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Water-soluble bis(1,10-phenanthroline) Octanedioate Cu2+ and Mn2+ Complexes with Unprecedented Nano and Picomolar in Vitro Cytotoxicity: Promising Leads for Chemotherapeutic Drug Development

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Water-soluble Cu^{II} and Mn^{II} bis-phenanthroline octanedioate complexes with unprecedented nano and picomolar *in vitro* cytotoxicity as promising leads for chemotherapeutic drug development

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Dinuclear Cu^{II} and Mn^{II} bis-phenanthroline octanedioate complexes exhibit rapid, unprecedented nano and picomolar *in vitro* cytotoxicity against colorectal cancer lines and are less toxic than cisplatin when examined *in vivo*. The complexes are potent generators of cellular reactive oxygen species, avid DNA binders and induce O_2 dependent cleavage of DNA. The Cu(II) complex was found to have self-cleaving nuclease activity.

Introduction

The development of self-cleaving chemical nucleases is regarded as the paradigm of redox-active metal-based chemotherapeutics. DNA targeted agents capable of inducing single stranded or double stranded scission have found clinical application within cancer chemotherapy.[1] Other applications within this class include; probing of DNA-specific structures, mapping of protein and DNA interactions, gene regulation and signal transduction.[2, 3] Thus, explorations toward the discovery and development of natural or synthetic chemical nucleases are major topics of interest. Redox active transition-metal-based chemical nucleases are particularly important due to their catalytic potential to support the one-electron oxidation/reduction reactions necessary to drive activation of C-H deoxyribose bonds.

In the presence of Cu^{2+}, the oxidative formation of π radical cations within marine-based products tambjamine E,[4] prodigiosin[5] and pyrimol,[6] have recently been shown to mediate self-cleaving DNA damage, i.e. scission which does not require the presence of added oxidant or reductant. These Cu^{2+} compounds have also demonstrated significant *in vitro* chemotherapeutic potential against leukaemia and ovarian cancer cells, some of which had cisplatin resistance.[5, 6]

The discovery of the first synthetic chemical nuclease, $[Cu(phen)_2]^{2+}$ (phen = 1,10-phenanthroline, ***Figure 1***), has sparked intensive effort toward the development of new bis-phen agents capable of enhanced DNA cleaving compared to the parent complex.[7] The DNA cleaving limitations of $[Cu(phen)_2]^{2+}$ include (*i*) a high dissociation constant of the second coordinated phenanthroline ligand[8] and (*ii*) the need for exogenous reductant to generate the active species $[Cu(phen)_2]^{+}$. The dissociation problem (*i*) was solved by Meunier, Pitie *et al* through the advent of clip-phen, whereby two phenanthroline ligands are connected at the 2' or 3' position by a serinol bridge.[9-11] Recently this laboratory has reported the first self-cleaving bis-phen system, [Cu(phen)$_2$(phthalate)] (phthalate = *o*-, *m*-, *p*-phthalate, ***Figure 1***), capable of inducing single-stranded DNA scission in the absence of exogenous reductant or oxidant.[12]

While [Cu(phen)$_2$(phthalate)] complexes displayed excellent chemotherapeutic potential against, colon (HT29), breast (MC-F7) and prostate (DU145) cancer lines, their water solubility is poor. Of the phthalate group, the cationic, dinuclear species $[Cu_2(phen)_4(\mu_2\text{-}p\text{-phthalate})]^{2+}$ was the most active DNA-binding, self-cleaving and chemotherapeutic agent. To that end, in the current study, we have investigated the application of water soluble Cu^{2+} and Mn^{2+} dinuclear, cationic, bis-phen octanedioate (oda) (***Figure 1***) systems as nuclease mimetics and their ability to induce cancer cell death through the redox-generation of reactive oxygen species (ROS). The possible formation of a π carboxyl radical within the Cu^{2+} system, which cleaves DNA by self-activation, is also discussed.

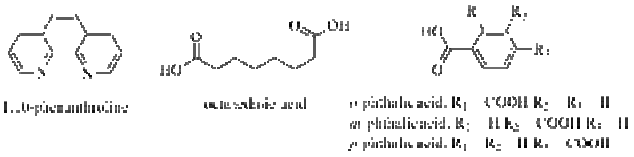

Figure 1. Molecular strucutres of phen, odaH_2 and the phthalates

The title complexes $[Cu_2(\mu_2\text{-oda})(phen)_4](ClO_4)_2$ (**1**) (***Figure 2***), $[Mn_2(\mu_2\text{-oda})(phen)_4(H_2O)_2(oda)_2]^{2-}[Mn_2(\mu_2\text{-oda})(phen)_4(H_2O)_2]^{2+}$ (**2**) (***Figure 3***), were prepared according to the literature methods by McCann and Devereux *et al*.[13, 14] The coordination environment about both Cu^{2+} ions in **1** is approximately square-pyramidal with both metals being linked via a bridging oda ligand. In the double salt complex **2**, the environment about each Mn^{2+} is octahedral with both metals in the cationic and anionic groups being bridged by oda in a similar fashion to **1**. Whereas the Mn^{2+} centres of the anionic subunit in **2** each contain a singly bound oda dianion coordinated in the apical position, the cationic unit contains bound water at these equivalent positions.

Figure 2. Molecular strucutre the $[Cu_2(\mu_2\text{-oda})(phen)_4]^{2+}$ cation in complex **1**

Figure 3. Molecular strucutres of the dimeric cation and anion subunits in the Mn^{II} double salt complex $[Mn_2(\mu_2\text{-oda})(phen)_4(H_2O)_2(oda)_2]^{2-}[Mn_2(\mu_2\text{-oda})(phen)_4(H_2O)_2]^{2+}$ (**2**)

DNA Binding Studies

In order to examine the interaction of compounds **1** & **2** with DNA, competitive ethidium bromide displacement and fluorescence quenching experiments with calf thymus DNA were conducted (***Table 1***).† Complexes **1** & **2** were found to have high apparent DNA binding constants (K_{app}), and low fluorescence quenching values (Q), indicating that both systems have a high affinity for binding to DNA.

Table 1. Apparent DNA binding constants (K_{app}) and Q values for **1** and **2**.† Assay conditions; C_{50}/K_{app}: final volume 2 mL, 1.2 μ*M* EtBr, 1 μ*M* CT-DNAp, 10 m*M* TES, 0.1 m*M* Na_2EDTA, pH 7.0; Q: final volume 2 mL, 2.0 μ*M* EtBr, 20 μ*M* CT-DNAp, 2 m*M* NaOAc buffer, 9.3 m*M* NaCl, 0.1 m*M* Na_2EDTA, pH 5.0

Complex	C_{50}* (μ*M*)	K_{app}**	Q‡
1	46.89	2.55 x 10^5	22.64
2	28.46	4.20 x 10^5	43.13

* C_{50} = concentration required to reduce fluorescence by 50% (Competitive)
** $K_{app} = K_e$ x $1.26/C_{50}$ where K_e = 9.5 x 10^6 $M(bp)^{-1}$
‡ Q = equivalent concentration required to reduce fluorescence by 50% (Quenching)

DNA Cleavage Reactions

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 **1** & **2**.† To examine for DNA self-cleaving ability, SC DNA was exposed to both complexes over a concentration range of 1-50 μ*M* for 20 hours in the absence of added H_2O_2 or reductant (***Figure 4(a)***). Complex **1** shows concentration-dependant self-cleaving of SC (Form I) DNA to OC (Form II) with complete depletion of the parent SC band (I→II) between 10-20 μ*M* (lane 4 & 5 (*a*)). In the presence of added reductant (ascorbate) (***Figure 4(b)***) both complexes exhibit enhanced DNA scission during a shorter time-frame (2hr). While both **1** & **2** induced complete relaxation to OC Form II (lanes 4 & 8 (*b*)) at 10 μ*M*, the Cu^{2+} complex was found to induce efficient double stranded scission (I→III) at a concentration of 20 μ*M*, characterised by the absence of a band in lane 5 (*b*). In order to identify what role molecular O_2 and the metal cations (Cu^{2+}/Mn^{2+}) play within DNA cleavage, experiments were conducted under anaerobic conditions in an atmosphere saturated with argon and, separately, in the presence of 100 m*M* Na_2EDTA (***Figure 4* (*c*) & (*d*)**). The Mn^{2+} system was examined at 20 μ*M* over two hours in the presence of ascorbate while the Cu^{2+} system was examined at 20 μ*M* over 20 hr without added reductant. Both complexes did not cleave DNA in the absence of oxygen but complex **1** was found to induce some DNA damage in the presence of excess metal chelator (EDTA).

Figure 4. Relaxation of pUC18 by **1** and **2**.† Cleavage was carried out at 37°C then analyzed by agarose gel electrophoresis† (*a*) 20hr incubation in the absence of added oxidant or reductant, lane 1: DNA alone; lanes 2-6: 1, 5, 10, 20, 50 μ*M* **1**; lanes 7-10: 5, 10, 20, 50 μ*M* **2**. (*b*) 2hr incubation in the presence of added ascorbate (at twice complex concentration), lane 1: DNA alone; lanes 2-5: 1, 5, 10, 20 μ*M* **1**; lanes 6-9: 1, 5, 10, 20 μ*M* **2**. (*c*) 20hr incubation of 20 μ*M* **1** in the absence of added oxidant or reductant, lane 1: + 100 m*M* Na_2EDTA; lane 2: sat. Ar atmosphere. (*d*) 2hr incubation of 20 μ*M* **2** with added ascorbate (at twice complex concentration), lane 1: + 100 m*M* Na_2EDTA; lane 2: sat. Ar atmosphere.

Redox Activity

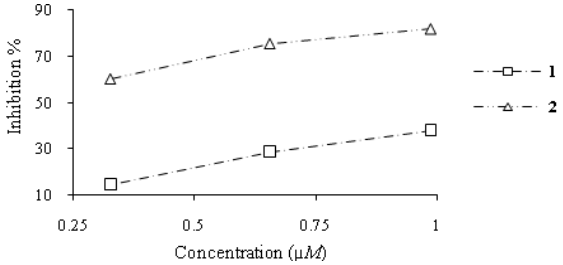

Figure 5. SOD activity profiles for complexes **1** and **2**

Table 2. SOD concentrations of complexes **1** and **2** equivalent to effect 1U of Bovine Erythrocyte SOD activity (50% Inhibition) and Catalase mimetic potential of **1** and **2** examined as a function of H_2O_2 disproportionation

Complex	Concentration Equivalent to 1U Bovine SOD (μ*M*)	Number of H_2O_2 molecules disproportionated by one molecule of complex in the first 5 min[14]
1	1.300	0
2	0.024	6 x 10^3

Since the catalytic interaction of $[Cu(phen)_2]^{2+}$ and its reduced form, $[Cu(phen)_2]^+$, with the superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are imperitive for cleaving the phosphodiester backbone in DNA,[15-17] we have examined the interaction of complexes **1** and **2** with both these species. Superoxide was generated enzymatically by the xanthine/xanthine-oxidase system and quantified photometrically by the detector nitro-blue-tetrazolium (NBT). Both complexes **1** and **2** show potent SOD (superoxide dismutase) mimetic activity with the Mn^{2+} system being an exceptional catalyst (1U SOD = 24.6 n*M*) (***Figure 5*** & ***Table 2***). The catalase mimetic activity of complexes **1** & **2** were examined as a function of oxygen evolution. After the addition of a 30% v/v solution of H_2O_2 to **1** & **2** the O_2 gas evolved was quantified volumetrically (***Table 2***). Only the Mn^{2+} complex (**2**) was capable of decomposing H_2O_2 and its activity can also be described as exceptional in this regard (6 x 10^3 H_2O_2 molecules disproportionated in 5 min). Overall, both complexes **1** & **2** interacted with superoxide to produce hydrogen peroxide (*I*), but, only the Mn^{2+} complex (**2**) appears capable of disproportionates the resulting peroxide (*II*).

$$I. \quad 2O_2^{\bullet-} + 2H^+ \rightarrow 2H_2O_2 + O_2 \quad (SOD)$$
$$II. \quad 2H_2O_2 \rightarrow 2H_2O + O_2 \quad (CAT)$$

Antitumour Activity

Table 3. LD_{50} (at ± 95% CI) values for complexes **1** and **2**, the free ligand phen and the antitumour agent cisplatin, against colorectal cancer lines HT29, SW480 and SW620 over a period of 24 and 96 hours[†] **Alan do we need ±SD values here? Also can you include the HaCaT results here?**

	Antitumour Activity LD_{50} (μ*M*)					
	HT29		SW480		SW640	
	24 hr	96 hr	24 hr	96 hr	24 hr	96 hr
phen	<200	9.240	<200	10.70	160.00	10.700
1	9.610	>0.001	11.30	0.220	31.00	1.220
2	108.00	0.092	7.460	0.261	58.50	0.342
cisplatin	166.00	4.810	<200	1.290	<200	7.030

The cytotoxicity of **1** & **2** along with the free phen ligand and the clinical antitumour agent cisplatin, were measured at 24 and 96hr intervals using a standard MTT assay against three progressive colorectal human-derived tumour cell lines (***Table 3***).[†] Both complexes display remarkable cytotoxicity against all three lines. Low-micromolar LD_{50} activities for both complexes were found after 24 hrs of exposure and, significantly, these activities reached the nano- and picomolar level after 96 hrs. As the colorectal tumour lines progress (HT29→SW480→SW620) activity of complex **1**, over 96hrs, reduces from pico- to low micomolar, while the activity of **2** remained consistently in the low-mid nanomolar region. It is worth noting that while cisplatin displays significant low-micromolar cytotoxicity against all tumour lines after 96hrs, the activity of **1** & **2**, in this period, is superior to this agent by a factor between $1x10^1 – 1x10^5$.

Cellular Reactive Oxygen Species (ROS) Study

In order to elucidate the relationship between cytotoxicity and ROS generation, complexes **1** & **2**, along with phen and cisplatin, were exposed to colorectal cancer cells, HT29, pre-treated with the intracellular ROS indicator 2′,7′-dichlorofluorescin diacetate (DCFH-DA).[†] In the presence of endogenously generated ROS, DCFH-DA becomes oxidised to release the fluorophore 2′,7′-dichlorofluorescin (DFC). Results are expressed relative to the fluorescent response of the positive control (+Ctrl), hydrogen peroxide, which is considered a potent generator of ROS. Corrections were made using a negative control (-Ctrl), which represents the natural level of cellular ROS generated. Results were recorded at 15, 30, 60, 120 and 180 min intervals and are shown in ***Figure 6***. Complex **2** was found to be an exceptional generator of ROS with greater activity relative to H_2O_2 (+Ctrl) across the concentration range 1000-250 n*M* and approximate equal activity to peroxide at 125 n*M*. It is worth commenting that this species was almost seven times more active than the next most effective ROS generator, phen. The activity of the copper(II) complex (**1**) was somewhat lower than both free phen and complex **2**, indeed **1** needed to be assessed across a higher concentration range (100,000-195 n*M*). The clinical agent cisplatin was the least active of all tested compounds. This is unsurprising considering it is non-catalytic, it has also been well established that cisplatin only becomes cytotoxic upon its hydrolysis to $[Pt(NH_3)_2(OH)_2]$, which generally occurs between 48-96 hrs.

Figure 6. Generation of endogenous reactive oxygen species (ROS) within the cancer cell line HT29 after exposure to; (*a*) the free ligand 1,10-phenanthroline, (*b*) the clinical antitumour agent cisplatin, (*c*) complex **1** and (*d*) complex **2**.

In vivo Drug Tolerance

Larvae of the insect *Galleria mellonella* were employed to assess the *in vivo* cytotoxic tolerance of complexes **1** and **2**, the ligand phen and the clinical antitumour agent cisplatin. Larvae of *G. mellonella* (the greater wax moth) have been widely used as a convenient and inexpensive *in vivo* screening model to assess the therapeutic potential of novel antimicrobial drugs.[18, 19] They have yielded results that are considered comparable to those obtained using mammalian models.[20] The innate defences of insects, including *G. mellonella*, like those of mammals consist of structural and passive barriers as well as humoral and cellular responses within the haemolymph (analogous to the blood of mammals).[21] Indeed cellular responses within the haemolymph are often activated by signal transduction systems comparable to mice.[22]

Table 4. % Kill of *G. mellonella* larvae after exposures of 5000-100 μg/mL of complexes **1** and **2**, the ligand phen and the clinical antitumour drug cisplatin over the period 72 hours[†] **Pauraic/Malachy we need to include ±SD values in this table.**

	% Kill of *Galleria mellonella* larve (72 hr)				
	Concentration μg/mL (mg/kg body weight)				
	5000 (333)	**2000 (133)**	**1000 (67)**	**500 (33)**	**200 (13)**
phen	100	90	80	0	0
1	93.3	86.7	50	0	0
2	93.3	93.3	40	0	0
cisplatin	100	100	100	60	0

Testing was carried out in triplicate using ten healthy *G. mellonella* larvae in the 6th developmental stage. Compounds were tested across the concentration range 5000-100 μg/mL (333-13 mg/kg average body weight) with sterile test solutions being administered via hypodermic injection. Larvae were incubated at 30°*C* for 72 hr with survival being monitored at 24 hr intervals and significance being determined using the log rank (Mantel-Cox) method. Death was assessed by the lack of movement in response to stimulus together with discolouration. Results are presented (***Table 4***) as the mean % kill (± standard deviation) resulting from exposure to tested compound. *Galleria* exposed to high drug concentrations of 5000 and 2000 μg/mL showed poor tolerance to all tested species. However, at lower concentration ranges 1000-200 μg/mL significant differences were observed. Tolerance of *Galleria* larvae exposed to complexes **1** and **2** was highest of all tested species (50 and 40% kill at 67 mg/kg respectively, and 0% kill at 33 mg/kg). The clinical antitumour agent cisplatin was the poorest tolerated tested species with high toxicity (60% kill) being observed at 500 μg/mL (33 mg/kg). Encouragingly, the well-established rat LD_{50} of cisplatin (oral exposure) is reported as 25.8 mg/kg body weight, thus, it appears some agreement between these two models does exist.

Proposed DNA Self-Cleaving Mechanism

To conclude we would like to propose a mechanism by which of complex **1** self-activates the phosphodiester backbone in DNA via formation of a π carboxylate radical, concomitantly leading to the reduced formation of $[Cu(phen)_2]^+$ (***Figure 7***). Aliphatic carboxylate radicals are known to react by hydrogen abstraction in close competition with decarboxylation.[23, 24] Generation of a π carboxylate radical within complex **1** would depend on the strength of the HOMO *d* orbital overlap on Cu^{2+} with the oxygen carboxylate. X-ray crystallography has revealed that the bond length between Cu-O in **1** is 1.974Å and is significantly shorter than the equivalent Mn-O bond in **2** which is 2.147 Å.[13, 14]

However, since the Cu-O bond length may be altered when **1** binds with DNA, it is impossible to know its exact length *in vivo*.

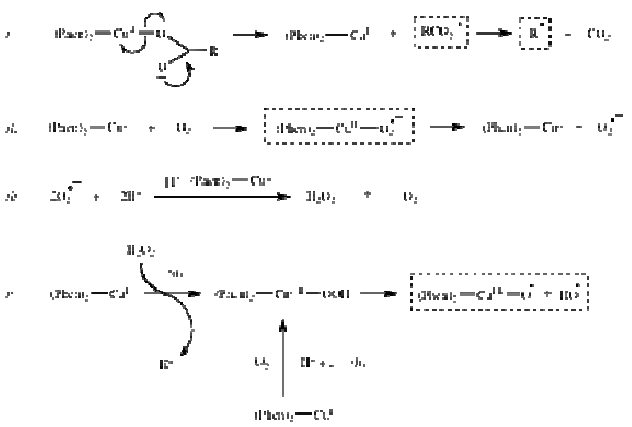

Figure 7. Proposed mechanism of generating C-H bond activators (---)

It is proposed the mechanism proceeds by the following steps: (*i*) after the binding of **1** to DNA, homolytic cleavage at the Cu-O bond generates the π carboxyl radical (---) and the reduced d^{10} complex $[Cu(phen)_2]^+$, the resultant carboxyl π radical undergoes rapid conversion to σ which decarboxylates to generate CO_2 and $R^{\bullet}$ (---); (*ii*) $[Cu(phen)_2]^+$ reacts, as is known, with O_2 to generate the superoxide radical through an intermediate (---) yielding $[Cu(phen)_2]^{2+}$; (*iii*) complex **1** or $[Cu(phen)_2]^{2+}$ subsequently react with $O_2^{\bullet-}$ to generate H_2O_2 (as shown in ***Figure 5*** & ***Table 2***); (*iv*) since **1** does not disproportionate hydrogen peroxide (shown in ***Table 2***) a subsequent reaction with H_2O_2 in (*a*) or O_2 in (*b*) can generate known metal-oxo (---) and hydroxyl radical (---) bond activators. Since nuclease activity was detected in the presence of the chelator EDTA (***Figure 4*** (***c***)) it would stand to reason that the carboxylate radical or its breakdown product ($R^{\bullet}$) appears capable of abstracting $H^{\bullet}$ from the phosphodiester backbone in DNA. In the absence of oxygen, the self-cleaving nuclease potential of **1** is diminished (***Figure 4*** (***c***)), and since, in the proposed mechanism, steps (*ii*)-(*iv*) are dependant on molecular oxygen, it also stands to reason that the nuclease potential should be reduced in the absence of these aerobically generated oxo- and hydroxo- bond activators. Futher efforts are currently underway in our laboratory to find further evidence for this proposed mechanism.

Conclusions

In summary, dinuclear Cu^{2+} and Mn^{2+} bis-phen complexes of the dicarboxylate, octanedioate, represent a significant advancement compared to existing metal-phenanthroline adducts. Their application as new water-soluble DNA-targeted chemotherapeutics is highly significant given their powerful and unprecedented cytotoxicity, encouraging *in vivo* drug tolerance and unique modes of action. While both complexes are avid binders of DNA, additionally, the copper(II) system has the capacity to self-cleave DNA, possibly through the generation of a π carboxyl radical, while the manganese(II) system is an exceptional redox catalyst that generates unprecedented levels of intracellular ROS within colon cancer cells.

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Notes and references

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[c] ???

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† Electronic Supplementary Information (ESI) available: Experimental procedures, X-ray crystallographic data and biological evaluation studies See DOI: 10.1039/b000000x/

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