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A novel series of G-quadruplex ligands with selectivity for HIF-expressing osteosarcoma and renal cancer cell lines

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ABSTRACT

A series of naphthalene derivatives with disubstituted triazole side-arms have been assembled by click chemistry. Lead compounds show a high level of selectivity for renal, osteo- and Ewing's sarcomas that express the HIF-1α transcription factor. They also interact selectively with the quadruplex DNAs located in the promoter of the HIF genes and it is suggested that the mechanism of action involves inhibition of transcription by drug-mediated quadruplex stabilization in these regions.

Quadruplex nucleic acid structures can be formed from repeats of short G-tracts, in both DNA and RNA sequences.[1,2] In principle these can result in impediments to transcription, translation or telomere maintenance, depending on their context within a genome.[3,4] Crystallographic and NMR studies have revealed a wide diversity of folds for quadruplexes[5,6] and it is currently not possible to predict tertiary fold from primary sequence. Quadruplex stability is normally enhanced by small-molecule binding[7] and a large number of quadruplex-binding compounds have been reported,[8,9] an increasing number with selectivity for quadruplex over duplex DNA.[10] Initial studies focused on telomeric quadruplexes as therapeutic targets,[7–9] but more recently promoters in a number of oncogenes (*c-myc*, *c-kit*, *src* in particular) containing G-quadruplex sequences have also been targeted,[11–13] as have several quadruplex RNA sequences in 5′-untranslated regions of genes such as *bcl*-2.[14] Several challenges remain to be overcome. Firstly, few of these small molecules are able to discriminate between different quadruplexes, thus their cellular effects tend to be non-specific. Secondly, the majority of such molecules disclosed to date comprise a polycyclic heteroaromatic core that is relatively non-drug-like and development of such compounds into therapeutic agents is inherently more challenging than for drug-like compounds.[8]

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that plays a critical role in mediating cellular responses to hypoxia.[15–17] HIF-1 activates genes leading to increased glycolysis, resistance to apoptosis and increased angiogenesis. The activity of HIF-1 is primarily determined by levels of its alpha subunits. HIF-1α protein is expressed in many human tumors but is absent in most normal tissues. Over-expression has been associated with poor prognosis in patients with renal cancers,[16] osteosarcomas and chondrosarcomas.[18–20] Levels of serum VEGF, a HIF target gene, are significantly higher in Ewing's sarcoma patients than healthy controls and are an independent prognostic factor for survival. HIF-1α is thus an attractive molecular target for the development of novel drugs for these cancers in particular.

A quadruplex sequence has been located in the promoter region of both the HIF-1α and the HIF-2α genes and both have been previously shown to form stable quadruplex structures by means of biophysical methods.[21] We have developed a screening strategy for small-molecule targeting of these two quadruplexes since their detailed tertiary structures are currently unknown and structure-based drug design is therefore not feasible at present. This manuscript discloses a library of compounds arising from this project, with lead members showing high cellular selectivity for HIF-expressing cancer cells. Their detailed cell and molecular biology will shortly be reported elsewhere.

The library design incorporated several key groupings. (i) A planar aromatic core. A naphthalene moiety was employed, as

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conceived to be sufficiently small to overcome some of the druggability problems associated with large polycyclic groups, yet of sufficient surface area to potentially cover part of a terminal G-quartet. (ii) Side-arms with triazole rings assembled with click chemistry, which have been previously shown to be an effective approach to incorporating this highly polarized group, well-suited for effective quadruplex binding by stacking onto terminal G-quartets, [22] and (iii) cationic end-groups to enhance electrostatic interactions with quadruplex grooves. The synthesis of the library (Fig. 1) is shown in the (Scheme 1). Building-blocks were synthesized as previously described for the side-arms.[23] Full details of the experimental procedures and of the synthesized molecules are given in the (Supplementary data); the synthesis of the lead compound **13** (**CL67**) is outlined below.[24]

Surface plasmon resonance (SPR) was used to assess the binding of selected compounds in the series to the two HIF promoter quadruplexes, and to a randomized duplex DNA control sequence:
HIF-1α: 5′-GCGCGGGGAGGGGAGAGGGGGCGGGAGCGCG-3′
HIF-2α: 5′-GGGGCGGGCACGGGGCGGG-3′
Random DNA duplex: 5'-GCGAATTCGCTCTCGAATTCGC-3′

The methodology used is closely similar to published procedures with 5′-biotin on the DNAs for immobilization.[25] Table 1 lists the derived binding data. The quadruplex-binding SPR sensorgrams (see the Supplementary data) for compounds **7** and **10** were fitted to a single-site model for both quadruplexes, with both compounds having higher affinity for the HIF-1 α quadruplex. The data for compound **CL67** fits a two-site binding model to both HIF quadruplexes, albeit with slightly weaker binding. The binding free energy for the strongest site with **10** is −9.9 kcal/mole while it is −8.9 kcal/mole for **CL67** per site but with a total binding free energy of twice that. It may be that somewhat weaker binding with the ability to stack on both quadruplex ends gives **CL67** a biological advantage in cancer over normal cells. Compound **14** has higher affinity for a single site on the HIF-1α quadruplex and is accommodated in a two-site model for the HIF-2α quadruplex. None of the compounds showed significant binding to the control duplex DNA sequence (Table 1). A typical set of SPR sensorgrams for **10** with all three DNAs and the corresponding equilibrium binding plot with the quadruplex sequences is given in the (Supplementary data).

The circular dichroism (CD) spectrum of the HIF-1 α quadruplex, in 25 mM K^+ solution, titrating in increasing molar ratios of compound **CL67**, is shown in Figure 2. This experiment confirms that the quadruplex structure is retained on binding the compound. The form of the spectra, with a maximum ellipticity at 265 nm and a negative minimum at 240 nm, is consistent with (but does not prove) that this quadruplex has a folded parallel topology, which is retained on ligand binding.

The effects of the compounds in this small focused library on the growth characteristics of a panel of cancer cell lines[26] were assessed by means of the sulforhodamine B (SRB) 96 h assay (Fig. 3a and Table 2).[27] Remarkably the majority of the compounds show only moderate or weak activity against breast, lung or pancreatic carcinoma cell lines whereas they all show high to moderate activity against all the renal carcinoma lines studied here. Compound **CL67** has been highlighted, and subjected to further study, not only because it shows the greatest selectivity overall for the renal lines, but also because its activity in these lines is some 40-fold greater than in the normal human fibroblast line WI38, indicative of a potential therapeutic window. Figure 3a highlights this selectivity of compound **CL67**, as well as in the two human renal cancer cell lines RCC4 and RCC4VHL. Significant activity is also notable in the PC-3 human prostate cancer cell line. Over 80% of renal cancer cells demonstrate over-activation of the HIF-1 pathway due to disruption of the VHL tumor suppressor gene.[26] Compound **CL67** shows two-fold increased sensitivity in RCC4 cells lacking the VHL gene

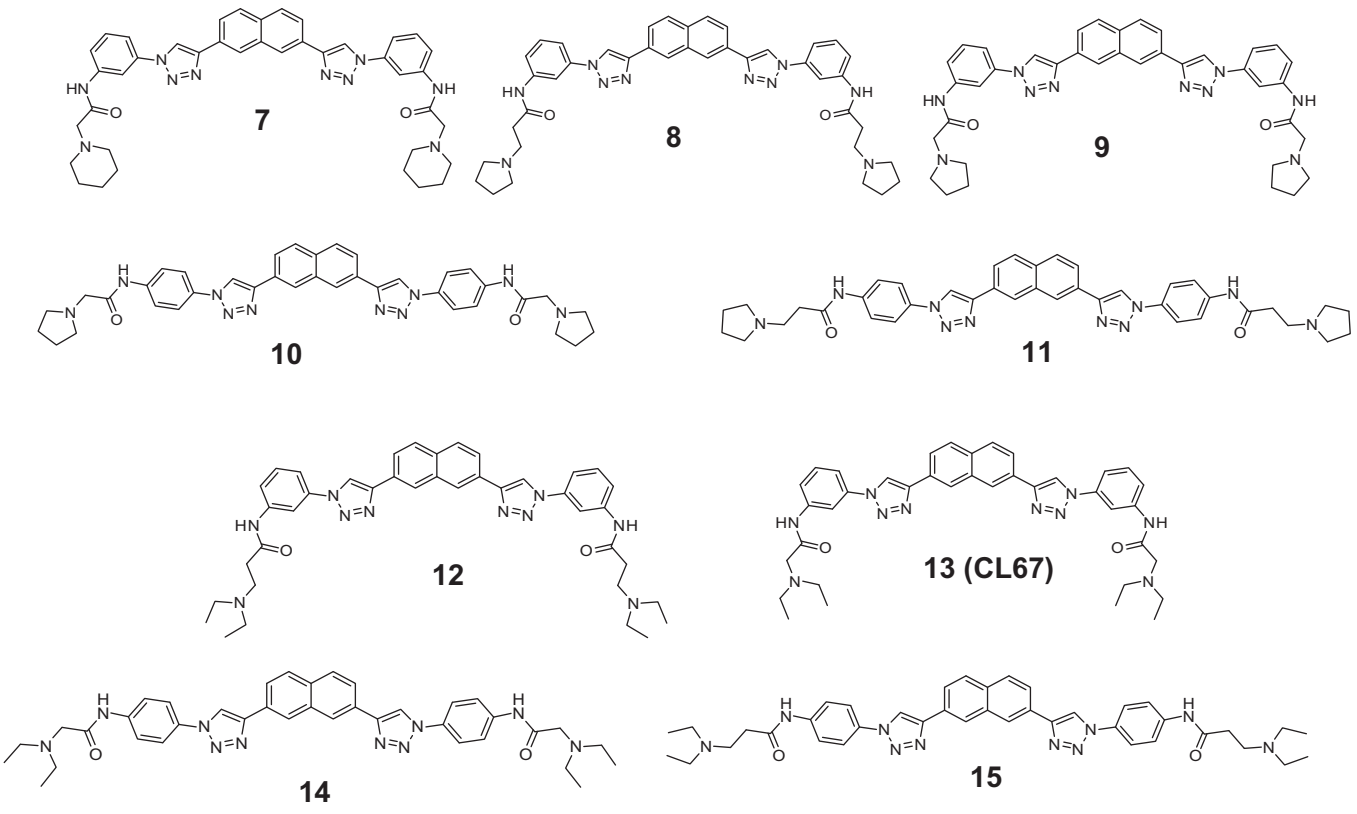
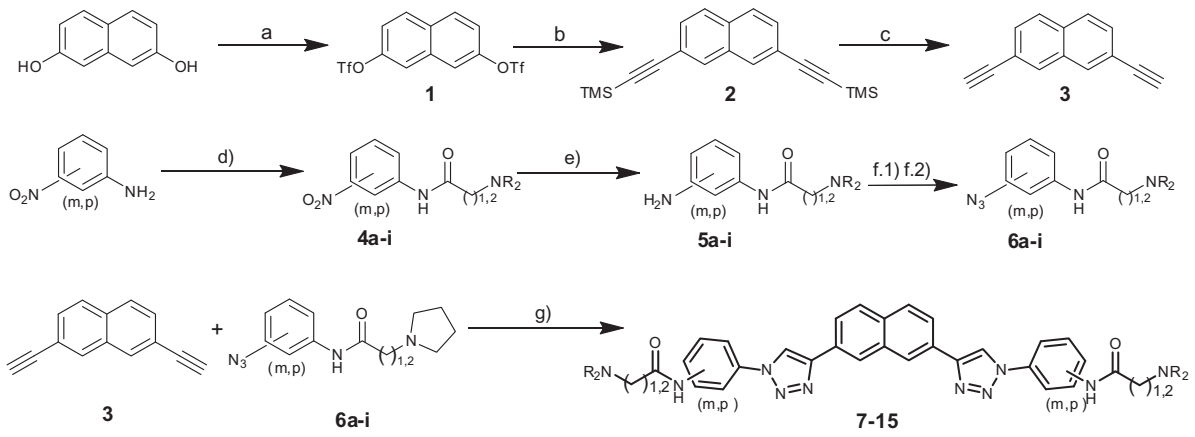


Figure 1. Structures of the members of the naphthalene *bis*-triazole click library.



Scheme 1. *General scheme for the synthesis of compounds* **7–15**. (a) TF_2O, DMAP, 2,6-lutidine, anhydrous THF, anhydrous DCM, molecular sieves, Ar, −78 °C 2 h, 0 °C 5 h; (b) Ethynyltrimetilsilane, CuI, $Pd(PPh_3)_4$, PPh_3, piperidine, molecular sieves, Ar, reflux overnight; (c) NaOH 1M in H_2O, THF, rt, 2 h; d.1) TEA, acetyl chloride (**4a–e**)/3-chloropropionyl chloride (**4f–i**), THF, 4 °C to rt, 2.5 h; (d.2) pyrrolidine (**4a**, **d**, **f**, **h**)/piperidine (**4b**)/diethylamine (**4c**, **e**, **g**, **i**), 4 °C to rt, overnight; (e) H_2, Pd/C, anhydrous THF, N_2, rt, overnight; (f.1) HCl (conc.), ^{t}BuONO, THF, 4 °C, 1.5 h; (f.2) NaN_3, H_2O, 4 °C to rt, overnight; (g) $CuSO_4{\cdot}5H_2O$, sodium ascorbate, bathophenanthrolinedispufonic acid disodium salt hydrate, 50% H_2O–50% ^{t}BuOH, microwave irradiation, 110 °C, 15 min.

Table 1
Binding constants derived from SPR data, for four compounds in the series. See the References and notes section for further detail on the analyses

	HIF-1α ($K \times 10^7$ M^{-1})	HIF-2α ($K \times 10^7$ M^{-1})	DNA duplex ($K \times 10^7$ M^{-1})
7	0.81	0.59	No binding
10	1.49	0.97	No binding
13 (CL67)	0.29, 0.29	0.27, 0.34	$<10^5$
14	1.25	0.63, 0.15	No binding

compared to RCC4 cells transfected with the wild-type VHL gene (RCC4VHL) (IC_{50} of 1.3 μM and 2.6 μM, respectively). Cell viability is also preferentially reduced in RCC4 cells compared with RCC4VHL (data not shown here) cells, particularly under hypoxic conditions (1% oxygen; 2.1-fold decreased cell viability in RCC4 compared to RCC4VHL cells) suggesting that **CL67** may affect cell growth and viability by interfering with the HIF-1 pathway.

Compound **CL67** also preferentially inhibited the growth of a panel of osteosarcoma and Ewing's sarcoma cells in vitro (IC_{50} values 0.9–7.9 μM) (Fig. 3a and Table 3).[28] These sarcomas are also characterized by over-activation of the HIF-1 pathway. We have also found that compound **CL67** inhibited HIF-1α protein in a dose-dependent manner under both normoxic (20% O_2) and hypoxic (1% O_2) conditions in the osteosarcoma cell line U2OS and that HIF-1α protein levels were reduced within 2 h of treatment with **CL67** ($5 \times IC_{50}$) (Fig. 3b). HIF-1α mRNA abundance in U20S cells was reduced to 79.7% of the untreated control following 4 h exposure to **CL67** at the same concentration. HIF-1 transactivation was also reduced in a dose-dependent manner to 7% of pre-treatment activity after 4 h.

The small size of the chemical library precludes any definite conclusions at present on structure-activity relationships. There are indications of some trends from the cell growth inhibition data, though not as yet from the small amount of quadruplex-binding data (solubility problems have precluded a more extensive SPR study at present). We can however conclude that:

1. Shorter side-arms with one $–CH_2–$ unit are generally associated with greater activity, that is, lower IC_{50} values, in the sensitive cell lines.
2. Comparing compounds **14** and **15** shows that selectivity is completely lost by the addition of one $–CH_2–$ unit to each side-arm.

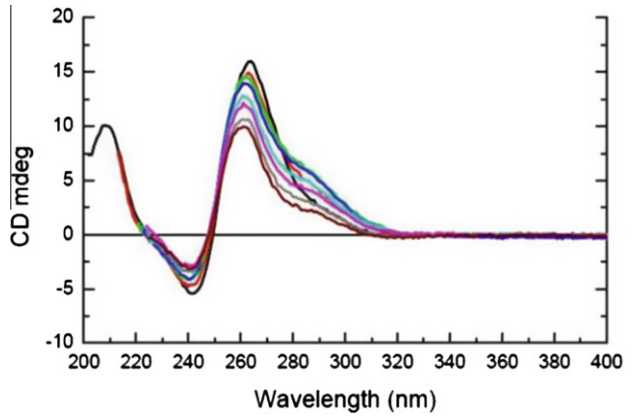


Figure 2. CD spectra of the HIF-1α quadruplex, titrated with increasing concentrations of compound **CL67**.

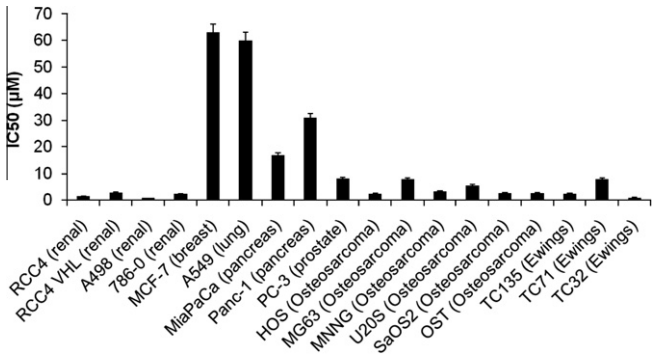


Figure 3a. Plot of IC_{50} values (in μM) for compound **CL67,** assayed in a panel of cancer cell lines, for 96 h exposure using the SRB method.

Table 2
IC_{50} values (in μM) for the nine compounds in the library, across a panel of normal human fibroblast (WI38) and cancer cell lines. The lines comprise breast, lung, pancreatic, renal and prostate carcinomas (see References and notes section for further details)

	HOS	MG63	MNNG	U20S	SaOS2	OST	TC135	TC71	TC32
7	47.0	90	23.0	22.0	16	37.0	14.0	81.0	6.6
8	7.1	>100	2.9	13.0	2.7	2.2	2.5	8.9	1.9
9	2.0	16	9.0	22.0	5.8	7.1	11.0	75.0	3.0
10	2.6	>10	9.1	18.0	9	14.0	3.2	14.0	2.5
11	0.6	2.8	2.1	2.2	1.8	0.9	2.2	16.0	0.7
12	ND	ND	3.1	7.8	2.8	2.1	2.6	28.0	2.5
13 (CL67)	2.4	7.9	3.2	5.5	2.6	2.6	2.4	7.8	0.9
14	75.0	81	25.0	22.0	95	37.0	23.0	>100	29.0
15	5.2	7	2.8	5.1	2.5	1.9	2.2	7.5	2.1

Table 3
IC_{50} values (in μM) for the nine compounds in the library, across a panel of osterosarcoma and Ewing's sarcoma cell lines (see References and Notes section for further details)

	WI38	MCF7	A549	Mia-PaCa	Panc1	HPAC	PC-3	786-0	A498	RCC4	$RCC4_{VHL}$
7	>10	>100	>100	>100	>100	96	>100	17.5	19.0	2.6	2.7
8	18.0	30	76	11	7.1	8.0	9.4	5.85	7.5	4.2	6.8
9	17.0	>100	72	>100	29	9.0	25	6.9	3.1	3.2	2.4
10	26.0	>100	80.5	25.5	88	9.5	91	2.6	3.5	5.1	6.4
11	3.9	8.1	17	7.2	7.8	1.9	7.1	6.5	1.9	2.5	2.6
12	10.6	21	29	17.5	7.4	ND	7.8	9.8	9.9	1.6	2.7
13 (CL67)	91.0	63	60	16.8	31	7.3	8.05	2.3	2.5	1.4	2.6
14	9.0	100	>100	>100	>100	52.0	>100	23	8.0	22	26
15	7.4	9.2	7.6	12.5	8	6.9	8.2	5.6	6.9	1.9	2.4

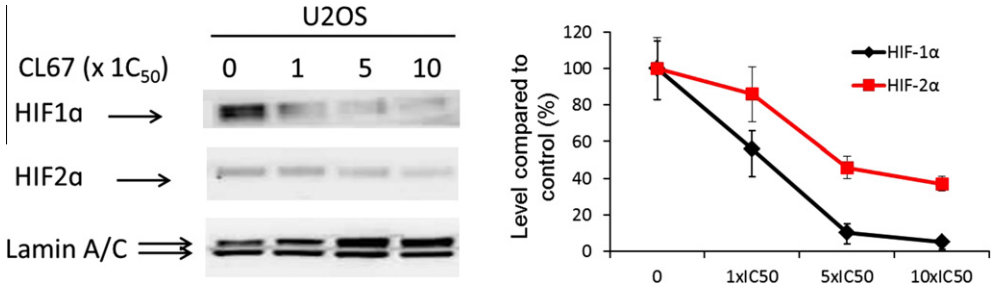


Figure 3b. U20S osteosarcoma cells incubated in hypoxia (1% oxygen) for 16 h were then treated for 4 h with increasing concentrations of **CL67** as indicated. Cells were lysed and Western blotting shown was performed probing for HIF-1α and HIF-2α. Lamin A/C were used as loading controls and protein expression was quantified by densitometry relative to control. Quantification of the effect of exposure to increasing concentrations of **CL67** on HIF-1α protein expression is shown in the rhs plot.

3. A comparison of compounds **CL67** and **14** suggest that *meta* substitution on the phenyl rings results in greater potency than *para*.
4. The size of the end-group may be a factor, since compound **7** is overall the least active compound (while still showing selectivity), and the behavior of compound **9** is intermediate between it and compound **CL67**.

The selective effect of these compounds, especially the lead compound, **CL67**, on HIF-1 expressing cancer cell lines, may be due to inhibition at any one of a number of steps in the HIF pathway. The down-regulation of HIF-1 expression in both renal carcinoma and osteosarcoma cell lines (Figs. 3a and b), though not fully validating the hypothesis that the HIF-1 promoter is a target, is consistent with this, and thus with the concept that the HIF-1α quadruplex sequence in particular, is stabilized by these compounds. It is implausible to suggest that compound **CL67** and its analogues are selective solely for the HIF quadruplexes, and other genomic quadruplex targets within the HIF pathway (and indeed other categories of quadruplex and non-quadruplex target) may also be involved.[4,14] Current studies are aimed at addressing these questions, and also at improving the druggability of compound **CL67**. Although a number of other inhibitors of the HIF pathway have been reported in the literature,[29–33] none to our knowledge are structurally related to the compounds presented here, or are known to function at the quadruplex level. It remains the case that remarkably few HIF inhibitors have to date progressed through to clinical trial, possibly because of the challenges posed by developing potent inhibitors of either HIF transcription or HIF protein–protein recognition.

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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.2012.07.009.

References and notes

1. Burge, S.; Parkinson, G. N.; Hazel, P.; Todd, A. K.; Neidle, S. *Nucleic Acids Res.* **2006**, *34*, 5402.
2. Phan, A. T.; Kuryavyi, V.; Luu, K. N.; Patel, D. J. *Nucleic Acids Res.* **2007**, *35*, 6517.
3. Huppert, J. L.; Balasubramanian, S. *Nucleic Acids Res.* **2007**, *35*, 406.
4. Balasubramanian, S.; Hurley, L. H.; Neidle, S. Nat. Rev. Drug *Discovery* **2011**, *10*, 261.
5. Neidle, S. *Curr. Opin. Struct. Biol.* **2009**, *19*, 1.
6. Adrian, M.; Heddi, B.; Phan, A. T. Methods 2012, doi.org/10.1016/j.ymeth.2012.05.003.
7. Monchaud, D.; Teulade-Fichou, M.-P. *Org. Biomol. Chem.* **2008**, *6*, 627.
8. Neidle, S. *Therapeutic Aspects of Quadruplex Nucleic Acids*; Academic Press: San Diego, 2011.
9. Yang, D. Z.; Okamoto, K. *Future Med. Chem.* **2010**, *2*, 619.
10. See for example: (a) Sparapani, S.; Haider, S. M.; Doria, F.; Gunaratnam, M.; Neidle, S. *J. Amer. Chem. Soc.* **2010**, *132*, 12263; (b) Tran, P. L.; Largy, E.; Hamon, F.; Teulade-Fichou, M. P.; Mergny, J.-L. *Biochimie* **2011**, *93*, 1288; (c) Dash, J.; Das, R. N.; Hegde, N.; Pantoș, G. D.; Shirude, P. S.; Balasubramanian, S. *Chemistry* **2012**, *18*, 554.
11. (a) Siddiqui-Jain, A.; Grand, C. L.; Bearss, D. J.; Hurley, L. H. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11593; (b) Brown, R. V.; Danford, F. L.; Gokhale, V.; Hurley, L. H.; Brooks, T. A. *J. Biol. Chem.* **2011**, *286*, 41018.
12. (a) 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; (b) McLuckie, K. I.; Waller, Z. A.; Sanders, D. A.; Alves, D.; Rodriguez, R.; Dash, J.; McKenzie, G. J.; Venkitaraman, A. R.; Balasubramanian, S. *J. Amer. Chem. Soc.* **2011**, *133*, 2658.
13. Rodriguez, R.; Miller, K. M.; Forment, J. V.; Bradshaw, C. R.; Nikan, M.; Britton, S.; Oelschlaegel, T.; Xhemalce, B.; Balasubramanian, S.; Jackson, S. P. *Nat. Chem. Biol.* **2012**, *8*, 301.
14. (a) Wieland, M.; Hartig, J. S. *Chem. Biol.* **2007**, *14*, 757; (b) Bugaut, A.; Balasubramanian, S. *Nucleic Acids Res.* **2012**. http://dx.doi.org/10.1093/nar/gks068.
15. Greer, S. N.; Metcalf, J. L.; Wang, Y.; Ohh, M. *EMBO J.* **2012**, *31*, 2448.
16. Jonasch, E.; Futreal, A.; Davis, I.; Bailey, S.; Kim, W. Y.; Brugarolas, J.; Giaccia, A. J.; Kurban, G.; Pause, A.; Frydman, J.; Zurita, A.; Rini, B. I.; Sharma, P.; Atkins, M.; Walke, C.; Rathmell, W. K. *Mol. Cancer Res.* **2012**. http://dx.doi.org/10.1158/1541-7786.MCR-12-0117.
17. Keith, B.; Johnson, R. S.; Simon, M. C. *Nat. Rev. Cancer* **2011**, *12*, 9.
18. Yang, Q. C.; Zeng, B. F.; Dong, Y.; Shi, Z. M.; Jiang, Z. M.; Huang, J. *Jpn J Clin Oncol.* **2007**, *37*, 127.
19. Mizobuchi, H.; García-Castellano, J. M.; Philip, S.; Healey, J. H.; Gorlick, R. *Clin. Orthop. Relat. Res.* **2008**, *466*, 2052.
20. Chen, C.; Zhou, H.; Wei, F.; Jiang, L.; Liu, X.; Liu, Z.; Ma, Q. J. *Orthop. Res.* **2011**, *29*, 143.
21. De Armond, R.; Wood, S.; Sun, D.; Hurley, L. H.; Ebbinghaus, S. W. *Biochemistry* **2005**, *44*, 16341.
22. Moorhouse, A. D.; Santos, A. M.; Gunaratnam, M.; Moore, M.; Neidle, S.; Moses, J. E. *J. Amer. Chem. Soc.* **2006**, *128*, 15972.
23. Lombardo, C. M.; Sánchez Martínez, I.; Haider, S.; Gabelica, V.; De Pauw, E.; Moses, J. E.; Neidle, S. *Chem. Commun.* **2010**, *46*, 9116.
24. Synthesis and analysis of compound **13 (CL67)**. 2,7-Diethynylnaphthalene (20 mg, 0.11 mmol) was dissolved in H_2O (1.5 ml) and ^tBuOH (1.5 ml), then the azide (84 mg, 0.34 mmol), $CuSO_4$ (2 mg, 0.005 mmol), sodium ascorbate (11 mg, 0.05 mmol) and bathophenanthrolinedispufonic acid disodium salt hydrate (clicl catalyst) (6 mg, 0.01 mmol) were added, to give compound **CL67** as a brown solid crude (126 mg, quantitative). Prep HPLC gave a beige solid; HPLC Rt 12.06 minutes, purity 97%; mp 97–99 °C; δH (d_6-DMSO, 400 MHz) 10.01 (2H, s, 2 × N*H*), 9.44 (2H, s, 2 × C═C*H*), 8.61 (2H, s, 2 × Ar*H*), 8.44–8.43 (2H, m, 2 × Ar*H*), 8.17–8.09 (4H, m, 4 × Ar*H*), 7.82–7.80 (2H, m, 2 × Ar*H*), 7.68–7.65 (2H, m, 2 × Ar*H*), 7.61–7.57 (2H, m, 2 × Ar*H*), 3.23 (4H, s, 2 × CH_2), 2.64 (8 H, quartet, *J* = 7.2 Hz, 4 × CH_2), 1.05 (12 H, t, *J* = 7.2 Hz, 4 x CH_3); δC (d_6-DMSO, 100 MHz) 170.39 (2 × C═O), 147.13 (2 x C═CH), 139.76 (2 × Ar-*C*), 136.84 (2 × Ar-*C*), 133.25 (Ar-*C*), 132.36 (Ar-*C*), 130.22 (2 × Ar-CH), 128.61 (2 × Ar-*C*), 128.36 (2 × Ar-CH), 123.97 (2 × Ar-CH), 123.89 (2 × Ar-CH), 120.15 (2 × *C*═CH), 119.30 (2 × Ar-CH), 114.74 (2 × Ar-CH), 110.88 (2 × Ar-CH), 57.25 (2 × CH_2), 47.81 (4 × CH_2), 11.83 (4 × CH_3); HRMS *m/z* calc. $C_{38}H_{43}N_{10}O_2$ $[M+H]^+$ 671.3571, found $[M+H]^+$ 671.3594.
25. Experiments were performed on a BIACORE T200 instrument at 25 °C with buffer containing 10 mM HEPES, 100 mM KCl, 3 mM EDTA and 0.05% P20 surfactant with the final pH adjusted to 7.3. The steady-state response obtained (RUobs) was plotted versus the unbound ligand concentration (Cfree) in the flow solution and fitted to either a single-site or a two-site model: RUobs= RUmax/site × $(K_1C_{free} + 2K_1K_2C_{free}^2)/(1 + K_1C_{free} + K_1K_2C_{free}^2)$; K_2= 0 for a single site.
26. Cells and reagents: A498 human renal carcinoma, 786–0 human renal carcinoma, MCF-7 human breast carcinoma, Mia-Pa-Ca human pancreatic carcinoma, Panc-1 human pancreatic carcinoma, and PC-3 human prostate carcinoma cells were obtained from American Type Culture Collection (Rockville, MD). Human RCC4 and RCC4/VHL cells were obtained from Professor P. Maxwell (University College London, UK) (Maxwell, P. H.; Wiesener, M. S.; Chang, G. W.; Clifford, S. C.; Vaux, E. C.; Cockman, M. E.; Wykoff, C. C.; Pugh, C. W.; Maher, E. R.; Ratcliffe, P. *J. Nature,* **1999**, *399*, 271). RCC4 cells lack pVHL therefore express constitutively high levels of HIF-1 α. These cells were stably transfected with a wild-type pVHL gene, as previously described (Cockman, M. E. et al., *J. Biol. Chem.* **2000**, *275*, 25733). All cells were grown in humidified 95% air, 5% CO2 at 37°C in Dulbecco's modified Eagle's medium (DMEM), RPMI or MEM medium supplemented with 10% fetal bovine serum (FBS) and 2mM L-Glutamine according to advice from ATCC (all reagents were obtained from Gibco, Invitrogen, UK). HIF-1α and HIF-2α antibodies were obtained from BD Transduction Labs, AKT antibody was obtained from Cell Signalling Biotechnology, and lamin A/C, actin, HIF-1β, HSP-90, and Raf-1 antibodies were from Santa Cruz Biotechnology.
27. Short-term antiproliferative activity was evaluated by means of the sulforhodamine B assay, and calculated as IC_{50} values for 96 h exposure Vichai, V.; Kirtikara, K. *Nature Protocol.* **2006**, *1*, 1112.
28. Osteosarcoma (HOS, MNNG, MG63, U2OS, SaOS2, OST) and Ewing's-(TC32, TC71, TC135) sarcoma cell lines were treated with compound **CL67** (with 1 ×, 5 × or 10 × IC_{50}) for various times in humidified air, 5% CO_2 (normoxia) or 1% O_2, 5% CO_2, 94% N_2 (hypoxia) using an In Vivo Hypoxia Workstation 400. Cell growth inhibition was measured using the sulphorhodamine B assay, HIF-1α protein using Western blotting, HIF-1 transactivation using transient transfection of cells with a plasmid expressing luciferase under the control of multiple copies of the hypoxia-response element, and HIF-1α mRNA using RT-PCR, as previously described Koh, M. Y.; Spivak-Kroizman, T.; Venturini, S.; Welsh, S.; Williams, R. R.; Kirkpatrick, D. L.; Powis, G. *Mol. Cancer Ther.* **2008**, *7*, 90.
29. Wendt, B.; Mulbaier, M.; Wawro, S.; Schultes, C.; Alonso, J.; Janssen, B.; Lewis, J. *J. Med. Chem.* **2011**, *54*, 8471.
30. An, H.; Kim, N. J.; Jung, J. W.; Jang, H.; Park, J. W.; Suh, Y. G. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 6297.
31. Mooring, S. R.; Jin, H.; Devi, N. S.; Jabbar, A. A.; Kaluz, S.; Liu, Y.; Van Meir, E. G.; Wang, B. *J. Med. Chem.* **2011**, *54*, 8471.
32. Xia, Y.; Choi, H. K.; Lee, K. *Eur. J. Med. Chem.* **2012**, *49*, 24.
33. Welsh, S.; Williams, R.; Kirkpatrick, L.; Paine-Murrieta, G.; Powis, G. *Mol. Cancer Ther.* **2004**, *3*, 233.