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Sequences in the HSP90 promoter form G-quadruplex structures with selectivity for disubstituted phenyl bis-oxazole derivatives

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ABSTRACT

The HSP90 protein is an important target in cancer. We report here that stable quadruplex DNAs can be formed from a promoter sequence in the HSP90 gene, on the basis of melting, circular and NMR studies, and show that these can be selectively targeted by non-macrocyclic quadruplex-stabilizing phenyl bis-oxazole derivatives. These do not bind significantly to duplex DNA and show low stabilization of the human telomeric quadruplex. These results suggest an approach to targeting HSP90 at the DNA level.

Guanine-rich sequences of nucleic acids can form a wide variety of inter- or intramolecular higher-order structures containing repeats of four guanines hydrogen-bonded together, the G-quartet motif.[1] G-tract repetitive telomeric DNA and RNA sequences at the end of eukaryotic chromosomes can form quadruplexes under the influence of small molecules, mostly based on polycyclic heteroaromatic motifs.[2] These telomeric quadruplex-small molecule complexes can inhibit the action of the telomerase enzyme, which is involved in telomere maintenance in many cancer cell types. Since telomerase is a key driver of tumorigenesis and cellular immortalization, this can result in anticancer effects.[3] Putative quadruplex sequences in the human genome[4] occur within the promoter regions of many genes[5] and in 5′-untranslated regions.[6] These quadruplexes have been studied as selective targets for down-regulating individual genes in cancer and other diseases, using quadruplex-binding organic[7] and metallo-containing[8] small molecules to stabilize the target quadruplex and hence to elicit a biological response. Down-regulation of transcription has been demonstrated for a number of promoter quadruplexes, including the *c-myc*,[9a] *c-kit*[9b] and *src* genes.[9c]

A number of small molecules have been identified with >10^3 or more selectivity for quadruplexes over genomic duplex DNA.[2] The basis for selectivity has not generally been explored but may arise as a result of differences in steric requirements between duplex and quadruplexes. However selectivity for one quadruplex over another remains a largely unsolved challenge that needs to be addressed in order to ensure that off-target effects are minimised in any therapeutic application of quadruplex targeting. Structural data on human genomic quadruplexes is available for telomeric G4s[10] and those from the *c-myc*,[11] *c-kit*,[12] *bcl*-2,[13] hTERT[14] and RET promoters and attempts at structure-based design are currently[15] restricted to using those quadruplexes. We report here on results from an alternative library-based strategy to generate quadruplex-selective ligands, in which we have screened a small panel of quadruplexes using a high-throughput quadruplexes-melting assay.

HSP90 is a well-established anticancer target at the protein level. It is involved in ensuring correct folding of many cellular proteins, and plays this role in rapidly-proliferating cells, especially in many cancers. Many small molecules have been developed as inhibitors of the HSP90 protein and are in clinical trial.[16] Resistance to such inhibitors occurs via mutations in the ATP binding site.[17] Targeting the HSP90 gene rather than the gene product offers the possibility of a distinct profile of activity and reduced susceptibility to resistance. HSP90 also plays a role in telomere maintenance and telomerase regulation so HSP90 down-regulation may have a consequence for these pathways.[18]

A search of the human genome using the Ensemble database and in-house software shows that the sequence occurs 77

aGGGCGGGCCAAAGGGAAGGGGTGGGc

nucleotides upstream of the transcription start-site of the HSP90 gene and contains the sp1 promoter consensus sequence. This suggests a functional role for the sequence. The presence of five short guanine tracts (highlighted in red) in the sequence also suggest

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that either it is polymorphic, capable of forming several inter-related quadruplexes, or that it corresponds to a single structurally complex quadruplex.

Analogy with the *c-myc* promoter sequence,[7,11] which similarly contains >4 short G-tracts, suggests that the former is more likely. We have examined the full-length sequence and two shorter, overlapping ones, each with four G-tracts (see below). Spectroscopic studies demonstrate that these are quadruplex-forming sequences in ionic conditions analogous to those in cells.

Quadruplexes in solution have distinct circular dichroism (CD) spectra depending on their topologies. Parallel-folded quadruplexes show a maximum positive signal at 260 nm with a negative signal at 240 nm. Anti-parallel topologies produce a positive signal at 295 nm with a negative peak at 260 nm. Two 21-mer G-rich subsections (HSP90A and B)

HSP90A: d(**GGG**CCAAA**GGG**AA**GGGG**T**GGG**)
HSP90B: d(**GGG**C**GGG**CCAAA**GGG**AA**GGGG**)

and the full sequence (HSP90C) were analyzed in K^+ and Na^+ phosphate-buffer at 10 mM and 200 mM concentrations. All three sequences have absorption properties consist with quadruplex topologies.

Both sub-sequences and the complete 27-mer sequence (HSP90C) form quadruplex structures in high K^+ concentration, with CD spectra indicative of mixed topology: a positive signal in the CD spectrum at 260 nm, a negative signal at 240 nm and some features around 295 nm (Fig. 1). The HSP90B sequence showed a single positive absorbance maximum in 200 mM Na^+ buffer (pH 7.0), suggesting an anti-parallel conformation (Fig. 1b). The HSP90 and the HSP90A sequences remain in a predominantly parallel arrangement under these conditions.

1-D NMR experiments on all three sequences show imino region of the spectra having the characteristic signals arising from G-quartets with patterns of imino resonances in the 11–12 ppm region, consistent with a quadruplex core comprising three stacked G-quartets (Fig. 2) .[10–12] The integration of this region indicates that multiple conformations are probably present.

In view of the indications that the HSP90 promoter sequences form stable quadruplex arrangements, these were included in the panel of quadruplexes used for small-molecule screening, with a human telomeric and *c-kit*[12] and *k-ras*[19] promoter quadruplex sequences.

BRACO-19 and telomestatin are well-studied and effective quadruplex stabilizing compounds (Fig. 3) BRACO-19 has a polyheteroaromatic acridine platform together with two cationic 'handles' (pyrrolidines), which are significant contributors to quadruplex binding.[20] The hepta-oxazole telomestatin has high quadruplex affinity and quadruplex:duplex selectivity.[21] Telomestatin has a largely planar structure. BRACO-19 contains the acridine planar structural motive but is not macrocylic and all three substituents interact strongly with quadruplex grooves and loops.[20] Telomestatin can interact only with the planar G-quartet at the terminus of a quadruplex and thus has a stringent surface area requirement. We have generated a focused library of phenyl bis-oxazoles with terminal tertiary amines of the type found in BRACO-19 and other quadruplex-binding ligands.[2] The hypothesis was that these acyclic compounds would bind to a range of quadruplex structural types, analogous to acyclic 'click' chemistry compounds.[22]

The side-arms were also envisioned to improve the solubility, and overall drug-likeness compared to a purely macrocyclic quadruplex ligand with either triazole[22] or oxazole groups. We have utilized a regioselective and atom-economic transition

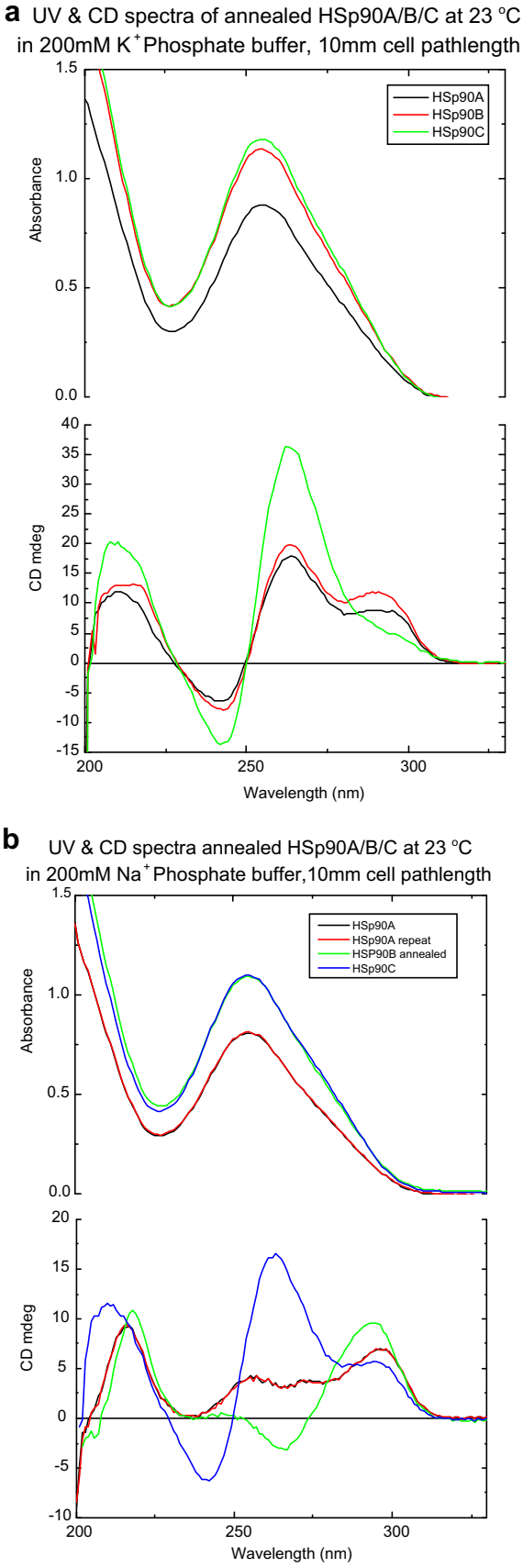

Figure 1. CD spectra of the three HSP90 sequences, (a) in 200 mM K^+ and (b) in 200 mM Na^+ solution.

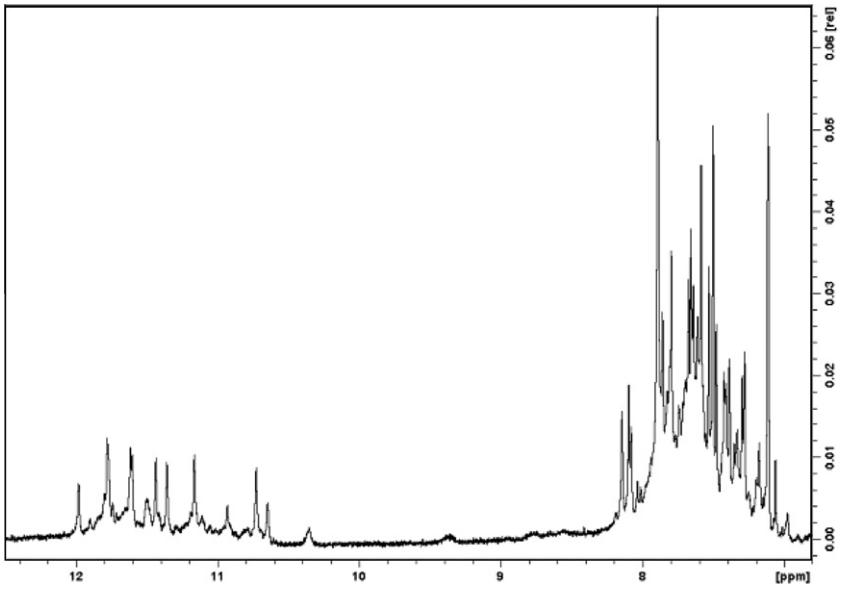

Figure 2. 1-D NMR spectrum of the HSP90A sequence, taken on a Bruker Avance 500 MHz instrument. Experiments in H_2O used excitation suppression of the water signal. Samples were made up in 10 mM KCl solution with 10 mM potassium phosphate pH 7.0 buffer. Samples were annealed by heating to 90 °C followed by slow cooling over a period of 15 h; 540 μL of sample and 60 μL of D_2O were then mixed to give a strand concentration of 200 μM with a 10% D_2O content.

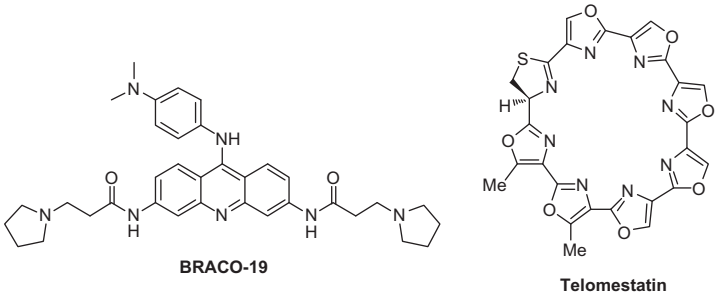

Figure 3. Structures of BRACO-19 and the hepta-oxazole macrocycle telomestatin.

metal-catalyzed direct arylation approach (Fig. 4) for the synthesis of a small library based on the phenyl bis-oxazole framework instead of the traditional sp^2–sp^2 cross-coupling reactions normally used for oxazoles.[23] Prior to the arylation step, 5-substituted phenyl bis-oxazole precursor **1** was synthesized from cheap and commercially available isophathalaldehyde using the tosylmethylisocyanate-procedure.[24] The *meta*-substituted phenyl bis-oxazole building block 1 was then arylated twice, either with *para*- or *meta*-substituted arylbromides or aryliodides.[25] Most of the arylhalides were freshly prepared or commercially available as the iodides and C2-regioselective and very mild 'on-water' oxazole arylation conditions were utilized.[26] The synthetic procedure and analytical data for compound **2f** is given below.[27] That for the other compounds is provided in the Supplementary data.

Concentration-dependent FRET (Fluorescence Resonance Energy Transfer) melting assays[28] using doubly labelled (Fam-Tamra) short G-rich DNA sequences revealed (Fig. 4) an unexpected selectivity profile for the compounds in K^+ buffer. Compounds **2a–i** showed significant stabilization of the two HSP90 quadruplexes A and B, yet no stabilization of a human telomeric quadruplex DNA sequence (Table 1). The ω-*N*-methyl piperazine bis-oxazole analogue **2f**, which at physiological pH is likely to be doubly-charged, is the best compound in this series with high selectivity for the HSP90A over HSP90B quadruplexes and especially over the human telomeric quadruplex DNA sequence (G4 h-tel DNA) (ΔT_m values for the HSP90A quadruplex of 14 °C and 23 °C at 1 and 2 μM). No significant stabilization of duplex DNA was observed for **2a–i**. Even at ligand concentrations as high as 3 μM the ΔT_m value for duplex DNA are <1 °C, showing that compound **2f** discriminates between quadruplexes. Selectivity over duplex DNA was demonstrated using a 300-fold excess of duplex by a competition assay in which increasing excess of calf thymus duplex DNA was titrated into the HSP90A quadruplex FRET assay (Fig. 5).

The selectivity of compound **2f** was examined further with other promoter quadruplex (Table 2), and showed modest stabilisation of the *K-ras* promoter quadruplex but none with the *c-kit*2 quadruplex. Compound **2f** also shows low-micromolar cell growth inhibitory activity in cancer cells,[29] yet only modest selectivity compared to its effect on normal WI38 fibroblast cells (Table 2). Only a modest change in HSP90 expression was observed in these cell lines (data not shown), suggesting that either the HSP90 promoter quadruplexes are not readily formed, are inaccessible to compound **2f**, or possibly that the affinity for these complexes is not sufficient for effective transcription factor/polymerase inhibition. This compound is also not an inhibitor of human telomerase activity (unpublished observations). We have previously reported that a structurally unrelated naphthalene diimide compound has exceptionally high affinity for HSP90 (and other) quadruplexes,[30] with a ΔT_m at 1.0 μM ligand

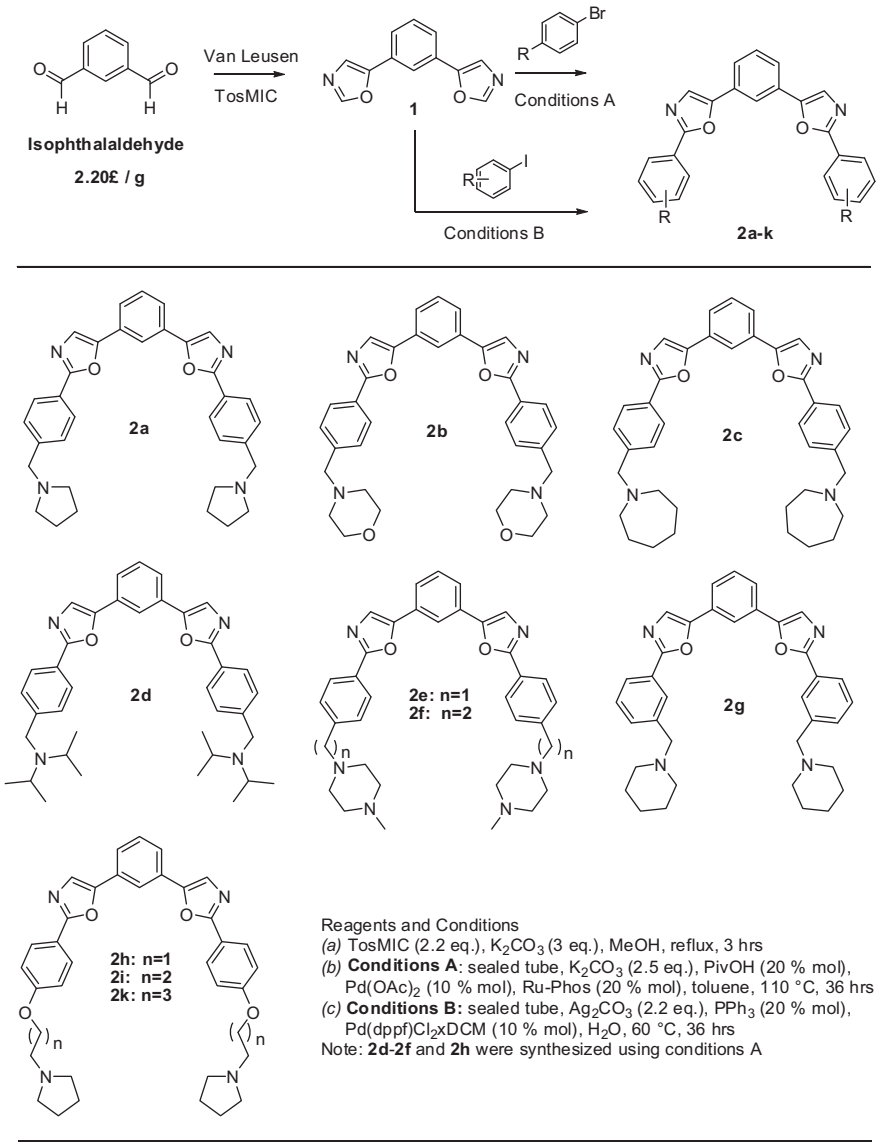

Figure 4. Structures (**2a–k**) of the final compounds generated via the double direct arylation of the C2-position of phenyl bis-oxazole **1**.

Table 1
Melting data for compounds **2a–k** with the human telomeric and HSP90 quadruplexes. Average esds are ±0.1 °C from triplicate experiments

Compd	G4 h-tel DNA		HSP-90A		HSP-90B		ds DNA	
	1 μmol	2 μmol	1 μmol	2 μmol	1 μmol	2 μmol	1 μmol	2 μmol
2a	2	6	7	14	2	4	0	0
2b	<2	2	6	6	5	6	0	0
2c	3	7	8	9	9	11	1	1
2d	<2	2	8	8	8	9	1	1
2e	<2	2	7	16	3	12	0	0
2f	<2	2	14	23	2	7	0	0
2g	<2	<2	5	8	3	6	1	1
2h	<2	<2	7	14	2	6	0	0
2i	<2	<2	7	14	2	6	0	0
2k	2	15	5	19	2	5	0	0

Note: All values are ΔT_m in degree Celsius (rounded to the nearest degree based on triplicate runs in 60 mmol potassium cacodylate buffer at pH-7.4).

concentration, of 36.3 °C for the HSP90A quadruplex and 32.0 °C for the HSP90B quadruplex. This particular compound also produces down-regulation of HSP90 expression in the Mia-Pa-Ca-2 pancreatic cancer cell line and in treated Mia-Pa-Ca-2 xenograft tumours, consistent with the hypothesis that a threshold level of quadruplex binding is needed in order to produce a significant cellular effect (although at this stage we cannot rule out other mechanisms of down-regulating HSP90 expression).

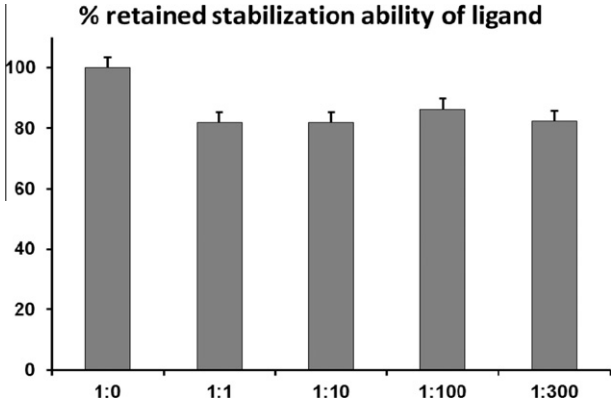

Figure 5. FRET competition plot for compound **2f**. Points 1–5 represent HSP90A:-calf thymus DNA molar ratios of 1:0, 1:1, 1:10, 1:100 and 1:300 respectively. With this duplex DNA no significant decrease in ΔT_m values for the HSP90A quadruplex were observed. Experiments were performed in triplicate.

Table 2
Results of studies on compound **2f**. Top: FRET melting studies using a range of quadruplex sequences and a duplex (ds) DNA probe, with two concentrations for compound **2f** (ΔT_m values in °C ± 0.1 °C.). **F21T** is the 21-mer human telomeric quadruplex. ***c-kit2*** and ***k-ras*** are sequences from the promoter of the *c-kit* and *k-ras* oncogenes. Bottom: data on inhibition of cell growth proliferation following 96 h exposure using a panel of cancer cell lines and a normal fibroblast line (WI38). All experiments were performed in triplicate

Sequence	1 μmol	2 μmol
F21T	<2	2
*c-kit*2	<2	2
k-ras	7	17
HSP90A	14	23
HSP90B	2	7
ds DNA	0	0
Cell line		IC_{50}, in μmol
A549		1.02 ± 0.13
MCF7		1.32 ± 0.29
RCC4		0.94 ± 0.08
786-o		1.33 ± 0.21
Mia-Pa-Ca2		1.25 ± 0.04
WI38		2.59 ± 0.63

The *para* regio-isomer (compound **2l**) of the *meta*-substituted phenyl bis-oxazole **2f** was also synthesized. Analysis of its quadruplex stabilization profile using all five quadruplex sequences showed no significant increase in melting temperature (ΔT_m at 2 μmol <5 °C) for any of the selected sequences compared to controls. This suggests, as previously shown with other acyclic quadruplex ligands,[22] that meta-substitution of the core phenyl bis-oxazole **1** is crucial for optimal quadruplex stabilization. The small size of the present library, together with the persistence of HSP90 binding across the series, does not enable a structure-activity relationship to be deduced at this stage. However it is apparent from a comparison of compounds **2e** and **2f**, that the slightly longer side-chains of the latter do improve HSP90 stabilization. More surprising is the significant activity of the weakly basic di-morpholine derivative **2b**, which is at variance with the behaviour of the majority of other quadruplex ligands that have been reported.[2]

During the course of this work a report has appeared on analogous quadruplex-targeted oxazole compounds but which lack charged side-arms.[31] Interestingly these also fail to bind to the human telomeric quadruplex in K^+ conditions, although binding does take place in Na^+ solution.

The HSP90 promoter quadruplexes are of especial topicality in view of the current interest in HSP90 at the protein level as a therapeutic target for human cancer. Targeting expression via a promoter quadruplex could circumvent resistance induction, although the current lead compound **2f** will require optimization so that its affinity for the HSP90 quadruplexes is significantly higher in order for this concept to be fully validated. Structural studies of small-molecule-HSP90 quadruplex complexes, analogous to those reported for BRACO-19[20] and naphthalene diimide derviatives with human telomeric quadruplexes[32] and quindoline with a c-*myc* promoter quadruplex,[33] will undoubtedly aid optimization. None of these structures provide obvious insight into the structural basis of the selectivity shown by the present compounds, in particular compound **2f**, binding to HSP90 quadruplexes. One can speculate, in the absence of structural data, that the sequence CCAAA present in the HSP90 quadruplexes, may form a long loop that folds into a binding cleft, analogous to that found in the *c-kit*1 quadruplex.[12] More extensive studies on the complete and partial HSP90 quadruplex sequences will also clarify which (or other variants) are the more biologically relevant.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.07.065.

References and notes

1. (a) Patel, D. J.; Phan, A. T.; Kuryavyi, V. *Nucleic Acids Res.* **2007**, *35*, 7429; (b) Burge, S.; Parkinson, G. N.; Hazel, P.; Todd, A. K.; Neidle, S. *Nucleic Acids Res.* **2006**, *34*, 5402; (c) Neidle, S. *Curr. Opin. Struct. Biol.* **2009**, *19*, 1.
2. See for example (a) Sun, D.; Thompson, B.; Cathers, B. E.; Salazar, M.; Kerwin, S. M.; Trent, J. O.; Jenkins, T. C.; Neidle, S.; Hurley, L. H. *J. Med. Chem.* **1997**, *40*, 2113; (b) Monchaud, D.; Teulade-Fichou, M.-P. *Org. Biomol. Chem.* **2008**, *6*, 627; (c) Yang, D. Z.; Okamoto, K. *Future Med. Chem.* **2010**, *2*, 619; (d) Neidle, S. *Therapeutic Aspects of Quadruplex Nucleic Acids*; Academic Press: San Diego, 2011.
3. Shay, J. W.; Wright, W. E. *Nature Rev. Drug Discov.* **2006**, *5*, 577.
4. (a) Huppert, J. L.; Balasubramanian, S. *Nucleic Acids Res.* **2005**, *33*, 2908; (b) Todd, A. K.; Johnston, M.; Neidle, S. *Nucleic Acids Res.* **2005**, *33*, 2901.
5. (a) Huppert, J. L.; Balasubramanian, S. *Nucleic Acids Res.* **2007**, *35*, 406; (b) Siddiqui-Jain, A.; Grand, C. L.; Bearss, D. J.; Hurley, L. H. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11593; (c) Balasubramanian, S.; Hurley, L. H.; Neidle, S. *Nature Rev. Drug Discov.* **2011**, *10*, 261.
6. (a) Wieland, M.; Hartig, J. S. *Chem. Biol.* **2007**, *14*, 757; (b) Bugaut, A.; Balasubramanian, S. *Nucleic Acids Res.* **2012**, *40*, 4727.
7. See for example: (a) Ou, T.-M.; Lu, Y.-J.; Zhang, C.; Huang, Z.-S.; Wang, X.-D.; Tan, J.-H.; Chen, Y.; Ma, D.-L.; Wong, K.-Y.; Tang, J. C.-O.; Sun-Chi, A.; Gu, L.-Q. *J. Med. Chem.* **2007**, *50*, 1465; (b) Waller, Z. A.; Sewitz, S. A.; Hsu, S. T.; Balasubramanian, S. *J. Am. Chem. Soc.* **2009**, *131*, 12628.
8. For example Alzeer, J.; Vummidi, B. R.; Roth, P. J.; Luedtke, N. W. *Angew. Chem., Int. Ed. Engl.* **2009**, *48*, 9362.
9. (a) Brown, R. V.; Danford, F. L.; Gokhale, V.; Hurley, L. H.; Brooks, T. A. *J. Biol. Chem.* **2011**, *286*, 41018; (b) Gunaratnam, M.; Swank, S.; Haider, S. M.; Galesa, K.; Reszka, A. P.; Beltran, M.; Cuenca, F.; Fletcher, J. A.; Neidle, S. *J. Med. Chem.* **2009**, *52*, 3774; (c) McLuckie, K. I.; Waller, Z. A.; Sanders, D. A.; Alves, D.; Rodriguez, R.; Dash, J.; McKenzie, G. J.; Venkitaraman, A. R.; Balasubramanian, S. *J. Am. Chem. Soc.* **2011**, *133*, 2658; (d) Rodriguez, R.; Miller, K. M.; Forment, J. V.; Bradshaw, C. R.; Nikan, M.; Britton, S.; Oelschlaegel, T.; Xhemalce, B.; Balasubramanian, S.; Jackson, S. P. *Nature Chem. Biol.* **2012**, *8*, 301.
10. (a) Parkinson, G. N.; Lee, M. P. H.; Neidle, S. *Nature* **2002**, *417*, 876; (b) Ambrus, A.; Chen, D.; Dai, J.; Bialis, T.; Jones, R. A.; Yang, D. *Nucleic Acids Res.* **2006**, *34*, 2723; (c) Luu, K. N.; Phan, A. T.; Kuryavyi, V.; Lacroix, L.; Patel, D. J. *J.Am. Chem. Soc.* **2006**, *128*, 9963.
11. (a) Phan, A. T.; Modi, Y. S.; Patel, D. J. *J. Am. Chem. Soc.* **2004**, *126*, 8710; (b) Mathad, R. I.; Hatzakis, E.; Dai, J.; Yang, D. *Nucleic Acids Res.* **2011**, *39*, 9023.
12. (a) Phan, A. T.; Kuryavyi, V.; Burge, S.; Neidle, S.; Patel, D. J. *J. Am. Chem. Soc.* **2007**, *129*, 4386; (b) Hsu, S. T.; Varnai, P.; Bugaut, A.; Reszka, A. P.; Neidle, S.; Balasubramanian, S. *J. Am. Chem. Soc.* **2009**, *131*, 13399; (c) Wei, D.; Parkinson, G. N.; Reszka, A. P.; Neidle, S. *Nucleic Acids Res.* **2012**, *40*, 4691.
13. Dai, J. X.; Chen, D.; Jones, R. A.; Hurley, L. H.; Yang, D. Z. *Nucleic Acids Res.* **2006**, *34*, 5133.

14. Lim, K. W.; Lacroix, L.; Yue, D. J.; Lim, J. K.; Lim, J. M.; Phan, A. T. *J. Am. Chem. Soc.* **2010**, *132*, 12331.
15. Tong, X.; Lan, W.; Zhang, X.; Wu, H.; Liu, M.; Cao, C. *Nucleic Acids Res.* **2011**, *39*, 6753.
16. Neckers, L.; Workman, P. *Clin. Cancer Res.* **2012**, *18*, 64.
17. Millson, S. H.; Chua, C. S.; Roe, S. M.; Polier, S.; Solovieva, S.; Pearl, L. H.; Sim, T. S.; Prodromou, C.; Piper, P. W. *FASEB J.* **2011**, *25*, 3828.
18. See for example: (a) Keppler, B. R.; Grady, A. T.; Jarstfer, M. B. *J. Biol. Chem.* **2006**, *281*, 19840; (b) Kim, R. H.; Kim, R.; Chen, W.; Hu, S.; Shin, K. H.; Park, N. H.; Kang, M. K. *Carcinogenesis* **2008**, *29*, 2425.
19. Cogoi, S.; Xodo, L. E. *Nucleic Acids Res.* **2006**, *34*, 2536.
20. Capbell, N. H.; Parkinson, G. N.; Reszka, A. P.; Neidle, S. *J. Am. Chem. Soc.* **2008**, *130*, 6722.
21. See also Nielsen (a) Kim, M.-Y.; Vankayalapati, H.; Shin-ya, K.; Wierzba, K.; Hurley, L. H. *J. Am. Chem. Soc.* **2002**, *124*, 2098; (b) Rzuczek, S. G.; Pilch, D. S.; Liu, A.; Liu, L.; LaVoie, E. J.; Rice, J. E. *J. Med. Chem.* **2010**, *53*, 3632; (c) Ulven, M. C. T. *Curr. Med. Chem.* **2010**, *17*, 3438. for a recent review of macrocyclic quadruplex ligands..
22. (a) Moorhouse, A. D.; Santos, A. M.; Gunaratnam, M.; Moore, M.; Neidle, S.; Moses, J. E. *J. Am. Chem. Soc.* **2006**, *128*, 15972; (b) Drewe, W. C.; Neidle, S. *Chem. Commun.* **2008**, 5295.
23. Reeder, M. R.; Gleaves, H. E.; Hoover, S. A.; Imbordino, H. R.; Pangborn, J. *J. Org. Process Res. Dev.* **2003**, *7*, 696.
24. van Leusen, A. M.; Hoogenboom, B. E.; Siderius, H. *Tetrahedron Lett.* **1972**, *23*, 2369.
25. Strotman, N. A.; Chobanian, H. R.; Gou, Y.; He, J.; Wilson, J. E. *Org. Lett.* **2010**, *16*, 3578.
26. Ohnmacht, S. A.; Mamone, P.; Culshaw, A. J.; Greaney, M. F. *Chem. Commun.* **2008**, *10*, 1241.
27. Synthesis and analytical data for compound **2f**, 1,3-bis(2-(4-(2-(4-methylpiperazine-1-yl)ethyl)phenyl)-oxazol-5-yl)benzene. A 5 mL microwave vial was charged with phenyl bis-oxazole (40 mg, 0.19 mmol, 1 equiv), 4-(4-iodobenzyl)-4-methylpiperazine (120 mg, 0.4 mmol, 2.1 equiv), $Pd(dppf)Cl_2 \cdot CH_2Cl_2$ (16 mg, 0.019 mmol, 10 mol%), Ag_2CO_3 (105 mg, 0.38 mmol, 2 equiv) and PPh_3 (10 mg, 0.038 mmol, 20 mol %). A magnetic stirrer bar was added and the mixture of solids was gently shaken for a few seconds to ensure all solids were well mixed. Distilled water (3 mL) was added and the vial was covered with a serum cap. The vial and its contents were then heated and stirred in a pre-heated oil bath at 60 °C for 36 h. After this time the reaction mixture was cooled down to room temperature. CH_2Cl_2 (4 ml) was added and the contents of the vial were filtered through a short pad of celite (in a Pasteur pipette with cotton wool at bottom). The vial was rinsed once with an additional 1 ml of CH_2Cl_2. The organic layer was separated, and the aqueous phase extracted once with CH_2Cl_2 (1 ml). The organic layers were combined and concentrated in vacuo. The residue was purified by flash chromatography (silica; CH_2Cl_2 / MeOH 9:1) to provide the title compound as an off-white solid in 78% yield. Best purification results were obtained when a gradient from CH_2Cl_2 (100%) to CH_2Cl_2 : MeOH (7:3) was applied. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (4H, d, J = 8 Hz, Ar-H), 8.00 (1H, s, Ar-H), 7.68 (2H, dd, J = 8 Hz, 8 Hz, Ar-H), 7.54–7.50 (3H, m, Ar-H), 7.35 (4H, d, J = 8 Hz, Ar-H), 2.91–2.87 (4H, m, 2 × CH_2), 2.68–2.50 (20H, m, 10 × CH_2), 2.31 (6H, s, 2 × CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.7 (quat), 150.5 (quat), 143.4 (quat), 129.6 (quat), 129.3 (CH), 128.9 (CH), 126.5 (CH), 125.3 (CH), 124.2 (CH), 124.0 (CH), 119.6 (quat), 60.0 (CH_2), 55.2 (CH_2), 53.2 (CH_2), 46.1 (CH_3), 33.6 (CH_2). HRMS (ES^+) calculated for $C_{38}H_{44}N_6O_2$ $(M+H)^+$ 617.3604, found 617.3626. Mp 191–193 °C (pale-yellow solid).
28. FRET DNA melting assays on compounds **2a–k** were performed as described previously (Guyen, B.; Schultes, C. M.; Hazel, P.; Mann, J.; Neidle, S. *Org. Biomol. Chem.* **2004**, *2*, 981) using a fluorescence resonance energy transfer (FRET) assay modified to be used as a high-throughput screen in a 96-well format. The labelled oligonucleotides had attached the donor fluorophore FAM: 6-carboxyfluorescein and the acceptor fluorophore TAMRA: 6-carboxytetramethylrhodamine. The FRET probe sequences were diluted from stock to the correct concentration (400 nM) in a 60 mM potassium cacodylate buffer (pH 7.4) and then annealed by heating to 95 °C for 10 min, followed by cooling to room temperature in the heating block (3 –3.5 h). The compounds were stored as a 1 mM stock solution in 10% DMSO / 90% 1 mmol HCl; final solutions (at 2 × concentration) were prepared using 60 mM potassium cacodylate buffer (pH 7.4). Relevant controls using BRACO-19 (in addition to blank runs) were also performed to check for quality of DNA samples (eg. F21T). 96-Well plates (MJ Research, Waltham, MA) were prepared by aliquoting 50 μl of the annealed DNA into each well, followed by 50 μl of the compound solutions. Measurements were made on a DNA Engine Opticon (MJ Research) with excitation at 450–495 nm and detection at 515–545 nm. Fluorescence readings were taken at intervals of 0.5 °C in the range 30–100 °C, with a constant temperature being maintained for 30 s prior to each reading to ensure a stable value. Final analysis of the data was carried out using a script written in the program Origin 7.0 (OriginLab Corp., Northampton, MA). The advanced curve-fitting function in Origin 7.0 was used for calculation of ΔT_m values. Esds in ΔT_m are ±0.1 °C.
29. Sulforhodamine B assay (SRB). Cells were counted and diluted to the required concentration in 20 mL medium. For cell lines MCF7, A549, MIA-Pa-Ca-2, RCC4 and 786-0, 1000–4000 cells with 160 μL media (WI38: 6000/well) were seeded into each well of a 96 well plate (Nunc, Denmark). After incubation for 24 h, the compound to be tested was dissolved in 40 μL of medium and was added in a range of concentrations, and the cells incubated for 96 h. The medium was then removed and the cells fixed by incubation with TCA (10%, Sigma-Aldrich, UK) for 30 min at 4 °C. After removal of the TCA, the cells were washed with deionised water 5 times and dried at 60 °C for 1 h. The cells were then incubated with sulforhodamine B (80 μL, 0.4 % in 1% acetic acid, Acros Organics, UK) for 15 min at RT. The SRB was removed, the wells washed with 1% acetic acid (200 μL), and dried at 60 °C for 1 h. Tris-base (100 μL, 10 mM, Acros Organics, UK) solution was added to each well, and the plates were gently shaken for 5 min. The absorbance at 540 nm was measured with a plate reader (Spectrostar Omega, BMG Labtech, Germany). The data were normalized to the value of 100 for the control experiment (untreated cells), and the IC_{50} values were obtained by interpolation from a plot with Origin (Version 7.0, OriginLab Corp.), as the concentration leading to an absorbance intensity of 50%.
30. Gunaratnam, M.; de la Fuente, M.; Hampel, S. M.; Todd, A. K.; Reszka, A. P.; Schatzlein, A.; Neidle, S. *Bioorg. Med. Chem.* **2011**, *19*, 7151.
31. Hamon, F.; Large, E.; Guédin-Beaurepaire, A.; Rouchon-Dagois, M.; Sidibe, A.; Monchaud, D.; Mergny, J.-L.; Riou, J.-F.; Nyugen, C.-H.; Teulade-Fichou, M.-P. *Angew. Chem., Int. Ed.* **2011**, *50*, 8745.
32. Collie, G. W.; Promontorio, R.; Hampel, S. M.; Micco, M.; Neidle, S.; Parkinson, G. N. *J. Am. Chem. Soc.* **2012**, *134*, 2723.
33. Dai, J.; Carver, M.; Hurley, L. H.; Yang, D. *J. Am. Chem. Soc.* **2012**, *134*, 17673.