This journal is © The Royal Society of Chemistry 2012

Supporting Information

Accompanying the manuscript

Potent Oxidative DNA Cleavage by the Di-Copper Cytotoxin: $[Cu_2(\mu\text{-terephthalate})(1,10\text{-phen})_4]^{2+}$

Andreea Prisecaru,[a] Michael Devereux,[b] Niall Barron,[c] Malachy McCann,[d] John Colleran,[d] Alan Casey,[b] Vickie McKee[e] and Andrew Kellett*[a]

List of Contents:

S-1: **Preparation of the complexes.**

S-2: **DNA cleavage experiments.**

S-3: **Cytotoxicity experiments.**

S-4: **Intracellular reactive oxygen species experiments.**

S-5: **Electrochemical experiments**

S-6: **Conclusion**

This journal is © The Royal Society of Chemistry 2012

S-1: Preparation of the Complexes.

Materials: Chemicals and reagents of analytical grade were purchased from Sigma-Aldrich Ireland and used without further purification.

Synthesis of [Cu_2(μ-terephthalate)(1,10-phen)$_4$](μ-terephthalate) (S1): The complex was prepared and characterized according to the method reported by Kellett et al.[1]

Synthesis of Cu(phen)$_2$Cl$_2$.3.5H_2O (S2): An ethanolic solution of copper(II) chloride dihydrate (0.50 g, 2.9 mmol) was treated with 1,10-phenanthroline monohydrate (1.2 g, 6.0 mmol) and refluxed under constant stirring at 90 ^oC for 2 hours. The green suspension was cooled on ice then filtrated with the resulting green powder being washed with a minimum amount of cold ethanol before being air-dried. Yield: 1.09 g (68.5 %), Elemental analysis: **T%:** C, 51.67; H, 4.16; N, 10.04, **F%:** C, 51.59; H, 4.56; N, 10.01, IR (KBr): 3392, 1624, 1517, 1427, 851, 722 cm^{-1}.

S-2: DNA cleavage experiments.

General procedure: Reactions were carried out according to the literature procedure reported by Melvin *et al.*[2] with slight modifications; in a total volume of 20 µL using 80 mM HEPES buffer (Fisher) at pH = 7.2 with 25 mM NaCl, an aliquot of the stock complex (**S1** initially prepared in DMF and **S2** and $CuCl_2.2H_2O$ in MeOH) was mixed with 400 ng of pBR322 (Roche). Samples were incubated at 37 °C before being quenched with 6x loading dye (Fermentas), containing 10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol, 60% glycerol and 60 mM EDTA, then loaded onto agarose gel (1%) containing 2.0 µL of GelRed™ (10,000X). Electrophoresis was completed at 80 V for 1.5 h using a wide mini-sub cell (BioRad) in 1XTAE buffer (Millipore). Experiments with exogenous reagents were conducted using 1 µL of 20 mM Na-L-ascorbate and/or 1 µL of 20 mM H_2O_2.

Experiments with restriction enzymes. General procedure: In a total volume of 20 µL using 2 µL of 100 mM HEPES buffer (pH = 7.2), 2 µL of 10x NEBuffer 3 (New England BioLabs), 0.2 µL 100x BSA (New England BioLabs), 2 µL 10x NEBuffer EcoRI (New England BioLabs) [used where appropriate], 1 µL of 20,000 U/mL EcoRV or 20,000 U/mL EcoRI (New England BioLabs) and 30 µM of the complex (from stock DMF) were mixed with 400 ng of pBR322 (Roche), and deionized H_2O. Samples containing the complex were incubated at 50 °C for 5 h then ice cooled prior to the restriction enzyme (EcoRI or EcoRV) being added. This mixture was further incubated at 37 °C for 1.5 h. 6x loading dye (Fermentas) was then added and samples were loaded onto agarose gel (1%) containing 2.0 µL of GelRed™ (10,000X). Electrophoresis was completed at 70 V for 2.0 h using a wide mini-sub cell (BioRad) in 1XTAE buffer (Millipore).

Religation experiments. In a total volume of 36 µL containing 30 µL of purified DNA (Qiagen QIAquick gel extraction kit as per manual protocol), 5 µL of 10x T4 ligation buffer (New England BioLabs) and 1 µL of T4 ligase (400,000 U/mL) (New England BioLabs) were incubated in a slush ice bath which was slowly allowed to reach room temperature overnight. 6x loading dye (Fermentas) was added and samples were loaded onto agarose gel (1%) containing 2.0 µL of GelRed™ (10,000X). Electrophoresis was completed at 70 V for 2.0 h using a wide mini-sub cell (BioRad) in 1XTAE buffer (Millipore).

This journal is © The Royal Society of Chemistry 2012

S-3: Cytotoxicity experiments.

Cell Culture.
Human Ovarian non-malignant cell line, HS-832 (passage 4 and 5, ATCC, USA), was grown in Dulbecco's modified eagle's medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS) containing penicillin-streptomycin (100 U/mL and 100 μg/mL) at 37 ºC in a humidified atmosphere with 5% CO_2.

Human Ovarian Cancer cell line, SK-OV-3 (passage 51 to 64, ATCC, USA), was grown in McCoys's 5a Medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS) containing penicillin-streptomycin (100 U/mL and 100 μg/mL) at 37 ºC in a humidified atmosphere with 5% CO_2.

Compounds were tested by the MTT assay following 24 h exposure of cells to **S1** and mitoxantrone (both prepared in DMSO). Each value represents the mean IC_{50} of three independent experiments +/- STD error.

MTT Assay. This method is based on the reduction of the tetrazolium salt, methylthiazolyldiphenyl-tetrazolium bromide (MTT), into a crystalline blue formazan product by the cellular oxidoreductases of viable cells.[3] The resultant formazan crystal formation is proportional to the number of viable cells. Cells were seeded at 4x 10^5 cells/mL in 96-well plates and incubated at 37 ºC in 5% CO_2 for 24 hours. Cells were treated with a four log range of concentration of the test compounds in triplicate from 0.1 to 500 μM or with a solvent control (0.5% DMSO) in complete medium. Following 24 h incubation, cells were incubated with 20 μL of MTT (5 mg/mL) in 0.1 M PBS, pH 7.4 at 37 ºC in a humid atmosphere with 5% CO_2 for 4 h. Media was then gently aspirated from test cultures and 100 μL of dimethyl sulphoxide (DMSO) was added to all wells. The plates were then shaken for 2 min and the absorbance was read at 550 nm in a Varioscan plate reader. The IC_{50} was defined as the concentration of test compound required to reduce the absorbance of the MTT-formazan crystals by 50%, indicating 50% cell deactivation.

This journal is © The Royal Society of Chemistry 2012

S-4: Cellular reactive oxygen species (ROS) experiments.

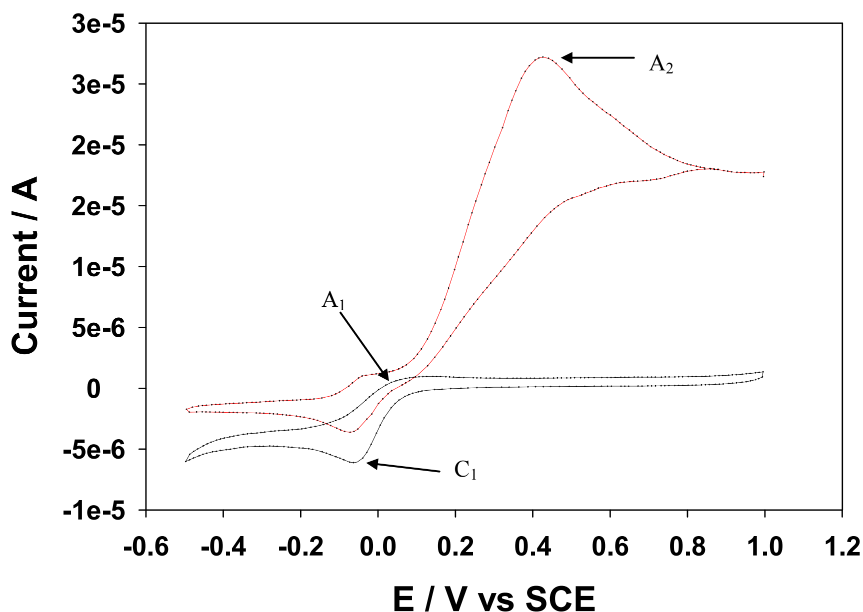
ROS activity. Intracellular oxidative stress.

Intracellular oxidative stress was quantified using 2′,7′-Dichlorofluorescin diacetate (DCFH-DA).[4] Briefly, healthy confluent A549 cells were harvested and seeded (1000 cells/well) into black bottomed 96 well plates (Nunc, Denmark) and allowed to attach for a period of 24 h prior to exposure. For ROS quantification, three independent experiments were performed. In each independent experiment, eight replicate wells were used for control, eight replicate wells were employed for the positive control and eight replicate wells were used for each test compound per micro-plate. A working stock of 20 μM DCFH-DA in PBS was prepared and all test compounds, unexposed controls and positive controls were prepared and exposed to the cells in this working stock. The first control consisted of the working stock solely, namely a 20 μM DCFH-DA solution in PBS, the positive control consisted of a 0.5 μM hydrogen peroxide (H_2O_2) prepared in the 20 μM DCFH-DA/PBS working stock solution and finally a concentration range (2000 – 2.0 nM) of the tested complex (**S1**) were prepared in the working stock of 20 μM DCFH-DA/PBS solution. After the initial 24 h attachment period, the media was removed, the cells were subsequently washed with 100 μL of PBS and treated with 100 μL of the control, positive control and the test compounds and then incubated. The rate of intracellular oxidative stress was then monitored by the emission at 529 nm (after 504 nm excitation) of the DCFH-DA dye at time intervals of 15 to 300 minutes (the exposure plates were re-incubated for the remaining time after each measurement had been recorded). Fluorescence measurements were conducted using a microplate reader (TECAN GENios, Grödig, Austria).

S-5: Electrochemical experiments

Experimental Set-up: A glassy carbon working electrode (3 mm diam.) and a platinum counter electrode were used to determine electrochemical parameters on a Solartron potentiostat, model 1875. Compounds were analysed in 1 mM concentrations and in a 1:2 ratio with added ascorbate at a scan rate of 100 mV s^{-1}. Aqueous experiments were performed 0.1 M KCl using saturated calomel electrode (SCE) as a reference. Non-aqueous voltammetry was performed in DMF with 0.1 M tetrabutylammonium hexafluorophosphate ($TBA^{+}PF6^{-}$). Prior to electrochemical measurements all solutions were purged with N_2 for 15 minutes. Experiments performed in DMF required the use of a Ag^{+}/Ag non-aqueous reference electrode [$E_{1/2}$ = 0.078V vs $Fe(Cp)^{+}$/Fe(Cp)] and potentials were corrected to SCE.[5] Electrodes were polished using 0.05 micron aluminium oxide paste on microcloth (both Buehler) and sonicated for ten minutes in ethanol prior to use.

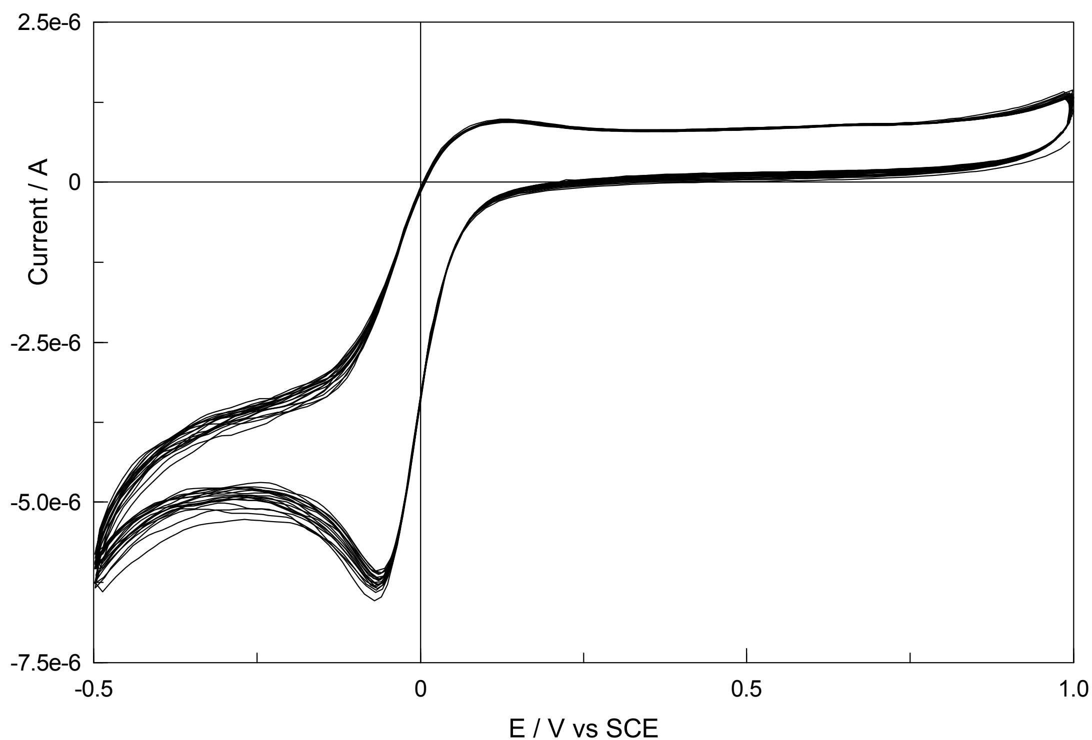
S-5, 1: Electrochemical behaviour of **S1** in aqueous solution in the absence and presence of ascorbate.



S-5, Figure 1: Enlargement of Figure 6 from manuscript; Typical cyclic voltammograms detailing the electrochemical behaviour of **S1** in the absence (black trace) and presence (red trace) of 2 mM ascorbate in 0.1 M KCl aqueous solution. The A_1/C_1 peaks describe the 1 electron redox couple of **S1**. Peak A_2 is the 2 electron, 1 proton oxidation of ascorbate. Glassy Carbon working electrode, platinum counter electrode, SCE reference electrode, and scan rate 100 mV s^{-1}. Little activity is lost after 30 potential cycles.

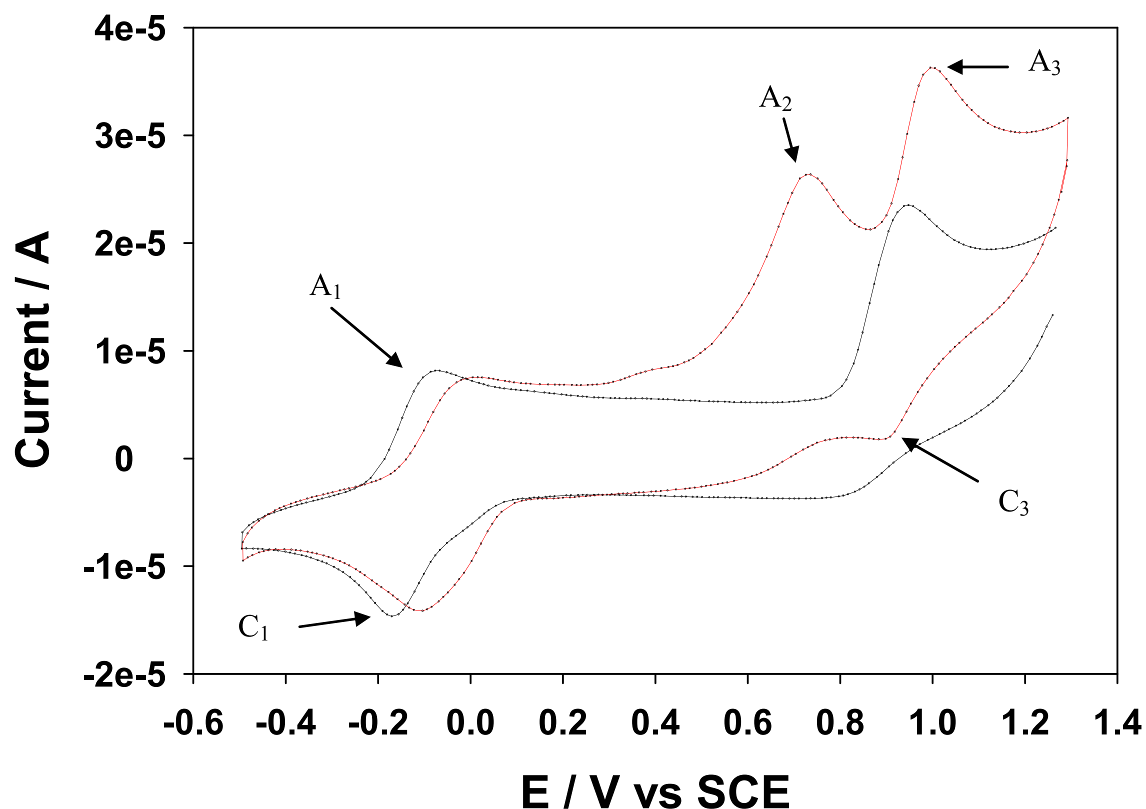
This journal is © The Royal Society of Chemistry 2012

S-5, 2: Electrochemical stability of **S1** with potential cycling in aqueous solution



S-5, 2: Typical cyclic voltammograms detailing the stability of 1 mM **S1**, with potential cycling, in a 0.1 M KCl aqueous solution. Little activity is lost after 30 potential cycles. Glassy Carbon working electrode, platinum counter electrode, SCE reference electrode, and scan rate 100 mV s^{-1}.

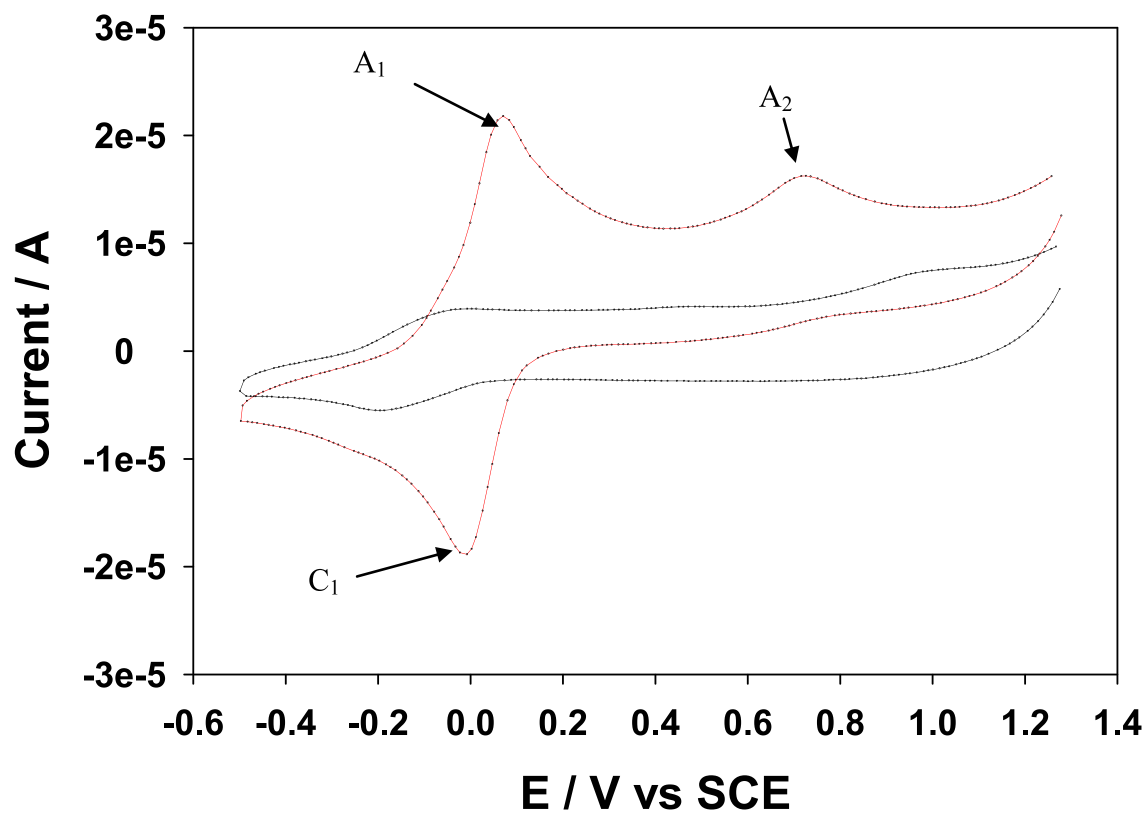
S-5, 3: Redox profile of $[Cu(phen)_2]^{2+}$ in DMF in the absence and presence of ascorbate



S-5, 3: Typical cyclic voltammograms detailing the redox behaviour of 1 mM $[Cu(phen)_2]^{2+}$ in the absence (black trace) and presence (red trace) of 2 mM ascorbate in 0.1 M $TBAPF_6$/DMF. The peaks correspond to the following processes; A_1/C_1 - $[Cu(phen)_2]^{2+}$ / $[Cu(phen)_2]^{+}$ couple, A_2 - ascorbate oxidation, A_3/C_3 - uncomplexed Cu^{2+}/Cu^{+} couple. Glassy Carbon working electrode, platinum counter electrode, Ag^{+}/Ag non-aqueous reference electrode, and scan rate of 100 mV s^{-1}.

This journal is © The Royal Society of Chemistry 2012

S-5, 4: Redox profile of **S1** in DMF in the absence and presence of ascorbate



S-5, 4: Typical cyclic voltammograms detailing the redox behaviour of 1 mM **S1** in the absence (black trace) and presence (red trace) of 2 mM ascorbate, in 0.1 M $TBAPF_6$/DMF. Glassy Carbon working electrode, platinum counter electrode, Ag^+/Ag non-aqueous reference electrode, and scan rate of 100 mV s^{-1}.

Tables S-5, (a) and **(b)** summarise the electrochemical parameters and all potentials are quoted *versus* SCE.[6]:

Table **S5 (a)**: Summary of the parameters calculated for **S1** in the absence and presence of 2 mM ascorbate in aqueous solution.

S1 Aqueous	**E_P (V)**	**ΔE_P (V)**	**E_P-$E_{P/2}$ (V)**	**n***	**α value**
Reduction, C_1	-0.062	-	0.066	1	0.71
Oxidation, A_1	0.097	0.159	0.138	1	0.66
With 2 mM AA, C_1	-0.072	-	-	-	-
With 2 mM AA, A_1	-0.031	0.041	-	-	-

Table S5,(a); Summary of the calculated parameters for the redox behaviour of **S1** in aqueous solution. In this table voltage values (V) are quoted versus the SCE calomel electrode.[6]

Table S5 (b): comparison of the redox behaviour of **S1** and$[Cu(phen)_2]^{2+}$ in DMF.

S1 DMF	**E_P (V)**	**ΔE_P (V)**	**E_P-$E_{P/2}$ (V)**	**S1 DMF**	**E_P (V)**	**ΔE_P (V)**	**E_P-$E_{P/2}$ (V)**
Reduction, C_1	-0.196	-	0.132	**Reduction, C_1**	-0.171	-	0.092
Oxidation, A_1	-0.007	0.189	0.133	**Oxidation, A_1**	-0.076	0.095	0.071
With 2 mM AA, C_1	-0.008	-	0.062	**With 2 mM AA, C_1**	-0.105	-	0.114
With 2 mM AA, A_1	+0.071	0.079	0.074	**With 2 mM AA, A_1**	+0.012	0.117	0.084

Table S2 (b); Summary of the calculated parameters for the redox behaviour of **S1** and $[Cu(phen)_2]^{2+}$, in the absence and presence of 2 mM ascorbate. Experiments performed in 0.1 M $TBAPF_6$ in DMF and potentials corrected to the SCE reference electrode.[5]

* To determine the important electrochemical parameters that describe an irreversible redox process we can use the following equation:[6]

For a reduction process

$$|E_P - E_{P/2}| = \frac{1.857\,RT}{\alpha nF}$$

and for an oxidation process.

$$|E_P - E_{P/2}| = \frac{1.857\,RT}{(1-\alpha)nF}$$

E_P is the Peak potential in Volts, $E_{P/2}$ is the potential at $I_{P/2}$, R is the gas constant, 8.314 J mol^{-1} K^{-1}, T is temperature = 295 K, n is the number of electrons involved in the process, F is the Faraday constant = 96,485 C mol^{-1}, and α is the transfer coefficient. The value of α must lie between 0 and 1, and is a measure of the symmetry of the redox process. For example a value of α = 0.5 is indicative of a transition state that lies midway between substrate and product. The value of α lies between 0.2 and 0.8 for irreversible redox processes.

This journal is © The Royal Society of Chemistry 2012

S-6 Conclusion:

In conclusion, we have demonstrated that $[Cu_2(\mu\text{-terph})(1,10\text{-phen})_4](\text{terph})$ is a potent oxidizer of duplex DNA which operates independently of exogenous reagents and with enhanced activity in their presence. To our knowledge, this is the first "self-active" oxidative copper(II)-bis-1,10-phen system to be reported. We have shown, using innovative experimental design, that this di-copper(II) reagent cleaves DNA in a non-sequence-specific fashion, potentially with slight preference at the minor-groove. The agent displays excellent *in vitro* chemotherapeutic activity toward cisplatin-resistant ovarian cancer and there is evidence of intracellular ROS production, at nano-molar exposure levels, within human-derived lung cancer cells. A major focus within our laboratory is to now develop probes suitable of inducing specific DNA cleavage, with high efficiency, at a predetermined locus. The innovative work by Barton *et al.* on the conjugation of sequence-specific proteins to DNA-targeting metal complexes may inform this effort.[7] While other Cu^{2+} "self-active" oxidative DNA cleavers known,[2, 8] none of these agents can be considered structurally similar to **S1**. Furthermore, it is our belief that the development of cleaving agents from readily available commercial ligands (1,10-phen and terephthalic acid in this case) is of broad chemical interest.

This journal is © The Royal Society of Chemistry 2012

References:

1. A. Kellett, M. O'Connor, M. McCann, M. McNamara, P. Lynch, G. Rosair, V. McKee, B. Creaven, M. Walsh, S. McClean, A. Foltyn, D. O'Shea, O. Howe and M. Devereux, *Dalton Trans.*, 2011, **40**, 1024-1027.
2. M. S. Melvin, J. T. Tomlinson, G. R. Saluta, G. L. Kucera, N. Lindquist and R. A. Manderville, *J. Am. Chem. Soc.*, 2000, **122**, 6333-6334.
3. T. Mosmann, *J Immunol Methods*, 1983, **65**, 55-63.
4. B. Halliwell and M. Whiteman, *Br J Pharmacol*, 2004, **142**, 231-255.
5. V. V. Pavlishchuk and A. W. Addison, *Inorg Chim Acta*, 2000, **298**, 97-102.
6. A. J. Bard and L. R. Faulkner, *Electrochemical Methods: Fundamentals and Applications*, Wiley, New York, 2001.
7. (a.) M. P. Fitzsimons and J. K. Barton, *J. Am. Chem. Soc.*, 1997, **119**, 3379-3380; (b.) K. D. Copeland, A. M. Lueras, E. D. Stemp and J. K. Barton, *Biochemistry*, 2002, **41**, 12785-12797.
8. (a.) S. Borah, M. S. Melvin, N. Lindquist and R. A. Manderville, *J. Am. Chem. Soc.*, 1998, **120**, 4557-4562; (b.) P. U. Maheswari, S. Roy, H. den Dulk, S. Barends, G. van Wezel, B. Kozlevcar, P. Gamez and J. Reedijk, *J. Am. Chem. Soc.*, 2006, **128**, 710-711; (c.) P. U. Maheswari, M. v. d. Ster, S. Smulders, S. Barends, G. P. v. Wezel, C. Massera, S. Roy, H. d. Dulk, P. Gamez and J. Reedijk, *Inorg. Chem.*, 2008, **47**, 3719-3727.