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COMMUNICATION

Bis-phenanthroline copper(II) phthalate complexes are potent *in vitro* antitumour agents with 'self-activating' metallo-nuclease and DNA binding properties[†]

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Three, structurally characterised, bis-phen Cu(II) complexes of the phthalate isomers display rapid, low micromolar *in vitro* cytotoxicity against a range of epithelial tumour cells. The complexes induce relaxation of supercoiled plasmid DNA in the absence of external reducing agents and display efficient CT-DNA, Poly $[d(A-T)]_2$ and Poly $[d(G-C)]_2$ binding.

The design of agents capable of controlled cleavage of DNA and RNA is of paramount importance due to their potential as DNAtargeted chemotherapeutic drugs and their potential applications in biology.^{1,2} Complexes of metals such as iron, copper and manganese have been shown to act as effective chemical nucleases through oxidative-induced DNA damage.3 The oxidative DNA cleavage activity of Cu2+-1,10-phenanthroline (phen, Fig. 1) in the presence of a reductant was reported by Sigman et al. in 1979 and the activity involves a succession of redox events with a copper-'oxo' species believed to be the active agent.⁴ More recently the ligands 2-clip-phen[†] and 3-clip-phen[†] were investigated as systems that maintain the 2:1 phen: Cu stoichiometry at low concentrations. The oxidative nuclease activity of copper complexes of these new ligands was found to be higher than that of phen itself and it would appear that they induce a different DNA oxidation profile to the parent $[Cu(phen)_2]^{2+}$.^{3,5} In these systems, the cleavage reactions of the copper bis-phen and copper clip-phen require initiation by an excess of exogeneous reducing agent and this



Fig. 1 Molecular structure of the phthalates and phen.

effectively limits their *in vivo* application. Only a few self-activating DNA cleavage systems have been reported including Fe(BLM) and Cu(BLM) (BLM = bleomycin) which require the presence of molecular oxygen.³ Bis(hydroxysalicylidene)ethylenediamine, Cutambjamine E† and its pyrrolidine derivatives, HPyramol† and 2-(2-pyridyl)benzthiazole have all been reported to cleave DNA in the presence of copper(II) and molecular oxygen and a number of them exhibit anticancer activity.⁶⁻⁹ To the best of our knowledge no copper bis-phen complexes have been reported to cleave DNA in the absence of added reductant. Karlin and co-workers and recently Pitié and Reedijk have demonstrated that nuclearity is an important factor in oxidative DNA cleavage.^{10,11} Copper bis-phen complexes are among a relatively small group of metallosystems that can bind DNA efficiently and selectively activate C–H deoxyribose bonds leading to strand scission.^{3,12}

Phen and its substituted derivatives have high affinities for copper ions^{13,14} and copper complexes of phen have been shown to exhibit excellent antitumour,15 anti-candida16 and antibacterial17 activities. The tumour suppressor gene p53 is known to become mutated in many human cancers and thus fails to function. It has been demonstrated that phen enhances p53 activity in vitro and can trigger apoptosis in p53 negative cell lines.^{18,19} Isomers of the phthalates; phthalic (phH₂), isopthalic (isophH₂) and terephthalic acid (terphH₂) (Fig. 1) and derivatives thereof are ubiquitous within the industrial plastics sector and extensive applications of metal-phthalate scaffolds are prominent within metal-organicframeworks (MOFs). There are very few reports describing the medicinal potential of the phthalates. Herein, we report the synthesis of Cu(II) complexes of these versatile ligands along with their chemotherapeutic potential, DNA binding and their ability to act as 'self-activating' chemical nucleases.

Complexes 1-6 were prepared (Scheme 1) and characterised by IR and Raman spectroscopy, elemental analysis, magnetic

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Scheme 1 Synthetic routes toward complexes $1-6^+$.

moment, molar conductivity and solid/solution-state electronic spectroscopy.† Crystals of **4–6**, suitable for X-ray analysis, were generated when **1–3** were treated with phen in ethanol (Fig. 2–4).† The Cu(II) centres in complexes **4–6** have N₄O ligation and all have approximately square-pyramidal geometries. Mononuclear complexes **4** and **5** are geometric isomers and both contain the ph and isoph dianion bound to the metal through one carboxylate group in a monodentate coordination mode. Complex **6** contains a binuclear [{Cu(phen)₂}₂(terph)]²⁺ dication which is identical to the cationic structure previously reported for the perchlorate salt, [Cu(phen)₂(terph)](ClO₄)₂.²⁰ The complexes were either soluble or partially soluble in DMF and DMSO. The molar conductivities in DMSO are low and this, combined with essentially identical solid and solution-state electronic spectroscopy suggests that the complexes remain intact in DMSO solution.



Fig. 2 X-Ray structure of [Cu(ph)(phen)₂] (4).



Fig. 3 X-Ray structure of [Cu(isoph)(phen)₂] (5).

The cytotoxicity of **1–6** along with their free ligands and the clinical antitumour agents cisplatin and mitoxantrone, were mea-



Fig. 4 X-Ray structure of the cation in $[{Cu(phen)_2}_2(terph)](terph)(6)$, atoms with a prime (') in the atom labels are at equivalent position (1 - x, 1 - y, 1 - z).

sured, using a standard MTT assay against three human-derived tumour cell lines (Table 1).† It should be noted that colorectal adenocarcinoma (HT29) and prostate epithelial (DU145) carcinomas are inherently resistant to cisplatin and they also contain mutant versions of the *p53* gene while MCF-7 contains a well characterised and normal wild-type *p53* gene.²¹⁻²³ Complexes **4–6** display rapid, low micromolar toxicities against all three tumour lines. Results for this group are comparable to mitoxantrone against HT29, however, significant improvements were noted in the DU145 line. Complex **6** showed the best broad-spectrum activity and was the most active complex against breast adenocarcinoma (MCF-7). The fact that **6** is dinuclear, and so contains two active copper units, may explain this heightened potency. It is also worth noting that the free ligands, and the non-phen complexes **1–3**, are essentially inactive against all tumour lines.

As the complexes exhibited low molar extinction coefficients (ϵ) or were only partly soluble in DMF/DMSO an indirect fluorometric method of examining their DNA binding capabilities was chosen. Competitive ethidium bromide (EtBr) displacement experiments were conducted with **1–6** along with the known intercalator, actinomycin D, and the minor groove binder, pentamidine, using calf thymus DNA (CT-DNA) (Fig. 5, Table 2).† Et-bound DNA is highly fluorogenic and in the presence of excess Et⁺, binding regions within the DNA polymer become saturated. Thus, during competitive displacement, an exogenous reagent must 'compete' at the binding site with Et⁺ resulting in a

Table 1 $\,IC_{\rm 50}$ values ($\mu M)$ for complexes 1–6, the free ligands and the antitumour agents, cisplatin and mitoxantrone, against breast cancer (MCF-7), prostate cancer (DU145) and colon cancer cells (HT29) over a period of 24 h

| | Activity IC_{50} (μM) 24 h | | |
|---------------------|-------------------------------------|------------------|-----------------|
| | MCF-7 | DU145 | HT29 |
| phen | 337.7 ± 64.7 | 272.5 ± 8.0 | 376.8 ± 9.2 |
| phH ₂ | > 500 | >500 | >500 |
| isophH ₂ | >500 | >500 | >500 |
| terphH ₂ | >500 | >500 | >500 |
| 1 | > 500 | >500 | >500 |
| 2 | 401.2 ± 54.1 | 422.5 ± 22.4 | >500 |
| 3 | 414.3 ± 26.0 | >500 | >500 |
| 4 | 44.9 ± 7.0 | 11.6 ± 4.5 | 6.0 ± 0.4 |
| 5 | 41.2 ± 1.4 | 10.6 ± 2.2 | 5.8 ± 0.2 |
| 6 | 7.9 ± 0.4 | 5.7 ± 0.2 | 5.4 ± 0.3 |
| Mitoxantrone | 7.5 ± 0.2 | 27.3 ± 5.6 | 8 ± 0.6 |
| Cisplatin | 90.8 ± 3.5 | >500 | 348.6 ± 80.6 |

Table 2Apparent DNA binding constants (K_{app}) of 4–6 along with
actinomycin D and pentamidine. Assay conditions: final volume 2 mL,
1.2 μ M EtBr, 1 μ M CT-DNAp, 10 mM TES, 0.1 mM Na₂EDTA, pH 7.0

| Drug | $C_{50}{}^{a}$ (μ M) | $K_{\mathrm{app}}{}^{b}$ |
|---------------|---------------------------|--------------------------|
| Actinomycin D | 12.35 | 9.69×10^{5} |
| Pentamidine | 19.72 | 6.07×10^{5} |
| 4 | 99.06 | 1.21×10^{5} |
| 5 | 99.79 | 1.20×10^{5} |
| 6 | 39.36 | 3.04×10^{5} |

^{*a*} C_{50} = concentration required to reduce fluorescence by 50%. ^{*b*} $K_{app} = K_e \times 1.26/C_{50}$ where $K_e = 9.5 \times 10^6 \text{ M(bp)}^{-1}$



Fig. 5 Competitive ethidium bromide displacement for actinomycin D, pentamidine and complexes **4–6** with CT-DNA.

sequential reduction in fluorescence. Complexes 1–3 are devoid of binding ability but the phen-containing complexes, 4–6, do interact with DNA. Actinomycin D and pentamidine are highly efficient in the displacement of Et⁺ bound DNA and, as expected, their apparent binding constants (K_{app}) are high (Table 2). Comparably, complexes 4–6 have lower, but notable, K_{app} constants with the cationic dinuclear 6 showing superiority over the neutral, mononuclear complexes 4 and 5. Thus the advancement of DNA binding in 6 may be explained by its expected stronger electrostatic interaction toward DNA.

In an effort to elucidate the binding mode of complexes **4–6**, fluorescence quenching (Q values) of duplex adenine–thymine (A–T) and guanine–cytosine (G–C) polymers were conducted (Table 3). Under conditions of limited Et bound to an excess of DNA, exogenous intercalating agents (*e.g.* actinomycin D) demonstrate high affinities toward G–C base pairs, while minor groove binding species (*e.g.* pentamidine) prefer A–T base paired regions. Complexes **4–6** display greater affinities toward poly[d(A–T)]₂ than pentamidine but low Q values are also evident

| Drug | $Q^{a} \text{ poly}[d(A-T)_{2}] (\mu M)$ | $Q \text{ poly}[d(G-C)_2] (\mu M)$ |
|---------------|--|------------------------------------|
| Actinomycin D | 314.0 | 4.2 |
| Pentamidine | 44.9 | 203.4 |
| 4 | 26.2 | 69.8 |
| 5 | 20.9 | 37.3 |
| 6 | 8.6 | 10.3 |

" Q = equivalent concentration required to reduce fluorescence by 50%

within poly[d(G–C)]₂, possibly indicating flexible or multimodal binding.

Relaxation of supercoiled (SC) pUC18 DNA (Form I) into open circular (OC, Form II) and linear (LC, Form III) conformations was used to quantify the relative cleavage efficiency of complexes **4–6**. SC DNA was exposed to **4–6** over a concentration range of 1–100 μ M for 5 h in the absence of added H₂O₂ or reductant (Fig. 6). All complexes show concentration-dependant relaxation of SC (Form I) DNA to OC (Form II) while some LC (Form III) activity is evident but only at lower concentrations. The overall trend in nuclease activity is $6 \gg 4 > 5$. Indeed, 6 displayed some activity at lower concentrations of 1–5 μ M (lanes 11 and 12) with almost complete depletion of the parent SC band (I \rightarrow II) being witnessed at higher concentrations (lanes 15 and 16).



Fig. 6 Relaxation of pUC18 by **4–6**. Cleavage was carried out at 37 °C for 5 h and then analyzed by agarose gel electrophoresis.† Lane 1: DNA alone; lanes 2–6: 5, 10, 20, 50, 100 μ M **4**; lanes 7–10: 10, 20, 50, 100 μ M **5**; lanes 11–16: 1, 5, 10, 20, 50, 100 μ M **6**.

In conclusion, copper(II) bis-phen complexes of the phthalates are the first 'self-activating' chemical nucleases of their class. Their potential application as DNA-targeted chemotherapeutics is significant given their binding affinities, efficient DNA cleavage and potent broad-spectrum cytotoxicity. Interestingly these complexes do not appear to induce cytotoxicity in a similar manner to the clinical Pt(II) drugs as evidenced by results against cisplatin resistant colorectal (HT29) and prostate (DU145) cell lines which lack the endogenous p53 tumour suppressor gene. Detailed mechanistic investigations, to help elucidate the nature of the excellent copper-mediated DNA cleavage activity in these novel bis-phen systems are currently in progress.

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