H). δ_C (125 MHz, MeOD) 172.86, 161.39, 158.13, 153.04, 145.31, 140.98, 136.09, 128.58, 120.96, 115.99, 113.11, 110.34, 55.57, 54.17, 46.42, 34.72. HRMS (FAB) for $C_{35}H_{41}N_7O_3$ ([M]$^+$): calcd: 607.3271: found: 608.3355 (100%). Found C, 69.38; H, 6.64; N, 16.20. Calcd C, 69.17; H, 6.80; N, 16.13%.

Compound 15. Yellow oil, 87.83% yield. HPLC run time 16 min, R_t = 9.309 min. δ_H (500 MHz, MeOD) 8.15 (d, J = 8.59 Hz, 4H), 8.03 (s, 2H), 7.89 (d, J = 8.59 Hz, 2H), 7.78 (d, J = 8.59 Hz, 4H), 7.47 (d, J = 7.56 Hz, 2H), 7.39 (t, J = 7.79 Hz, 2H), 7.33 (d, J = 7.45 Hz, 1H), 7.19 (d, J = 8.82 Hz, 2H), 5.20 (s, 2H), 3.25 (m, 8H, pip), 2.95 (t, J = 6.87 Hz, 4H), 2.88 (m, 8H, pip), 2.68 (t, J = 6.87 Hz, 4H). δ_C (125 MHz, MeOD) 172.92, 161.23, 158.16, 151.44, 138.50, 136.44, 132.98, 131.89, 129.57, 129.48, 128.97, 128.70, 128.59, 121.00, 116.61, 115.95, 71.06, 55.64, 54.47, 47.57, 34.66. HRMS (EI$^+$, 70 eV, 200 °C) for $C_{44}H_{49}N_7O_3$ ([M]$^+$): calcd: 723.3897: found: 724.3930 (100%). Found C, 73.09; H, 6.70; N, 13.60. Calcd C, 73.00; H, 6.82; N, 13.54%.

Compound 18. Yellow oil, 95% yield. HPLC run time 16 min, R_t = 5.924 min. δ_H (500 MHz, MeOD) 8.14 (d, J = 8.94 Hz, 4H), 8.11 (d, J = 8.71 Hz, 2H), 7.95 (d, J = 8.82 Hz, 2H), 7.85 (s, 2H), 7.72 (d, J = 8.71 Hz, 4H), 7.33 (m, 1H), 6.94 (m, 1H), 6.81 (m, 1H), 6.02 (s, 2H), 3.40 (m, 8H, pip), 2.82 (m, 8H, pip), 1.91 (m, 4H), 2.43 (m, 4H), 2.73 (m, 4H). δ_C (125 MHz, MeOD) 174.13, 161.45, 158.15, 151.08, 149.95, 140.98, 136.25, 134.01, 128.69, 122.23, 120.97, 116.90, 109.77, 108.41, 102.86, 59.53, 54.81, 46.12, 35.95, 23.36. HRMS (FAB) for $C_{40}H_{47}N_7O_4$ ([M]$^+$): calcd: 689.3690: found: 690.3782 (100%). Found C, 69.48; H, 6.77; N, 14.32. Calcd C, 69.64; H, 6.87; N, 14.21%.

Compound 19. Yellow oil, 90%. HPLC run time 15 min, R_t = 6.109 min. δ_H (500 MHz, MeOD) 8.13 (d, J = 8.71 Hz, 4H), 8.09

(s, 2H), 7.81 (m, 1H), 7.80 (d, J = 8.71 Hz, 4H), 7.40 (d, J = 3.55 Hz, 1H), 6.70 (dd, J = 3.55 Hz, 1.83 Hz, 1H), 3.41 (m, 8H, pip), 3.23 (m, 8H, pip), 2.95 (t, J = 6.64 Hz, 4H), 2.59 (t, J = 6.76 Hz, 4H), 2.04 (m, 4H). δ_C (125 MHz, MeOD) 174.13, 161.44, 158.16, 153.05, 145.26, 141.08, 136.02, 128.56, 120.93, 116.04, 113.11, 110.30, 59.44, 54.50, 46.00, 35.94, 23.34. HRMS (FAB) for $C_{37}H_{45}N_7O_3$ ([M]$^+$): calcd: 635.3584: found: 636.3686 (100%). Found C, 69.78; H, 6.99; N, 15.48. Calcd C, 69.90; H, 7.13; N, 15.42%.

Compound 21. Yellow oil, 69% yield. HPLC run time 27 min, R_t = 9.675 min. δ_H (500 MHz, MeOD) 8.08 (d, J = 8.80 Hz, 4H), 7.89 (d, J = 8.80 Hz, 2H), 7.79 (s, 2H), 7.67 (d, J = 8.80 Hz, 4H), 7.41 (d, J = 7.10 Hz, 2H), 7.35 (t, J = 7.70 Hz, 2H), 7.28 (d, J = 7.10 Hz, 1H), 6.84 (d, J = 8.80 Hz, 2H), 5.06 (s, 2H), 3.32 (m, 8H, pip), 3.30 (m, 4H), 2.81 (m, 4H), 2.40 (m, 8H, pip), 1.88 (m, 4H). δ_C (125 MHz, MeOD) 174.04, 161.44, 158.06, 150.81, 138.86, 138.44, 131.89, 130.34, 129.54, 129.44, 128.93, 128.58, 128.50, 120.91, 116.49, 115.90, 71.00, 59.49, 54.69, 46.07, 35.92, 23.33. HRMS (EI$^+$, 70 eV, 200 °C) for $C_{46}H_{53}N_7O_3$ ([M]$^+$): calcd: 751.4210: found: 752.4293 (100%). Found C, 73.53; H, 7.01; N, 13.17. Calcd C, 73.47; H, 7.10; N, 13.04%.

Compound 22. Yellow oil, 98% yield. HPLC run time 12 min, R_t = 6.123 min. δ_H (500 MHz, MeOD) 8.15 (d, J = 8.71 Hz, 4H), 8.01 (s, 2H), 7.81 (m, 1H), 7.79 (d, J = 8.48 Hz, 4H), 7.46 (s, 1H), 7.02 (d, J = 8.48 Hz, 1H), 6.07 (s, 2H), 3.25 (m, 8H, pip), 2.98 (m, 4H), 2.90 (m, 8H, pip), 2.70 (m, 4H). δ_C (125 MHz, MeOD) 172.88, 161.41, 158.10, 151.27, 149.98, 140.84, 136.33, 133.98, 128.70, 122.33, 120.97, 116.89, 109.77, 108.39, 102.91, 55.61, 54.30, 46.59, 34.72. HRMS (FAB) for $C_{38}H_{43}N_7O_4$ ([M]$^+$): calcd: 661.3377: found: 662.3467 (100%). Found C, 68.84; H, 6.46; N, 14.93. Calcd C, 68.97; H, 6.55; N, 14.82%.

FRET

General. Labelled oligonucleotides, F21T, F23T, FKrasT and FCkit2T and unlabelled oligonucleotides, ds26 and TG_5T were purchased from Eurogentec (Belgium). For the labelled oligonucleotides the stock solutions were diluted in purified water to obtain an initial concentration of 100 μM. TG_5T and ds26 were stored as concentrated stock solutions in purified water. Compounds were stored in purified water at a stock concentration of 300 μM and further diluted to 5 μM in purified water prior to conducting FRET assays.

All oligonucleotides were then further diluted in the relevant buffer and pre-annealed prior to conducting FRET assays. Pre-annealing of the labelled oligonucleotides and ds26 consisted of heating at 95 °C for 2 min and then cooling on ice. The pre-annealing procedure for TG_5T consisted of heating at 90 °C for 5 min and then storing at 4 °C for 48 h.

FRET-melting. The FRET assays were performed as a high-throughput screen in a 96-well format on a Stratagene Mx3005P real-time PCR machine. After a first equilibration step at 25 °C for 5 min, a stepwise increase of 1 °C every minute for 71 cycles to reach 96 °C was performed and measurements were made after each "cycle" with excitation at 492 nm and detection at 516 nm. Final analysis of the data was carried out using Excel and KaleidagGraph software. Emission of FAM was normalized between 0 and 1, and apparent T_m was defined as the temperature for which the normalized emission is 0.5.

Each well was duplicated and contained a total reaction volume of 25 μL, with the labelled oligonucleotide (0.2 μM) in K^+ or Na^+ buffer in the absence or presence of compound (1 μM). K^+ buffer contained lithium cacodylate (10 mM) at pH 7.2, KCl (10 mM) and LiCl (90 mM) and Na^+ buffer contained lithium cacodylate (10 mM) at pH 7.2 and NaCl (100 mM). The reported ΔT_m values are an average of three experiments ± standard deviation.

FRET competition with ds26 or TG_5T. Each well was duplicated and contained a total reaction volume of 25 μL, with the labelled oligonucleotide (0.2 μM) in K^+ or Na^+ buffer in the presence of compound (1 μM). For each compound there were wells containing ds26 or TG_5T competitor (3 μM), ds26 or TG_5T competitor (10 μM) and no competitor respectively. In addition there was a blank well in the absence of compound and competitor. K^+ buffer consisted of lithium cacodylate (10 mM) at pH 7.2, KCl (10 mM) and LiCl (90 mM). Na^+ buffer consisted of lithium cacodylate (10 mM) at pH 7.2 and NaCl (100 mM). The reported ΔT_m values are an average of two or more experiments ± standard deviation.

Dose response. Each well was duplicated and contained a total reaction volume of 25 μL, with the labelled oligonucleotide (0.2 μM) in K^+ or Na^+ buffer in the absence or presence of compound. Compound concentrations tested were 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 μM.

FID

FID experiments were performed with 96 well microplates. Each condition was tested in triplicate and at least 2 times, in a volume of 25 μL for each sample. Oligonucleotides tested were 3 G-quadruplexes (22AG, Ckit2, Kras) and a duplex (ds26). Samples were incubated in buffer containing 10 mM lithium cacodylate pH 7.2 and 100 mM KCl. The pre-annealed DNA target (0.5 μM strand concentration for G4-DNA and 1 μM strand concentration for ds26) was mixed with 1 μM of thiazole orange (TO). Compound concentrations tested were 1.25, 2.5 and 5 μM. The fluorescence of samples was measured at 25 °C in a qPCR (Stratagene Mx3005P) instrument. The temperature was kept constant with a thermostat cell holder (Peltier). The TO was excited at 492 nm and the emission was collected at 516 nm with 8-fold gain.

Cytotoxicity

Cell culture and reagents. The human cervical cancer cell line HeLa was incubated in DMEM medium supplemented with 10% fetal bovine serum in a 5% CO_2 humidified atmosphere at 37 °C. MTT kit (CellTiter 96TM) was purchased from Promega.

MTT assay. Cells were seeded on 96-well plates (3×10^3/well) and pre-incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 before exposure to different concentrations of ligands for 48 h. An MTT assay was used to measure cellular respiration taken as being proportional to cell proliferation activity. The assay is based on the conversion of a tetrazolium salt into a blue formazan product by mitochondria. Four hours before the end of the assay, 15 μl of the dye solution was added to each well.

The cells were re-incubated under previously described conditions. After 4 h, 100 µl of solubilization solution was added to each well and the absorbance at 570 nm was measured to estimate cell viability.

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