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Synthesis and X-ray crystal structure of $[Ag(phendio)_2]ClO_4$ (phendio = 1,10-phenanthroline-5,6-dione) and its effects on fungal and mammalian cells

Malachy McCann[1], Barry Coyle[1], Sinead McKay[1], Paul McCormack[1], Kevin Kavanagh[2], Michael Devereux[3], Vickie McKee[4], Paula Kinsella[5], Robert O'Connor[5] & Martin Clynes[5]
[1]*Chemistry Department and* [2]*Biology Department, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland (Fax: 353-1-7083815; Tel: 353-1-7083767; E-mail: mmcann@may.ie);* [3]*Dublin Institute of Technology, Cathal Brugha Street, Dublin, Ireland;* [4]*Chemistry Department, Loughborough University, Loughborough, Leics., LE11 3TU, UK;* [5]*National Institute for Cellular Biotechnology, Dublin City University, Glasnevin, Dublin 9, Ireland*

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Abstract

The Cu(II) and Ag(I) complexes, $[Cu(phendio)_3](ClO_4)_2{\cdot}4H_2O$ and $[Ag(phendio)_2]ClO_4$ (phendio = 1,10-phenanthroline-5,6-dione), are prepared in good yield by reacting phendio with the appropriate metal perchlorate salt. The X-ray crystal structure of the Ag(I) complex shows it to have a pseudo tetrahedral structure. 'Metal-free' phendio and the Cu(II) and Ag(I) phendio complexes strongly inhibit the growth of the fungal pathogen *Candida albicans*, and are more active than their 1,10-phenanthroline analogues. The simple Ag(I) salts, $AgCH_3CO_2$, $AgNO_3$ and $AgClO_4.H_2O$ display superior anti-fungal properties compared to analogous simple Cu(II) and Mn(II) salts, suggesting that the nature of the metal ion strongly influences activity. Exposing *C. albicans* to 'metal-free' phendio, simple Ag(I) salts and $[Ag(phendio)_2]ClO_4$ causes extensive, non-specific DNA cleavage. 'Metal-free' phendio and $[Ag(phendio)_2]ClO_4$ induce gross distortions in fungal cell morphology and there is evidence for disruption of cell division. Both drugs also exhibit high anti-cancer activity when tested against cultured mammalian cells.

Introduction

The yeast *Candida albicans* is an opportunistic fungal pathogen and causes a range of diseases in susceptible individuals (Pfaller *et al.* 1998). These can range from superficial infections involving the oral cavity, vagina or skin to severe life-threatening infections involving many essential organs. In recent years there has been a considerable increase in the incidence of disease attributable to this yeast, with the spread of AIDS, the widespread use of immuno-suppressive therapy and the prolonged survival of patients with critical illnesses (Lunel *et al.* 1999). Conventional therapy for the control of fungal infections relies upon the use of polyene (e.g. amphotericin B) and azole (e.g. ketoconazole) drugs. However, the emergence of *C. albicans* isolates resistant to these drugs has serious implications for the continued success of these prescription anti-fungal compounds (Van den Bossche *et al.* 1998; Kontoyiannis & Lewis 2002).

Metal-based drugs represent a novel group of anti-fungal agents with potential applications for the control of fungal infections. 1,10-Phenanthroline (phen) (Figure 1) and substituted derivatives, both in the metal-free state and as ligands co-ordinated to transition metals, disturb the functioning of a wide variety of biological systems (Butler *et al.* 1969). Furthermore, when the metal-free N,N′-chelating bases

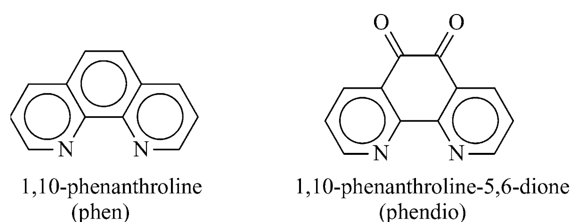

Fig. 1. Structures of phen and phendio.

are found to be bioactive it is usually assumed that the sequestering of trace metals *in situ* is involved, and that the resulting metal complexes are the active species (MacLeod, 1952; Dwyer *et al.* 1969). Previous work has demonstrated that in RPMI (Roswell Park memorial Institute) medium at 37 °C the metal-based drugs $[Cu(phen)_2(mal)] \cdot 2H_2O$, $[Mn(phen)_2(mal)] \cdot 2H_2O$ and $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ (phen = 1,10-phenanthroline; $malH_2$ = malonic acid) inhibit the growth of *C. albicans* by around 95% at a concentration of 5 μg/ml (McCann *et al.* 2000; Coyle *et al.* 2003a). It was established that both metal-free phen and the metal-phen complexes affect mitochondrial function, retard the synthesis of cytochromes b and c and uncouple respiration. Treatment of fungal cells with the Cu(II) and Ag(I) complexes resulted in a reduced amount of ergosterol in the cell membrane and subsequent increase in its permeability. Cells exposed to metal-free phen and the Cu(II) and Mn(II) complexes (but not the Ag(I) complex) demonstrated an elevation in oxygen uptake. The general conclusion was that the drugs damage mitochondrial function and uncouple respiration. Furthermore, the fact that the drugs were not uniformly active suggested that their bioactivity had a degree of metal-ion dependency.

In an attempt to enhance the anti-*Candida* activity of this class of phenanthroline drug current efforts have focused on the phenanthroline derivative 1,10-phenanthroline-5,6-dione (phendio) (Figure 1) and its Ag(I) and Cu(II) complexes. The phendio ligand, which has been known since 1947 (Smith & Cagle 1947), has two chelating functionalities, the quinonoid and the diimine (the N and O atoms are both sp^2 hybridised). The Cu(II) bis- and tris-N,N′ chelated phendio complexes, $[Cu(phendio)_2][PF_6]_2 \cdot 2H_2O$ (Goss & Abruña 1985) and $[Cu(phendio)_3](ClO_4)_2 \cdot 2H_2O \cdot 2MeCN$ (Liu *et al.* 2000), have been synthesized and the latter structurally characterized. The complex was reported to have an approximately ideal octahedral configuration with little static *Jahn-Teller* distortion. The structure of the five-coordinate complex $[CuCl(phendio)_2][PF_6] \cdot H_2O$ has recently been published (Yamada *et al.* 2002) and the mixed phendio/phen species $[Cu(phendio)(phen)]^{2+}$ has been synthesized (Wu *et al.* 2002). The preparations of Fe(II), Ru(II), Os(II), Co(II), Ni(II), Cu(I) and Zn(II) complexes incorporating phendio have also been documented (Goss & Abruña 1985; Lei *et al.* 1996; Eckert *et al.* 1982). The O,O′-bonded Pt(0) complex $[Pt(phendio)(PPh_3)_2]$ and the N,N′-bonded Pd(II) complex $[Pd(phendio)Cl_2]$ are also known (Girgis *et al.* 1975), as are heterobimetallic (Pt/Pd, Pt/Ru) species which use both of the O,O′ and N,N′ donor sets of the phendio ligand (Fox *et al.* 1991; Paw & Eisenberg 1997). In addition, an assortment of mononuclear, homobinuclear and heterobinuclear phendio complexes with Group 4 and 5 metals in both low and high oxidation states have been synthesized (Calderazzo *et al.* 1999).

Materials and methods

Chemistry

Chemicals were purchased from commercial sources and, unless specified, were used without further purification. Phendio, which was essentially insoluble in water, was prepared in accordance with the literature method (Yamada *et al.* 1992). Infrared spectra of solids (in a KBr matrix) were recorded in the region 4000–400 cm^{-1} on a Nicolet FT-IR Impact 400D infrared spectrometer. Cyclic voltammetry experiments were preformed using a low noise potentiostat (Biostat II, Electrochemical and Medical systems, Newbury, UK). Data acquisition was carried out using a Gateway GP6-350 computer, a Powerlab\400 interface system (AD Instruments Ltd, East Sussex, UK) and Echem for Windows software (AD Instruments Ltd). The Pt disk working electrode (5 cm length) was made from Teflon-insulated platinum/iridium (90%/10%) wire (125 μm bare diameter, 160 μm coated diameter (5T) obtained from Advent Research Materials, Suffolk, UK). A silver wire and a saturated calomel electrode (SCE) were used as the auxiliary and reference electrode, respectively. Fresh solutions (1 mmol) of the complexes were made up in the supporting electrolyte solution {0.1 mol dm^{-3} solution of tetraethylammonium perchlorate (TEAP) in dry, purified MeCN}. For reasons of water-insolubility voltammograms of $[Ag(phen)_2]ClO_4$ were run in DMF/TEAP.

The complex solutions were deaerated with O_2-free N_2 for 20 min prior to each experiment and cycles were run at a scan rate of 100 mV s^{-1}. Potentials are reported with respect to the SCE. Microanalytical data were provided by the Microanalytical Laboratory, National University of Ireland, Cork, Ireland.

[Cu(phendio)$_3$](ClO$_4$)$_2$ · 4H$_2$O

This complex was prepared, with improved yield, using a slight modification of the procedure used to synthesise [Cu(phendio)$_3$](ClO$_4$)$_2$ · 2H$_2$O · 2MeCN (Liu *et al.* 2000). To a pale blue solution containing Cu(ClO$_4$)$_2$ · 6H$_2$O (0.37 g, 1.0 mmol) in ethanol (20 cm^3) was added phendio (0.63 g, 3.0 mmol) and the resulting green solution was stirred at room temperature for 0.5 h. The precipitated green solid was filtered off, washed with ethanol and ether and then air-dried. The complex was soluble in water and in ethanol. Yield: 0.74 g (77%). % Found: C, 44.93; H, 2.01; N, 8.74; % Calc: C, 44.85; H, 2.72; N, 8.71. IR (KBr): 3445, 1707, 1640, 1584, 1486, 1436, 1313, 1264, 1104, 932,729, 630 cm^{-1}.

[Ag(phendio)$_2$]ClO$_4$

The following synthesis was carried out in the absence of light. To a solution of phendio (0.2 g, 1.0 mmol) in ethanol (20 cm^3) was added AgClO$_4$ (0.1 g, 0.4 mmol) and the resulting beige coloured solution was stirred at 30 °C for 0.5 h. After cooling to room temperature the precipitated orange product was filtered off, washed with small volumes of ethanol and ether, and then air-dried (with light excluded). The solid was recrystallised from hot acetonitrile and was not light-sensitive. The complex was soluble in hot acetonitrile and in DMSO, and was insoluble in water. Yield: 0.43 g (69%). % Found: C, 45.99; H, 1.91; N, 8.98; % Calc: C, 45.96; H, 1.93; N, 8.93. IR (KBr): 3432, 1695, 1640, 1578, 1467, 1430, 1307, 1104, 929, 818, 735, 627 cm^{-1}. NMR: ^{1}H NMR (ppm DMSO): 8.85 (dd, 2H), 8.46 (dd, 2H), 7.72 (dd, 2H).

[Ag(phen)$_2$]ClO$_4$

This complex was prepared using a modification of the literature procedure employed to make [Ag(phen)$_2$]NO$_3$ · H$_2$O (Murtha & Walton 1973). All operations were conducted in the absence of light. To a solution containing 1,10-phenanthroline (0.54 g, 3 mmol) in 20 cm^3 of ethanol, AgClO$_4$ (0.2 g, 1 mmol) was added. The yellow solution was refluxed for 0.5 h

Table 1. Crystal data and structure refinement for [Ag(phendio)$_2$]ClO$_4$.

Empirical formula	$C_{24}H_{12}AgClN_4O_8$	
Formula weight	627.70	
Temperature	150(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	2cm C2/c	
Unit cell dimensions	a = 13.069(5) Å	$\alpha = 90°$.
	b = 12.209(4) Å	$\beta = 108.334(4)°$.
	c = 14.517(5) Å	$\gamma = 90°$.
Volume	2198.7(13) Å^3	
Z	4	
Density (calculated)	1.896 Mg/m^3	
Absorption coefficient	1.102 mm^{-1}	
F(000)	1248	
Crystal size	0.35 × 0.23 × 0.17 mm^3	
Theta range for data collection	2.34 to 25.00°.	
Index ranges	−15<=h<=15, −14<=k<=14, −17<=l<=17	
Reflections collected	7375	
Independent reflections	1941 [R(int) = 0.0453]	
Completeness to theta = 25.00°	99.9 %	
Absorption correction	Multiscan	
Max. and min. transmission	1.00000 and 0.553911	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	1941 / 0 / 186	
Goodness-of-fit on F^2	1.070	
Final R indices [I>2sigma(I)]	R1 = 0.0379, wR2 = 0.0966	
R indices (all data)	R1 = 0.0461, wR2 = 0.1032	
Largest diff. peak and hole	1.617 and −1.041 e.Å^{-3}	

Table 2. Selected bond lengths [Å] and angles [°] for [Ag(phendio)$_2$]ClO$_4$.

Ag(1)-N(2)	2.347(3)	
Ag(1)-N(1)	2.362(3)	
Ag(1)-O(2)#1	3.329(3)	
N(2)-Ag(1)-N(2)#2		119.05(13)
N(2)-Ag(1)-N(1)#2		157.36(10)
N(2)-Ag(1)-N(1)	70.33(10)	
N(2)#2-Ag(1)-N(1)		157.36(10)
N(1)#2-Ag(1)-N(1)		109.36(14)
N(2)-Ag(1)-O(2)#1		126.29(8)
N(1)-Ag(1)-O(2)#1		70.09(8)
N(2)-Ag(1)-O(2)#3		89.08(8)
N(1)-Ag(1)-O(2)#3		71.58(8)

Symmetry transformations used to generate equivalent atoms: #1 x−1/2,−y+3/2,z−1/2 #2 −x,y,−z+3/2 #3 −x+1/2,−y+3/2,−z+2

and allowed cool. The yellow precipitate was filtered and washed with small volumes of ethanol and ether and air dried in darkness. This complex was insoluble in water and all common organic solvents. Yield: 0.40 g (70%). % Found: C, 51.08; H, 2.82; N, 9.90; % Calc: C, 50.77; H, 2.84; N, 9.90. IR (KBr): 3432, 3075, 1621, 1584, 1516, 1430, 1338, 1147, 1092, 843, 760, 723, 624 cm^{-1}.

X-ray crystallography

Crystal data for $[Ag(phendio)_2]ClO_4$ are summarized in Table 1. Data were collected using a Bruker SMART1000 diffractometer and the structure was solved by Patterson methods and refined by full-matrix least-squares on F^2 using all the data (Sheldrick 2001). All non-hydrogen atoms were refined with anisotropic atomic displacement factors and hydrogen atoms attached to carbon were inserted at calculated positions.

Supplementary data for $[Ag(phendio)_2]ClO_4$ are available from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, England (Fax: +44-1223-336033), on request quoting the deposition number CCDC 212685.

Anti-fungal susceptibility testing

C. albicans ATCC 10231 was obtained form the American Type Culture Collection, (VA., U.S.A.). Cultures were grown on Sabouraud dextrose agar (SDA) plates at 37 °C and maintained at 4 °C for short-term storage. Cultures were routinely sub-cultured every 4–6 weeks. Cultures were grown to the stationary phase (approximately 1×10^8 cells cm^{-3}) overnight at 30 °C and 200 rpm in minimal medium (MM) (2% w/v glucose, 0.5% w/v yeast nitrogen base (without amino acids or ammonium sulphate), 0.5% w/v ammonium sulphate). Solutions of the water-soluble complex $[Cu(phendio)_3](ClO_4)_2 \cdot 4H_2O$ were prepared by dissolving the complex (0.02 g) in distilled water (100 cm^3) to yield a stock solution with a concentration of 200 $\mu g\ cm^{-3}$. Doubling dilutions of this stock solution were made to yield a series of test solutions. The water-insoluble Ag(I) complexes, $[Ag(phendio)_2]ClO_4$ and $[Ag(phen)_2]ClO_4$, were made up as suspensions (0.02 g) in water (100 cm^3) to give stock suspensions. With vigorous agitation of these suspensions doubling dilutions were made to give a series of test suspensions. Minimum inhibitory concentrations (MIC's) were then determined using the broth microdilution method (McCann *et al.* 2000).

Extraction of DNA from C. albicans

Yeast cells were grown in the presence of drug (10 $\mu g\ cm^{-3}$) to the late exponential phase (18–24 h) in minimal medium at 30 °C in an orbital incubator. Cells were harvested by centrifugation and washed with 1 mM EDTA. Cells were resuspended in spheroplasting buffer (1 M sorbitol, 0.1 M EDTA, 6 mg cm^{-3} lyticase and 0.05 M dithiotheritol, pH 7.5) and incubated at 37 °C for 2 h. Spheroplasts were harvested by centrifugation, resuspended in lysing buffer (50 mM EDTA, 50 mM Tris (pH 8), 1% w/v SDS and 8 $\mu g\ cm^{-3}$ proteinase K) and incubated at 65 °C for 0.5 h. DNA was precipitated with two volumes of chilled ethanol (95% v/v) and incubated at −20 °C overnight. DNA was harvested by centrifugation, washed with ethanol (20% v/v), removed by centrifugation at 4000 × *g* for 25 min and allowed air dry. The DNA was resuspended in TE buffer (5.4% w/v Tris HCl, 2.75% w/v boric acid, 20 cm^3 0.5 M EDTA (pH 8.0)), 1 mg cm^{-3} RNase and incubated at 37 °C for 0.5 h. Ethanol (95% v/v) and 3 M sodium acetate solution (pH 5.2) was added and the sample stored at −20 °C overnight.

Purity and concentration of DNA was determined by UV spectroscopy (260–280 nm). DNA damage was assessed using gel electrophoresis. DNA from yeast cells was run at a concentration of 40 $\mu g\ cm^{-3}$ on a 0.8% (w/v) agarose gel at 40 V for 18 h. Following staining with ethidium bromide DNA was visualised using a UV transilluminator.

Electron microscopy of yeast cells

Primary fixation of stationary phase yeast cells was in a 3% solution of gluteraldehyde in 0.1 M phosphate buffer for 2 h. Secondary fixation was in a 2% solution of osmium tetroxide in 0.1 M phosphate buffer for 1 h. Dehydration of samples was in an alcohol series of 10%, 30%, 50%, 75%, 95% and 100% v/v, each for 15 min. Samples were embedded in Agar 100 resin (Agar Scientific Ltd., U.K.) and viewed using a Hitachi H-7000 Transmission Electron Microscope (TEM) operating at 100Kv accelerating voltage.

In vitro *toxicity testing on mammalian cells*

Sub-confluent DLKP cells were harvested by trypsinisation, washed and resuspended in phosphate buf-

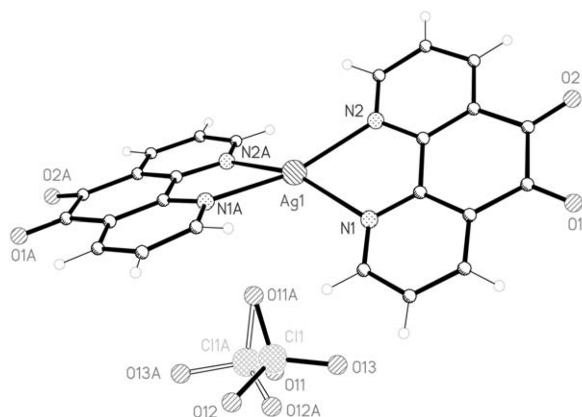

Fig. 2. X-ray crystal structure of $[Ag(phendio)_2]ClO_4$.

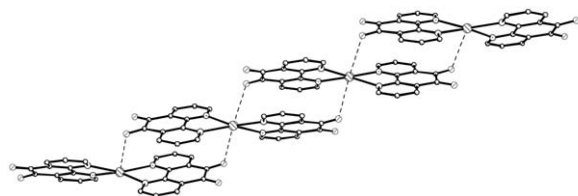

Fig. 3. Intermolecular and π–π stacking interactions in the structure of $[Ag(phendio)_2]ClO_4$.

fered saline (PBS, pH 7.2). Cells were enumerated microscopically and diluted with Minimal Essential Medium (MEM, Sigma Aldrich Chemical Co., UK) to give a final cell density of 2×10^4 cm^{-3}. Ninety six well plates (NUNC) were seeded with 100 μl of this suspension per well and incubated at 37 °C in a humidified atmosphere (5% CO_2) for 24 h to allow cell attachment. Water-insoluble metal-free phendio and $[Ag(phendio)_2]ClO_4$ were first dissolved in the minimum amount of DMSO and the resulting stock solution diluted in growth medium until the DMSO concentration was approximately 1%. A range of concentrations of the drugs were added to the rows of wells and the plates were re-incubated until controls reached 80–90% confluency (typically 5–6 days). Cell growth in toxicity assays was quantified as described previously (Martin & Clynes 1993).

Results and discussion

Chemical synthesis and structure

$[Cu(phendio)_3](ClO_4)_2 \cdot 4H_2O$ and $[Ag(phendio)_2]ClO_4$ were prepared in good yield by reacting phendio with the appropriate metal perchlorate salt. The Cu(II) complex has previously been made (Liu *et al.* 2000) and the purpose of the present synthesis was to procure a sample of the complex for anti-fungal testing.

The X-ray crystal structure of the metal-free phendio ligand has recently been published (Calderazzo *et al.* 1999) and shows the molecule to be almost planar and stacked by pointing their O atoms in opposite directions. The X-ray crystal structure of $[Ag(phendio)_2]ClO_4$ is shown in Figure 2 and important bond lengths and angles are given in Table 2. The metal lies in a pseudo tetrahedral environment and is ligated by the four nitrogens of two phendio ligands. There is a 2-fold axis passing through the silver ion and the uncoordinated perchlorate is disordered about the axis (the Cl is not exactly on the axis). There are longer interactions between the metal and the O(2) atoms of neighbouring molecules (Figure 3) so that the cations form 1-dimensional chains through the structure. In addition, there is a significant amount of π–π stacking also present throughout the unit cell (interplanar distance for the π–π stacking is 3.32 Å). The basic structure of $[Ag(phendio)_2]ClO_4$ is similar to those reported for $[Ag(dmphen)_2][BF_4] \cdot CH_2Cl_2$ and $[Zn(dmphen)_2][BF_4]_2$ (dmphen = 2,9-dimethyl-1,10-phenanthroline) (Pallenberg *et al.* 1997).

Cyclic voltammetry data for metal-free phendio, $[Ag(phendio)_2]ClO_4$, $[Ag(phen)_2]ClO_4$ and some simple Ag(I) salts are listed in Table 3. All of the silver-containing species showed a single, irreversible cathodic wave for the reduction process $Ag^+ \rightarrow Ag^0$ and also a large anodic stripping peak for the reoxidation of the plated silver metal ($Ag^0 \rightarrow Ag^+$). With respect to metal deposition it has been reported (Lei *et al.* 1996) that the Cu(II) complex $[Cu(phendio)_2]^{2+}$ gets reduced to Cu(I) at slightly less negative pots than that required to reduce the phendio ligand, and that copper metal plating on the electrode surface was frequently observed. Allowing for the differences in solvent (DMF and MeCN) it is interesting that reduction of Ag^+ to Ag^0 was more facile for $[Ag(phendio)_2]ClO_4$ than for $[Ag(phen)_2]ClO_4$ suggesting that the N atoms of the phen ligand are stronger electron donors than those of phendio. This fact is substantiated by data derived from studies of pK_a and metal ion binding constants for protonated phen and phendio, which showed that the carbonyl oxygens on the latter molecule lessen the electron density on the N atoms of the pyridine rings (Eckert *et al.* 1982). Furthermore, binding constants with the divalent metal ions, Zn(II), Co(II) and Ni(II) are known to be 2-6 orders of magnitude larger for phen than for phendio.

Table 3. Cyclic voltammetry data (potentials in mV v's SCE).[a]

	$Ag^+ \rightarrow Ag^0$	$Ag^0 \rightarrow Ag^+$	phendio $(E_{1/2})^A$	phendio $(E_{1/2})^B$
$AgCH_3CO_2$	+25	+282		
$AgNO_3$	+134	+310		
$AgClO_4$	+152	+288		
$[Ag(phen)_2]ClO_4$	−100	+482		
phendio			−471	−1042
$[Ag(phendio)_2]ClO_4$	+8	+298	−475	−1039

[a] $[Ag(phen)_2]ClO_4$ was run in DMF/TEAP and all other samples were run in MeCN/TEAP.

Unlike metal-free phen, which is electroinactive in the potential region +1000 to −1000 mV (versus SCE), an MeCN solution of uncomplexed phendio showed two, reversible, one-electron waves (A and B) with half-wave potential values of $(E_{1/2})^A = -471$ mV and $(E_{1/2})^B = -1042$ mV. This behavior is very similar to that previously reported (Eckert *et al.* 1982; Eckert & Bruice 1983; Goss & Abruña 1985; Hilt *et al.* 1982) for phendio in aprotic solvents, with the quinone being reduced firstly to the semiquinone (wave A) and then to the hydroquinone (wave B). Presumably, the protons required for these conversions are derived from traces of water in the MeCN solvent. The voltammogram of $[Ag(phendio)_2]ClO_4$ (Figure 4) shows the plating and stripping peaks for the metal and also the two sets of reversible, one-electron waves (A and B) for the phendio ligand, the $E_{1/2}$ values of the ligand redox waves being essentially the same as that of the metal-free phendio ligand. Thus, upon electrochemical reduction of the Ag^+ center in $[Ag(phendio)_2]ClO_4$ the resulting Ag^0 metal atom dissociates from the phendio ligand and this metal-free ligand then exhibits its normal two, Nernstian, one-electron redox waves.

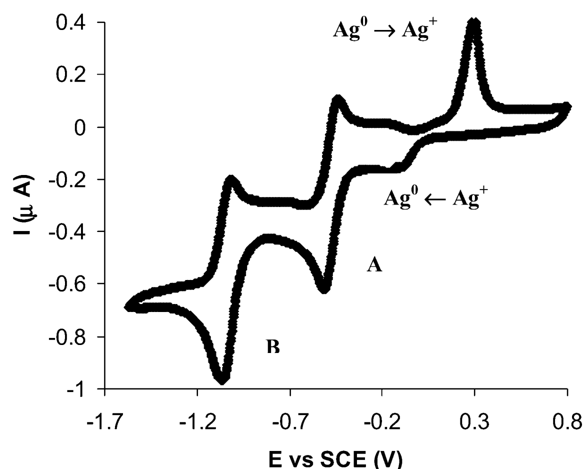

Fig. 4. Cyclic voltammogram of $[Ag(phendio)_2]ClO_4$.

Effect of drugs on fungal cell growth

MIC values for *in vitro* anti-*Candida* susceptibility testing are displayed in Table 4. In the first instance, 'metal-free' phendio is a more active drug than 'metal-free' phen. As previously suggested for 'metal-free' phen (McCann *et al.* 2000; Coyle *et al.* 2003a) it is thought that the biologically active form of chelating 1,10-phenanthroline-type ligands is an *in situ* generated metal complex of the ligand. The Cu(II) and Ag(I) complexes incorporating the phendio ligand are significantly more active than the most active phen complex, $[Ag_2(phen)_3(mal)]\cdot 2H_2O$ (Coyle *et al.* 2003a). The results for 'metal-free' phendio and the Cu(II) and Ag(I) complexes suggest that the two carbonyl appendages on the phenanthroline skeleton of the ligand play an important role in its biochemical activity. It is envisaged that the carbonyl oxygens might offer points of secondary interactions with fungal cell biomolecules containing electron-acceptor sites (e.g. polar $H^{\delta+}$ sites and other metal cations). In addition, the more facile electrochemical activity of the phendio ligand, compared to that of phen, opens up the possibility for phendio to participate destructively in cellular redox reactions.

As the simple Ag(I) salts, $AgCH_3CO_2$, $AgNO_3$ and $AgClO_4 \cdot H_2O$ are substantially superior anti-*Candida* drugs than analogous simple Cu(II) and Mn(II) salts (Geraghty *et al.* 2000) this implies that the type of metal ion greatly influences activity, with Ag(I) being particularly active. In this respect tetrahedral bis-diphosphine Ag(I) complexes have previously been shown to exhibit good anti-*Candida* and anti-cancer activity. (Berners-Price *et al.* 1988). Free Ag(I) ions are also known to be strongly fungicidal,

algicidal and bactericidal at comparatively low doses (Ratte 1999). The use of silver preparations in medicine and for sterilizing potable or swimming-pool water is based on a particular sensitivity of bacterial metabolism to Ag(I)-inhibiting thiol enzymes. Tilton and Rosenberg (Tilton & Rosenberg 1978) proposed three possible mechanisms for bacterial growth inhibition by Ag(I): interference with electron transport, binding to DNA and interaction with the cell membrane. A bactericidal effect has been reported for both metallic silver and Ag(I) salts, with the concentration of the metal ion being responsible for the disinfection capability (Thurman & Gerba 1989). Both $AgNO_3$ and the Ag(I) complex of 4-amino-*N*-2-pyrimidinyl-benzenesulfonamide (commercially available as the cream silvadene or flamazine) and used topically to prevent infections in cut and burn wounds) inhibit the activity of isolated fungal (*C. albicans*) and human phosphomannose isomerases (PMI). PMI are Zn(II)-containing metalloenzymes which catalyse the reversible interconversion of fructose 6-phosphate and mannose 6-phosphate (Wells *et al.* 1995). It was postulated that enzyme inhibition is due to binding of Ag^+ to either the imidazole N of histidine or to a free S on cysteine. Whereas the Ag(I)-sulfonamide complex showed little or no discrimination between fungal and human PMI $AgNO_3$ was more active against the latter enzyme. An *in vivo* study, which monitored the mortality in burned mice infected with *Pseudomonas aeruginosa* and treated with an oil/water cream containing an Ag^+ complex, showed that survival numbers were less for animals treated topically with $AgNO_3$ than for those with silvadene (Bult *et al.* 1981). However, no obvious relationships were found between the antibacterial activity and selected physical properties (stability constant, water solubility and conductivity) of the Ag(I) species. In the same study it was tentatively concluded that $AgNO_3$ was active *in vivo* as a consequence of the relatively high concentration of free Ag^+ ion formed upon dissolution of the salt. Given the very low water solubility of silvadene its good *in vivo* activity was attributed to the interaction of the whole molecule with the microorganism. If the above arguments are applied to the present results then it would seem that in the case of the simple Ag(I) salts, such as $AgNO_3$, the anti-*Candida* activity is due to the bioavailability of a relatively large concentration of Ag(I) ions. With the very potent, yet poorly water-soluble complexes, $[Ag_2(phen)_3(mal)]\cdot 2H_2O$ and $[Ag(phendio)_2]ClO_4$, it seems that their activity is due to the whole molecule. In the case of the highly water-insoluble complex $[Ag_2(imppn)_4](ClO_4)_2$ (imppn = (Z)-3-(1*H*-imidazol-1-yl)-2-phenylpropenenitrile) its poor anti-*Candida* activity is a feature of the whole molecule (McCann *et al.* 2003).

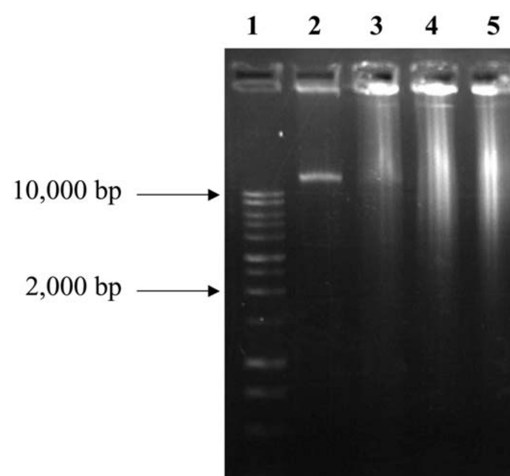

Fig. 5. DNA banding pattern of *C. albicans* cells treated with marker, control, phendio, $AgClO_4$. $[Ag(phendio)_2]ClO_4$. Lane 1: Molecular weight marker; Lane 2: Control; Lane 3: phendio; Lane 4: $AgClO_4$; Lane 5: $[Ag(phendio)_2]ClO_4$.

Effect of drugs on the integrity of fungal nuclear DNA

The effects of selected Ag(I) complexes and simple Ag(I) salts on the integrity of fungal nuclear DNA was investigated and compared to that of a non-drug-treated control. *C. albicans* exposed to metal-free phendio, $AgClO_4 \cdot H_2O$ and $[Ag(phendio)_2]ClO_4$ showed extensive smearing of DNA, indicating non-specific cleavage of the DNA (Figure 5). $AgCH_3CO_2$, $AgNO_3$ and $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ have previously been shown to have a similar effect on the fungal DNA (Coyle *et al.* 2003b),. In contrast, the metal-metal bonded dimer, $[Ag_2(imppn)_4](ClO_4)_2$ (McCann *et al.* 2003), which does not contain a chelating phen-type ligand and has only marginal anti-*Candida* activity, did not disrupt the fungal DNA. From studies of nucleic acid-Ag(I) interactions, conducted using isolated calf thymus DNA (Nordén *et al.*1986) and the free purine and pyrimidine bases found in DNA (Šponer *et al.* 1999; Nordén *et al.* 1986), there was compelling evidence for complexation of the metal with N atoms of the bases. However, in contrast to the present experiments, which show extensive disruption of *Candida* DNA by Ag(I) simple salts and complexes, results with the calf thymus DNA indicated that only

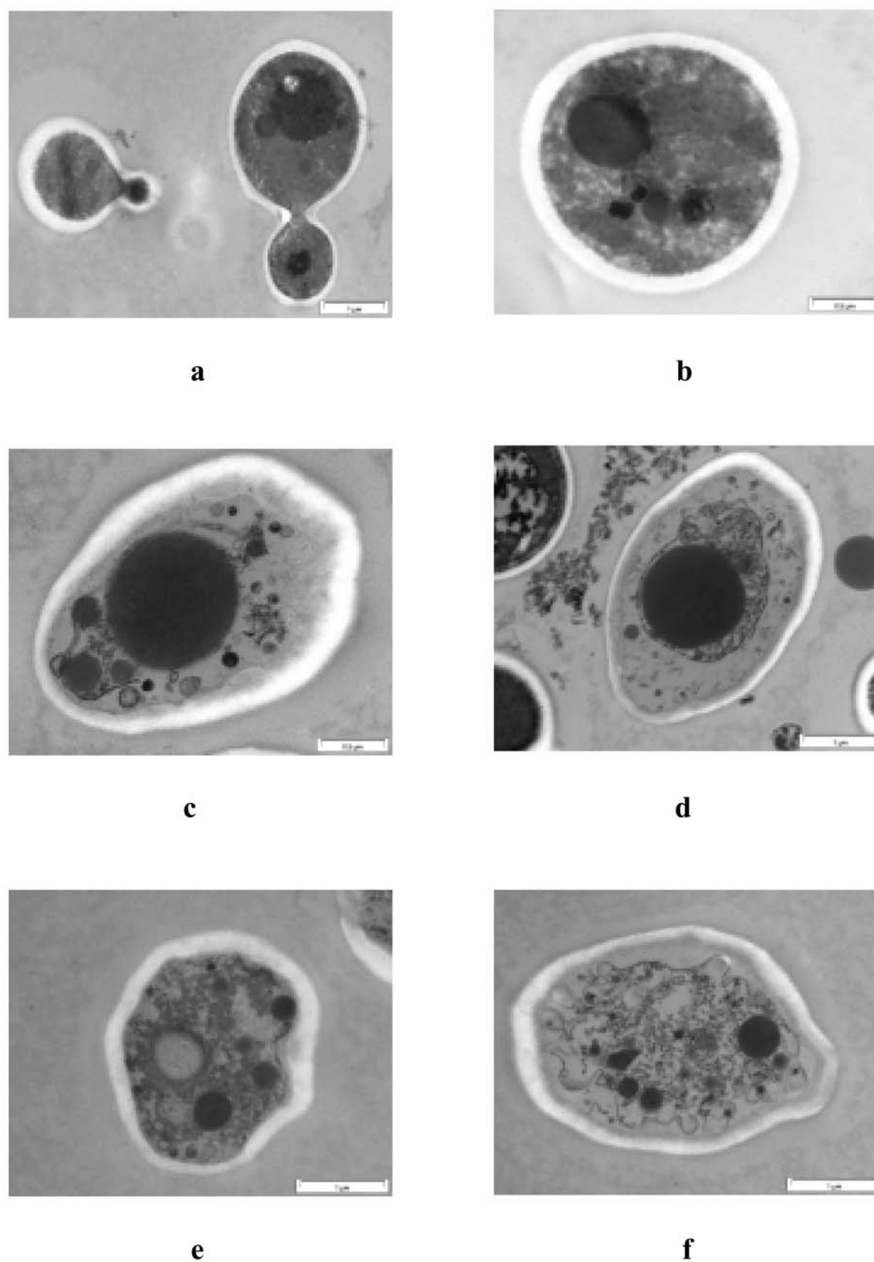

Fig. 6. Electron micrographs of *C. albicans* cells grown in the absence of drug (a) and (b), and in the presence of phendio (c and d) and $[Ag(phendio)_2]ClO_4$ (e and f).

relatively minor structural changes occurred upon exposure to Ag(I) ions (swelling at some base pair sites by coordination of the Ag(I) and reorientation of the DNA molecule into a strongly tilted conformation) (Nordén *et al.* 1986).

Effect of drugs on the internal structure of C. albicans

Cultures of *C. albicans* were grown to the stationary phase overnight in MM at 30 °C and 200 rpm in the presence of phendio and $[Ag(phendio)_2]ClO_4$ at a final drug concentration of 10 μg cm^{-3}. Cells were harvested by centrifugation, washed with PBS (pH 7.2) and placed on ice prior to preparation for TEM examina-

tion (as described above). Cells grown in the absence of drug showed normal cellular morphology. A high percentage (>70%) of budding cells were present in the population and all cells possessed a distinct cell wall, an intact nucleus and numerous membranous organelles (Figure 6a and 6b). In contrast, cells grown in the presence of metal-free phendio were significantly larger (see scale bars on figures) than cells grown in drug-free medium and they also lacked buds (Figure 6c and 6d). This indicated a possible drug-induced circumvention of the control of cell division. In addition, the cells exhibited a diffuse cell wall, ruptured internal organelles, the withdrawal of the cytoplasmic membrane from within the cell wall and an enlarged nucleus. Cells treated with [Ag(phendio)$_2$]ClO_4 had a distended cell wall, ruptured membranous organelles and, in some cases, a fragmented nucleus (Figure 6e). In addition, there was also evidence of withdrawal of the cytoplasmic membrane from within the cell wall (Figure 6f). Once again, the lack of buds suggests an interference in the process of cell division.

Previous work has demonstrated that [Ag_2(phen)$_3$(mal)] · $2H_2O$ has a profound effect on the physiology of *C. albicans* by lowering the respiration rate which, in turn, leads to a depletion of ergosterol in the fungal cell membrane (Coyle *et al.* 2003a). Recently it has been established that this Ag(I) complex induces many of the morphological changes evident in cells treated with [Ag(phendio)$_2$]ClO_4 and it also causes extensive, non-specific DNA fragmentation in *C. albicans* (Coyle *et al.* 2003b). Examination of the integrity of nuclear DNA following exposure of cells to [Ag(phendio)$_2$]ClO_4 indicates that this complex is capable of inducing non-specific DNA cleavage, a feature which is also analogous to the cell death pathway evidenced in *C. albicans* exposed to [Ag_2(phen)$_3$(mal)] · $2H_2O$. This ability to splinter DNA may be responsible for the appearance of nuclear fragments evident in the electron-micrographs (Figure 6e).

Mammalian cell studies

Preliminary experiments on cultured human cancer cells have produced IC_{50} values of 0.008 μg cm^{-3} (0.04 μM) and 0.025 μg cm^{-3} (0.40 μM) for metal-free phendio and [Ag(phendio)$_2$]ClO_4, respectively. Previous studies (Igdaloff *et al.* 1983) have shown that phendio and its isomer, 1,7-phenanthroline-5,6-dione, inhibit the growth of S49 mouse lymphona cells and S110 mouse cells, with phendio being the most active (EC$_{50}$ S49 = 0.056 μM; S110 = 0.042 μM). Although it was postulated that inhibition of DNA and RNA syntheses were major components of the cytotoxic effects the phenanthrolines were presumed to have more than one mode of action. Futhermore, since the effects of the two molecules on dXTP levels were qualitatively different, the molecules probably differed in at least one of their target sites. In this regard it was noted that whereas phendio can chelate metal ions, the 1,7-isomer is incapable of chelation (Igdaloff *et al.* 1983).

Table 4. MIC data for *C. albicans* {concentrations expressed as μM and as μg cm^{-3} (in brackets)}.

Compound	MIC μM (μg cm^{-3})
$AgCH_3CO_2$	30 (5)
$AgNO_3$	30 (5)
$AgClO_4.H_2O$	44 (10)
phen	14 (2.5)
phendio	3 (0.6)
[Cu(phendio)$_3$(ClO_4)$_2$]·$4H_2O$	1.3 (1.3)
[Ag(phendio)$_2$]ClO_4	0.5 (0.3)
[Ag(phen)$_2$]ClO_4	8.8 (5)

Conclusions

The Cu(II) and Ag(I) complexes, [Cu(phendio)$_3$](ClO_4)$_2$ · $4H_2O$ and [Ag(phendio)$_2$]ClO_4, can be prepared in good yield, and the latter has a pseudo tetrahedral structure. 'Metal-free' phendio and the Cu(II) and Ag(I) phendio complexes are potent anti-*Candida* agents and are more active than their phen analogues, suggesting an influential biochemical role for the carbonyl oxygen atoms of the phendio molecule. The simple Ag(I) salts, $AgCH_3CO_2$, $AgNO_3$ and $AgClO_4$ · H_2O, are much better anti-*Candida* drugs than analogous simple Cu(II) and Mn(II) salts, suggesting that the nature of the metal ion strongly influences activity. *C. albicans* exposed to metal-free phendio, simple Ag(I) salts and [Ag(phendio)$_2$]ClO_4 experience extensive and non-specific DNA cleavage. 'Metal-free' phendio and [Ag(phendio)$_2$]ClO_4 cause gross distortions in fungal cell morphology and there is evidence for disruption of cell division. Both drugs also have the ability to kill cultured mammalian cancer cells at relatively low concentrations (IC_{50} = 0.008 μg cm^{-3} and 0.025 μg cm^{-3}).

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