Highly cytotoxic iron(II) complexes with pentadentate pyridyl ligands as a new class of anti-tumor agents†

Ella Lai-Ming Wong, Guo-Su Fang, Chi-Ming Che* and Nianyong Zhu

Received (in Cambridge, UK) 1st June 2005, Accepted 15th July 2005
First published as an Advance Article on the web 19th August 2005
DOI: 10.1039/b507687k

The iron(II) complexes 1a and 2 with pentadentate pyridyl ligands are stable under physiological conditions and exhibit higher cytotoxicities toward a series of human carcinoma cell lines than cisplatin; 1a can significantly increase intracellular oxidant levels, cleave supercoiled plasmid DNA *in vitro* without addition of a reductant and induce apoptotic cell death in human cervical epithelioid carcinoma cells (HeLa) as observed in flow cytometric studies.

Iron is essential in many biological processes,[1] and is an important nutrient involved in the proliferation of cancer cells.[2] Current anti-cancer chemotherapy involves the use of bleomycin (BLM),[3] which is an iron containing glycopeptide[4] causing oxidative DNA damage in the presence of O_2 and H_2O_2.[5] Herein we present that the Fe(II) polypyridyl complexes **1a**, **1b** and **2** (Fig. 1) could be potential alternatives to bleomycin in killing cancer cells. They have acute cytotoxic effects (within 48 h) against human carcinoma cell lines (HeLa and HepG2) *in vitro* when compared with the clinically used anti-neoplastic drug, bleomycin. Moreover, they exhibit excellent cytotoxic effects on taxol resistant human hepatocellular carcinoma cell line, QGY-TR50. In this work, the polypyridine ligands were employed to form stable chelates with Fe(II) under physiological conditions.

Compounds $[Fe(R\text{-}qpy)(CH_3CN)_2](ClO_4)_2$ [qpy = 2,2′:6′,2″:6″,2‴:6‴,2⁗-quinquepyridine;[6] R = H (**1a**) and Ph (**1b**)] were prepared by reacting the corresponding ligand with $Fe(ClO_4)_2{\cdot}6H_2O$ in CH_3CN under N_2.‡ The crystal structure of **1a** shows a 7-coordinated Fe atom (Fig. S1, ESI†). The average $Fe\text{–}N_{pyridine}$ distance is 2.28 Å which is typical for high spin Fe(II)

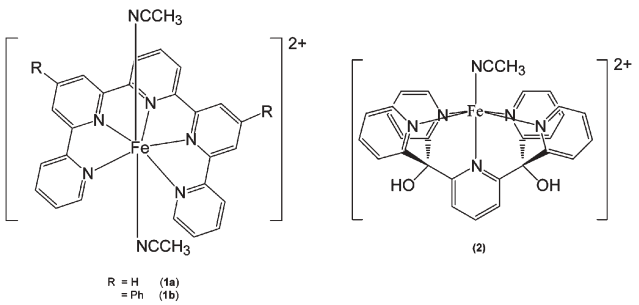

Fig. 1 Structure of complex cations of **1a**, **1b** and **2**.

Department of Chemistry and Open Laboratory of Chemical Biology of the Institute of Molecular Technology for Drug Discovery and Synthesis, The University of Hong Kong, Pokfulam Road, Hong Kong.
E-mail: cmche@hku.hk; Fax: +(852)-2857-1586
† Electronic supplementary information (ESI) available: Experimental and crystallographic details. See http://dx.doi.org/10.1039/b507687k

complexes.[7] Unlike the planar QP ligand in $[Fe^{II}(QP)(OH_2)_2](ClO_4)_2$ (QP = 2,2′:6′,2″:6″,2‴-quaterpyridine),[8] the pentapyridyl qpy ligand needs to be slightly twisted in order to accommodate the Fe atom. The $Fe\text{–}N_{CH_3CN}$ distances are 2.183 and 2.184 Å. A similar structure was found for **1b** (Fig. S2, ESI†). Ligand Py_5-OH and its iron complex $[Fe(Py_5\text{-}OH)(CH_3CN)](ClO_4)_2$ (**2**) were prepared according to literature method[9] and characterized by ESI-MS. The chloro-analogue of **2**, $[Fe(Py_5\text{-}OH)Cl](CF_3SO_3)$ has also been prepared and its structure was determined by X-ray crystallography (Fig. S3, ESI†). ESI-MS spectra of **1a**, **1b** and **2** in CH_3CN showed molecular ion peaks with *m*/*z* at 242.0, 317.7 and 271.8 corresponding to $[Fe(H\text{-}qpy)(CH_3CN)]^{2+}$, $[Fe(Ph\text{-}qpy)(CH_3CN)]^{2+}$ and $[Fe(Py_5\text{-}OH)(CH_3CN)]^{2+}$ respectively.

UV-vis spectra of **1a** and **1b** show a broad absorption band at 534 nm (ε = 630 $dm^3\ mol^{-1}\ cm^{-1}$) and 567 nm (ε = 890 $dm^3\ mol^{-1}\ cm^{-1}$) respectively. The complexes are stable in solid form and show no sign of ligand dissociation in CH_3CN for 6 days under air. In H_2O with 1% CH_3CN, **1a** is stable over 12 h with no observable change in its absorption spectrum. Depletion of the 534 nm absorption was followed by gradual formation of a shoulder at around 400 nm upon standing for 2 days. Complex **1b** is less stable in H_2O and demetalation was found upon standing in aqueous medium for 2 days. Complex **2** is stable in CH_3CN and aqueous solutions even with standing for 6 days. In 2 mM GSH solutions, **1a** and **2** are stable with no sign of demetalation for 48 h. Apparently, oxidation of Fe(II), characterized by depletion of the 534 nm band in **1a**, was retarded in the presence of GSH.

At room temperature **1a** is paramagnetic with a μ_{eff} of 4.94 B.M., corresponding to an $S = 2$ ground state (calculated: $S = 2$, μ_{eff} = 4.90 B.M.). This observation matches with the $Fe\text{–}N_{pyridine}$ distance of 2.28 Å typical for high-spin Fe(II) complexes. Cyclic voltammetry of **1a** in 0.1 M nBu_4NPF_6 CH_3CN solution shows a reversible oxidation couple at +0.5 V *vs.* $Cp_2Fe^{+/0}$ attributed to the oxidation of Fe(II) to Fe(III). $[Fe(H\text{-}qpy)O]^{2+}$ was generated by reacting **1a** with 1 eq. of PhIO or 2 eq. of H_2O_2 in CH_3CN for 1 min and was characterized by ESI-MS with *m*/*z* = 229.6. Thus, the qpy ligand is capable of stabilizing Fe(II) in aqueous medium and generating high-valent Fe(IV)=O species.

The biological activities of the iron complexes have been examined. Treatment of 0.6 μM of **1a** with supercoiled plasmid DNA pCR 2.1 (0.06 μg μL^{-1}), with $[Fe]/[DNA_{bp}]$ (r) = 0.05, in the presence of air without the addition of reductant, at pH 7.5 and 37 °C, resulted in immediate formation of nicked DNA. Fig. 2 compares the cleavage of supercoiled plasmid DNA pCR 2.1 by **1a**, **1b**, **2**, $[Fe^{II}(QP)(OH_2)_2]^{2+}$ and Fe(BLM) using a 1.2% agarose gel. No cleavage was observed for **2** at 10 s and 60 min time

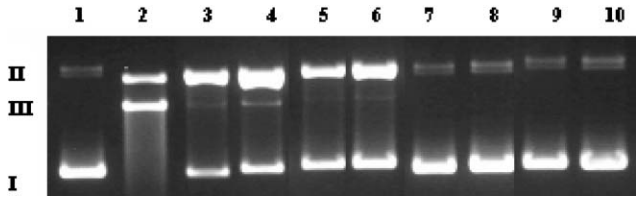

Fig. 2 Cleavage of pCR 2.1 supercoiled plasmid DNA (0.06 μg μL^{-1}) with 0.6 μM reagent at t = 10 s. Reactions were preformed in Tris buffer (10 mM, pH = 7.5) at 37 °C in the presence of air. **Lane 1**: DNA control; **Lane 2**: 0.06 μM Fe(BLM); **Lane 3**: 0.6 μM **1a**; **Lane 4**: 6 μM **1a**; **Lane 5**: 0.6 μM **1b**; **Lane 6**: 6 μM **1b**; **Lane 7**: 0.6 μM $[FeQP(OH_2)_2]^{2+}$; **Lane 8**: 6 μM $[FeQP(OH_2)_2]^{2+}$; **Lane 9**: 0.6 μM **2**; **Lane 10**: 6 μM **2**. I = supercoiled plasmid DNA; II = nicked DNA; III = linear DNA.

intervals. Significant DNA cleavage (t = 10 s) was observed for **1a**, **1b** (>50%) and Fe(BLM) (≈100%). Fe(BLM) afforded both nicked (47%) and linear (53%) DNA, whereas **1a** and **1b** mainly afforded nicked DNA (64% and 55% respectively), typical for a single-strand cleavage agent.[11] Experiments were performed to evaluate the effect of complex concentration and reaction time on the DNA cleavage activities. Increasing the concentration of iron complexes (0.6 μM–60 μM, r = 0.005–5) increased the extent of nicked and linear DNA formation for **1a** and **1b** when supercoiled plasmid DNA concentration (0.6 μg μL^{-1}) remained constant. The percentages of nicked and linear DNA formed at t = 10 s and 120 min were similar (metal complex 0.6 μM, DNA 0.06 μg μL^{-1}), showing that the DNA cleavage was an instant process. No DNA cleavage was observed for **2** at 0.6 μM concentration at t = 120 min or at 60 μM concentration at t = 60 min with 0.06 μg μL^{-1} DNA.

By means of the MTT assay, cytotoxicities of **1a**, **1b** and **2** toward some established human carcinoma cells, including some drug resistant variants were determined. The results are listed in Table 1. All the iron complexes are more potent than the clinically used platinum-based drug, cisplatin. Complex **1a** was 5-fold more potent than cisplatin toward HeLa and SUNE-1 cell lines; whereas **2** was 18- and 2-fold more potent respectively. The Fe(II) complexes appeared to be less toxic toward MCF-7 (human breast carcinoma) with IC_{50} ≈ 10–70 μM. More importantly, they exhibited acute cytotoxic effects when compared with Fe(BLM). In this work, using the HeLa and HepG2 cell lines with the same dosage range, Fe(BLM) was non toxic (IC_{50} > 100 μM), whereas **1a**, **1b** and **2** showed IC_{50} at micromolar range in the 48 h MTT assay. Fe(BLM) was effective only when incubation was prolonged to 5 days leading to an IC_{50} = 5.4 μM toward the HepG2 cell line.

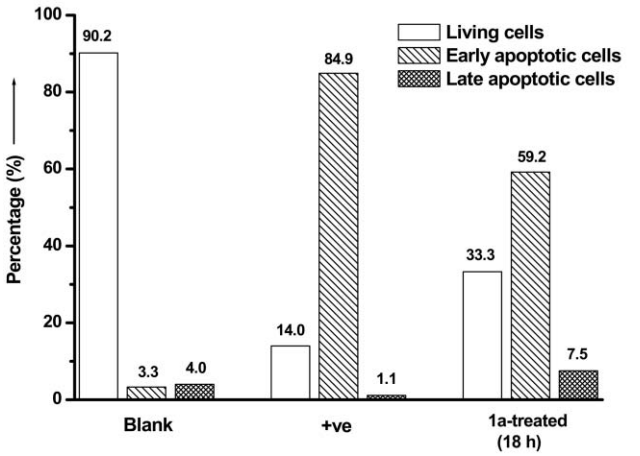

Fig. 3 Studies of **1a**-induced apoptosis of the HeLa cells using flow cytometry with an Annexin-V-FLUOS staining kit. Above shows the percentage of living, early apoptotic and late apoptotic cells in blank (untreated cells), positive control (*Staurosporine streptomyces* sp., t = 12 h) and **1a**-treated cells at t = 18 h.

Among the selected carcinoma cell lines, the Fe(II) complexes are more potent (IC_{50} ≈ 0.1–14 μM) toward hepatocellular carcinoma cell lines. Importantly, **1a** was potent even in taxol resistant human hepatocellular carcinoma QGY-TR50, and was 10-fold more cytotoxic than **2**.

The mechanism of cell death caused by **1a** was examined using HeLa cells. Different cell morphology was observed after drug treatment. Using an Annexin-V-FLUOS staining kit, apoptotic cells were quantified by flow cytometry. 59.2% of the HeLa cells were found to undergo early apoptosis when treated with **1a** for 18 h (Fig. 3). The cell cycle of **1a**-treated HeLa cells was evaluated at different time points by a propidium iodide (PI)-staining method and was analyzed using flow cytometry (Fig. 4). **1a**-treated cells tended to arrest at S-phase, which is responsible for DNA replication,[12] with prolonged treatment. This suggests that **1a** could cause damage of cellular DNA and the recovery mechanism of HeLa cells was activated for DNA repairment. As a result, most of the cells would arrest in S-phase before any DNA replication could proceed. These results suggest that the cytotoxic effect of **1a** could be attributed to its ability to damage DNA.

Intracellular production of oxidants upon treatment of HeLa cells with Fe(II) complexes was evaluated using dihydrorhodamine-123 (DHRh-123). Through the oxidation of non-fluorescent DHRh-123 to green fluorescence rhodamine-123^+ (Rh-123^+),

Table 1 Cytotoxicities (IC_{50}) of iron(II) complexes toward selected human carcinoma and normal cell lines[a,b]

		IC_{50} (μM) [48 h]						
Entry	Compound	HeLa	MCF-7	SUNE-1	HepG2	Hep3B	QGY-TR50	CCD-19Lu
1	**1a**	4.00 ± 0.02	12.20 ± 0.09	2.77 ± 0.17	0.74 ± 0.03	0.13 ± 0.01	0.67 ± 0.04	11.90 ± 0.37
2	**1b**	7.74 ± 0.04	68.50 ± 0.22	11.20 ± 0.76	0.13 ± 0.02	0.24 ± 0.03	ND[c]	17.70 ± 0.68
3	**2**	1.17 ± 0.14	27.40 ± 0.08	6.01 ± 0.22	0.93 ± 0.09	13.50 ± 0.36	6.75 ± 0.19	40.70 ± 0.65
4	Cisplatin (in H_2O)	20.50 ± 0.15	78.80 ± 0.19	14.20 ± 0.18	32.70 ± 0.07	11.82 ± 0.07	31.01 ± 0.96	117.30 ± 0.55

[a] IC_{50} of the corresponding ligands are >100 μM. HeLa = human cervical epithelioid carcinoma; MCF-7 = human breast carcinoma; SUNE-1 = human nasopharyngeal carcinoma; HepG2 = human hepatocellular carcinoma; Hep3B = human hepatocellular carcinoma (with p53 knock out); QGY-TR50 = human hepatocellular carcinoma (taxol resistance), IC_{50} for taxol toward QGY-TR50 in a 96 h XTT assay = 6.04 μM;[10] CCD-19Lu = normal human lung fibroblast cells; values are mean ± SD (μM) from at least three independent experiments. [b] IC_{50} for Fe(BLM) toward i) HeLa = 1.0 μM; ii) HepG2 = 5.4 μM were based on a 120 h MTT assay in this work. [c] ND = not determined.

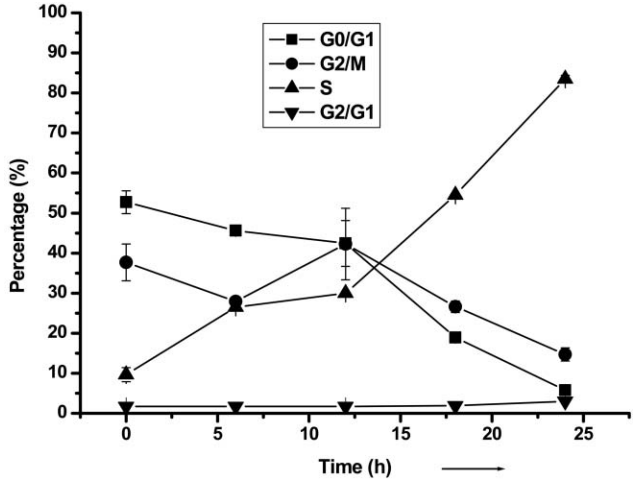

Fig. 4 Cell cycle evaluation of **1a**-treated HeLa cells. Cells were fixed with 70% ethanol, stained with PI and analyzed by flow cytometry; G0/G1 (■), G2/M (●), S (▲) and G2/G1 (▼) phases.

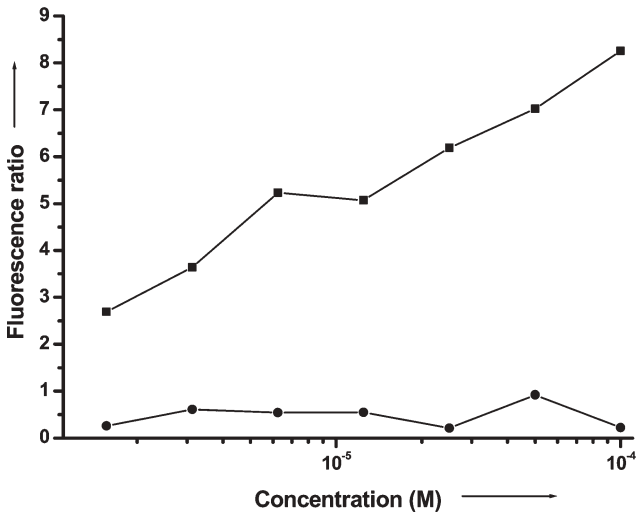

Fig. 5 Detection of ROS generated in HeLa cells after 60 min of drug treatment (1.5–100 μM); **1a** (■) and **2** (●). Fluorescence intensity has been corrected with untreated cells.

generation of intracellular reactive oxygen species (ROS) was measured. HeLa cells were incubated in the presence of DHRh-123 (5 μM L^{-1}) and **1a** (1.5–100 μM) for 60 min. The intensity of Rh-123$^+$ formed was measured using spectrofluorometry (Fig. 5). Significant increase of Rh-123$^+$ fluorescence was detected for **1a**-treated cells (8-fold more than controlled cells). This result supports that **1a** could lead to the generation of ROS, which could be responsible for its cytotoxic properties. On the contrary, **2**-treated cells did not show any observable increase in fluorescent intensity of Rh-123$^+$ even with an increase of its dosage up to 100 μM and/or on prolonging the incubation period.

Both Fe(BLM) and **1a** can cleave supercoiled plasmid DNA and generate ROS *in vitro*, but their activities toward selected carcinoma cells are different. Also both **1a** and **2** are cytotoxic, but their cytotoxic mechanism(s) seems to be different. It is apparent that the biological activities of iron(II) chelates could be modulated through variation of the auxiliary polypyridyl ligands.§

We acknowledge financial support from the University of Hong Kong (University Development Fund), the Area of Excellence Scheme (AoE) administered by the University Grants Council (HKSAR, China) and the Hong Kong Research Grants Council (HKU7384/02P). We also acknowledge Dr Y. Xie for his generosity in providing the QGY-TR50 cell line.

Notes and references

‡ Synthesis of **1a** and **1b**: A mixture of ligand (0.13 mmol) and $Fe(ClO_4)_2{\cdot}6H_2O$ (0.2 mmol) in a 50 mL Schlenk tube was degassed and the tube filled with N_2. Freshly distilled and degassed acetonitrile (20 mL) was added to the reaction mixture. The reaction mixture was gently warmed (50 °C) for 12 h. Crystals were obtained after keeping the reaction mixture at 0 °C for 4 days under an N_2 atmosphere.

§ *Crystal data:* **1a**·1.5CH_3CN: $C_{32}H_{27.50}Cl_2FeN_{8.50}O_8$, M = 785.88, triclinic, $P\bar{1}$, a = 11.732(2) Å, b = 12.435(3) Å, c = 13.997(3) Å, α = 94.77(3)°, β = 113.14(3)°, γ = 104.39(3)°, V = 1783.5(7) Å^3, T = 301(2) K, Z = 2, μ = 0.634 mm^{-1}, 4652 collected reflections, 3030 independent reflections [R(int) = 0.051], final R indices R = 0.094, wR = 0.25. **1b**: $C_{41}H_{31}Cl_2FeN_7O_8$, M = 876.48, triclinic, $P\bar{1}$, a = 13.072(3) Å, b = 13.181(3) Å, c = 14.660(3) Å, α = 76.69(3)°, β = 67.58(3)°, γ = 62.77(3)°, V = 2071.5(8) Å^3, T = 253(2) K, Z = 2, μ = 0.554 mm^{-1}, 2805 collected reflections, 2193 independent reflections [R(int) = 0.073], final R indices R = 0.12, wR = 0.29. [$Fe(Py_5$-OH)Cl](CF_3SO_3): $C_{29}H_{25}ClF_3FeN_5O_6S$, M = 719.90, triclinic, $P\bar{1}$(#2), a = 8.570(2) Å, b = 12.315(3) Å, c = 15.762(3) Å, α = 102.03(3)°, β = 97.89(3)°, γ = 108.04(3)°, V = 1510.1(6) Å^3, T = 253(2) K, Z = 2, μ = 0.728 mm^{-1}, 6218 collected reflections, 3711 independent reflections [R(int) = 0.046], final R indices R = 0.052, wR = 0.14. CCDC 274157–274159. See http://dx.doi.org/10.1039/b507687k for crystallographic data in CIF or other electronic format.

1 S. J. Lippard and J. M. Berg, *Principles of Bioinorganic Chemistry*, University Science Books, Mill Valley, CA, USA, 1994.
2 (*a*) C. P. Siegers, D. Bumann, H. D. Trepkau, B. Schadwinkel and G. Baretton, *Prog. Clin. Biol. Res.*, 1991, **369**, 439; (*b*) A. Kicic, A. C. G. Chua and E. Baker, *Anti-Cancer Drug Des.*, 2001, **16**, 195; (*c*) E. D. Weinberg, *Cancer Invest.*, 1999, **17**, 507; (*d*) M. Franchini and D. Veneri, *Hematol. J.*, 2004, **5**, 381.
3 L. M. Mir, O. Tounekti and S. Orlowski, *Gen. Pharmacol.*, 1996, **27**, 745.
4 H. Umezawa, *Antimicrob. Agents Chemother.*, 1965, **5**, 1079.
5 (*a*) J. Stubbe and J. W. Kozarich, *Chem. Rev.*, 1987, **87**, 1107; (*b*) J. Stubbe, J. W. Kozarich, W. Wu and D. E. Vanderwall, *Acc. Chem. Res.*, 1996, **29**, 322; (*c*) R. M. Burger, *Chem. Rev.*, 1998, **98**, 1153; (*d*) C. A. Claussen and E. C. Long, *Chem. Rev.*, 1999, **99**, 2797; (*e*) R. M. Burger, *Struct. Bonding*, 2000, **97**, 287; (*f*) S. T. Hoehn, H.-D. Junker, R. C. Bunt, C. J. Turner and J. Stubbe, *Biochemistry*, 2001, **40**, 5894.
6 (*a*) E. C. Constable and J. V. Walker, *J. Chem. Soc., Chem. Commun.*, 1992, 884; (*b*) E. C. Constable, M. A. M. Daniels, M. G. B. Drew, D. A. Tocher, J. V. Walker and P. D. Wood, *J. Chem. Soc., Dalton Trans.*, 1993, 1947.
7 Y. Zang, J. Kim, Y. Dong, E. C. Wilkinson, E. H. Appelman and L. Que, Jr., *J. Am. Chem. Soc.*, 1997, **119**, 4197.
8 (*a*) E. C. Constable, S. M. Elder, J. Healy and D. A. Tocher, *J. Chem. Soc., Dalton Trans.*, 1990, 1669; (*b*) C.-M. Che, C.-W. Chan, S.-M. Yang, C.-X. Guo, C.-Y. Lee and S.-M. Peng, *J. Chem. Soc., Dalton Trans.*, 1995, 2961; (*c*) E. C. Constable, S. M. Elder, M. J. Hannon, A. Martin, P. R. Raithby and D. A. Tocher, *J. Chem. Soc., Dalton Trans.*, 1996, 2423.
9 (*a*) M. E. de Vries, R. M. La Crois, G. Roelfes, H. Kooijman, A. L. Spek, R. Hage and B. L. Feringa, *Chem. Commun.*, 1997, 1549; (*b*) R. T. Jonas and T. D. P. Stack, *J. Am. Chem. Soc.*, 1997, **119**, 8566.
10 J. Zhou, S. C.-S. Cheng, D. Luo and Y. Xie, *Biochem. Biophys. Res. Commun.*, 2001, **280**, 1237.
11 (*a*) J. Bernadou, G. Pratviel, F. Bennis, M. Girardet and B. Meunier, *Biochemistry*, 1989, **28**, 7268; (*b*) G. C. Silver and W. C. Trogler, *J. Am. Chem. Soc.*, 1995, **117**, 3983.
12 L. H. Hartwell and M. B. Kastan, *Science*, 1994, **266**, 1821.

