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Induction of apoptosis in yeast and mammalian cells by exposure to 1,10-phenanthroline metal complexes

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Abstract

1,10-Phenanthroline (phen) and metal–phen complexes display fungicidal and fungiststic activity, disrupt mitochondrial function and induce oxidative stress. We have examined the effect of these drugs on the structure of yeast and mammalian cell organelles and the integrity of cellular DNA. Exposure of *Candida albicans* to $[Mn(phen)_2(mal)].2H_2O$ or $[Ag_2(phen)_3(mal)].2H_2O$ (mal H_2 = malonic acid) resulted in DNA degradation whereas exposure to phen or $[Cu(phen)_2(mal)].2H_2O$ did not. All drugs induced extensive changes to the internal structure of yeast cells including retraction of the cytoplasm, nuclear fragmentation and disruption of the mitochondrion. In the case of cultured mammalian cells $[Cu(phen)_2(mal)].2H_2O$ induced apoptosis as evidenced by the ladder pattern of DNA fragments following gel electrophoresis and also the blebbing of the cell membrane. The other drugs produced non-specific DNA degradation in mammalian cells. In conclusion, phen and metal–phen complexes have the potential to induce apoptosis in fungal and mammalian cells. Given their distinct mode of action compared to conventional anti-fungal drugs, phen and metal–phen complexes may represent a novel group of anti-fungal agents for use either in combination with existing drugs or in cases where resistance to conventional drugs has emerged.

Keywords: Apoptosis; *Candida*; Metal-based drug; Fungicidal; Fungistatic

1. Introduction

Fungal pathogens are a serious cause of infection and death in patients immuno-compromised as a result of disease (e.g. leukaemia) or therapeutic procedures (e.g. broad spectrum antibiotics, immuno-suppression prior to organ transplantation) (De Pauw, 1997). The yeast *Candida albicans* is an opportunistic fungal pathogen which 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. There has been a considerable increase in the incidence of disease attributable to this yeast in recent years 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 or azole drugs. The most widely used polyene anti-fungal drug is amphotericin B which functions by binding to ergosterol in the fungal cell membrane creating pores through which intracellular constituents leak (Abu-Salah, 1996). Azoles target the ergosterol biosynthetic pathway leading to cells depleted in ergosterol and with elevated levels of toxic intermediates which prove fatal to the cell. The emergence of *C. albicans* isolates resistant to anti-fungal drugs has serious implications for the continued success of conventional anti-fungal therapy (Van den Bossche et al., 1998; Kontoyiannis & Lewis, 2002).

Abbreviations: mal, malonic acid; MIC, minimum inhibitory concentration; MM, Minimal medium; Phen, 1,10-phenanthroline.

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Metal-based drugs represent a novel group of anti-fungal agents with potential applications for the control of fungal infections. 1,10-Phenanthroline (phen) 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 are found to be bioactive it is usually assumed that the sequestering of trace metals 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 medium at 37 °C the metal-based drugs $[Cu(phen)_2(mal)].2H_2O$, $[Mn(phen)_2(mal)].2H_2O$ and $[Ag_2(phen)_3(mal)].2H_2O$ (phen = 1,10-phenanthroline; $malH_2$ = malonic acid) inhibit the growth of *C. albicans* by around 95% at a concentration of 5 μg/ml (Coyle et al., 2003). 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. Indeed, part of the mode of action of this group of drugs seems to lie in their ability to induce oxidative stress within the cell as evidenced by the decreased reduced:oxidized glutathione ratios (GSH:GSSG) and increased levels of lipid peroxides in *Candida* cells treated with $[Cu(phen)_2(mal)].2H_2O$ (McCann et al., 2000).

The aim of the work presented here was to further characterise the mode of action of these drugs in terms of their effects on the morphology of fungal and mammalian cells. Due to their different mode of action compared to the polyene and azole anti-fungal drugs (Coyle et al., 2003), metal based drugs may represent a novel group of anti-fungal agents with potential applications either alone or in combination with conventional anti-fungals. In addition, they may be applicable in situations where resistance to conventional anti-fungal drugs has emerged.

2. Materials and methods

2.1. Fungal strain and culture conditions

C. albicans ATCC 10231 was obtained form the American Type Culture Collection, (VA, USA). 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/ml) 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].

2.2. Human cell culture

The HEp-2 cell line (ATCC CCL23) was obtained from the American Type Culture Collection (VA, USA) and cells were grown in MEM (Sigma Aldrich Chemical Co., Dorset, UK) supplemented with 5% v/v foetal calf serum (Gibco, Paisley, UK), 4 mM L-glutamine and 1% v/v Penn-Strep (Sigma Adrich). The DLKP cell line was obtained from the National Cell and Tissue Culture Centre (Dublin, Ireland) and is derived from a poorly differentiated cell carcinoma from a lymph node metastasis of a primary lung tumour. DLKP cells were cultured under the same conditions as HEp-2 cells. Adherent cells were grown in 80 cm^2 culture flasks at 37 °C and 5% CO_2 in a humidified atmosphere and sub-cultured by trypsinisation every 3–4 days.

2.3. Drugs

Chemicals were obtained from commercial sources and used without further purification. $[Cu(phen)_2(mal)].2H_2O$, $[Mn(phen)_2(mal)].2H_2O$ and $[Ag_2(phen)_3(mal)].2H_2O$ were prepared as previously described (McCann et al., 2000).

2.4. In vitro toxicity testing

Sub-confluent DLKP cells were harvested by trypsinisation washed and resuspended in PBS. Cells were enumerated microscopically and diluted with MEM to give a final cell density of 2×10^4/ml. Ninety-six-well plates (NUNC) were seeded with 100 μl of this suspension per well and incubated at 37 °C and 5% CO_2 in a humidified atmosphere for 24 h to allow cell attachment. Subsequently, a range of concentrations of metal based drug was 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).

2.5. Extraction of DNA from C. albicans

Yeast cells were grown in the presence of drug (10 μg/ml) to the late exponential phase (18–24 h) in MM 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/ml 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 μg/ml 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), harvested by centrifugation at 4000×*g* for 25 min and allowed air dry. The DNA was resuspended in TE buffer, 1 mg/ml 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.

2.6. Extraction of DNA from cultured human cells

Sub-confluent HEp-2 cells that had been cultured in the presence of 3 μM of metal-based drug for 24 h were harvested by trypsinisation, washed and resuspended in PBS. Cells (1×10^6) were resuspended in lysis buffer [20 mM EDTA, 0.8% (w/v) sodium lauryl sarcosiate, 100 mM Tris (pH 8.0)] and 10 mg/ml RNase (Boehringer Manneheim, Sussex, UK) and incubated at 37 °C for 18 h. Proteinase K (1 mg/ml) was subsequently added and the samples were incubated for a further 2 h at 50 °C.

2.7. DNA gel electrophoresis

Purity and concentration of DNA was determined by UV spectroscopy (260–280 nm). DNA from yeast and mammalian cells was run at a concentration of 40 μg/ml 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.

2.8. Electron microscopy

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%, each for 15 min. Samples were embedded in Agar 100 resin (Agar Scientific Ltd., UK) and viewed using a Hitachi H-7000 Transmission Electron Microscope operating at 100 kv accelerating voltage.

3. Results

3.1. Determination of effect of metal-based drugs on yeast cell DNA

The aim of the work presented here was to establish whether drug-induced oxidative stress altered the structure of the cellular organelles and affected the integrity of yeast DNA. The minimum inhibitory concentration of each drug was used as determined previously (Coyle et al., 2003). Cells of *C. albicans* were grown to the stationary phase in MM containing 10 μg/ml of each drug for 24 h. Cells were harvested by centrifugation, the DNA was extracted as described and visualised by ethidium bromide staining following agarose gel electrophoresis. The DNA extracted from yeast cells exposed to $[Ag_2(phen)_3(mal)].2H_2O$ shows extensive degradation (Fig. 1). Smaller amounts of degradation, as demonstrated by smearing, are also visible in cells treated with $[Cu(phen)_2(mal)].2H_2O$ and $[Mn(phen)_2(mal)].2H_2O$, whereas incubation of cells with metal-free phen causes little or no DNA breakdown under the conditions employed here.

3.2. Electron microscopic examination of C. albicans following growth in the presence of metal-based drugs

Cultures of *C. albicans* were grown to the stationary phase overnight in MM medium at 30 °C and 200 rpm in the presence of each drug at a final concentration of 10 μg/ml. Cells were harvested by centrifugation, washed with PBS (pH 7.2) and placed on ice prior to preparation for TEM examination (as described). Cells grown in the absence of drug showed normal cellular morphology with a distinct cell wall, an intact nucleus and numerous membranous organelles (Fig. 2a). In contrast, cells grown in the presence of metal-free phen demonstrated a distended cell wall, ruptured internal organelles and the withdrawal of the cytoplasmic membrane from within the cell wall (Fig. 2b). Cells treated with $[Ag_2(phen)_3(mal)].2H_2O$ possessed a distended cell wall, ruptured organelles and, in some cases, a fragmented nucleus (Fig. 2c). The most obvious feature of $[Cu(phen)_2(mal)].2H_2O$-treated cells was the occurrence

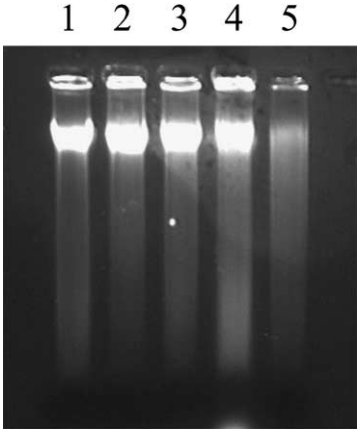

Fig. 1. DNA banding pattern of *C. albicans* cells treated with phen and metal-phen complexes for 24 h. Lane 1: DNA from control cells, Lane 2: phen, Lane 3: $[Cu(phen)_2(mal)].2H_2O$, lane 4: $[Mn(phen)_2(mal)].2H_2O$, Lane 5: $[Ag_2(phen)_3(mal)].2H_2O$.

of an enlarged nucleus which, in some cells, was crescent shaped (Fig. 2d). With this drug the internal organelles appeared intact but some shrinkage of the cytoplasm within the cells was apparent. Cells exposed to $[Mn(phen)_2(mal)].2H_2O$ had, in most cases, completely disrupted organelles. Some of these cells possessed enlarged nuclei while others appeared to contain distinct nuclear fragments (Fig. 2e).

3.3. Effect of metal-based drugs on integrity of mammalian DNA

Previous studies into the effect of metal-phen complexes on fungal cell viability indicated that the drugs disrupt mitochondrial function (Coyle et al., 2003). In vitro toxicity assays were performed to establish the concentration of 1,10-phen and $[Cu(phen)_2(mal)].2H_2O$

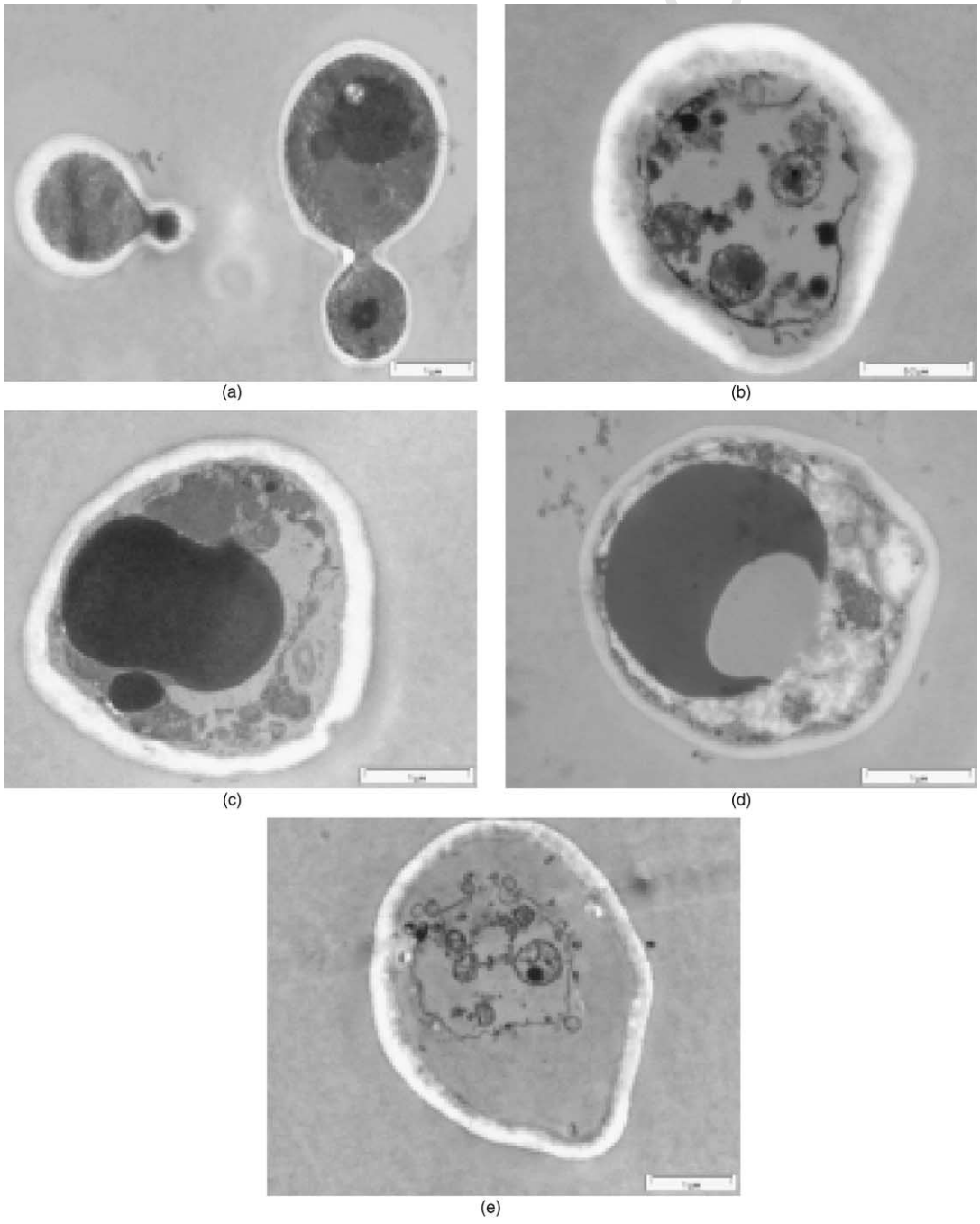

Fig. 2. Electron-micrographs of *C. albicans* cells exposed to phen and metal-phen complexes for 24 h. (a) Control, (b) Phen, (c) $[Ag_2(phen)_3(mal)].2H_2O$, (d) $[Cu(phen)_2(mal)].2H_2O$, (e) $[Mn(phen)_2(mal)].2H_2O$. (Bar = 1 μM).

capable of killing cultured human cells. The results indicated IC_{50} values of 0.002 μg/ml and 0.001 μg/ml for each drug, respectively against DLKP cells.

HEp-2 cells were chosen to determine whether the metal–phen complexes induced apoptosis since the appearance of the DNA fragmentation pattern associated with this mode of cell death (Verhaegen, 1998) is easier to visualise in this cell line than in the DLKP line. HEp-2 cells were cultured for 24 h in the presence of 3 μM of metal-free phen or the Cu(II), Mn(II) and Ag(I) phen complexes, and the DNA was subsequently extracted and separated by agarose gel electrophoresis. Degradation of high molecular weight DNA was evident in those cultures treated with Cu(II), Mn(II) and Ag(I) phen. Cells treated with metal-free phen also showed extensive degradation of DNA. Treatment of cells with the copper–phen complex produced a DNA pattern which was divided into distinct fragments. The appearance of this DNA fragmentation 'ladder' is indicative of apoptosis (or programmed cell death) occurring in the cells in response to the metal based drug. Cultures treated with Mn or Ag complexes demonstrated DNA degradation but did not produce a specific 'ladder' fragmentation pattern at this concentration.

3.4. Microscopic examination of cultured human cells grown in the presence of $[Cu(phen)_2(mal)].2H_2O$

Previous work has established that the metal-phen complexes affect the oxygen uptake rate of *C. albicans* and induced oxidative stress within the fungal cell (McCann et al. 2000; Coyle et al., 2003). We sought to determine whether these complexes affected the growth and cellular morphology of cultured mammalian cells in order to assess their toxicity in vitro towards cells derived from human tissue.

In order to examine the morphological changes taking place in cultured cells following exposure to the most toxic of the metal–phen complexes sub-confluent DLKP cells were exposed to $[Cu(phen)_2(mal)].2H_2O$ (3μM) and incubated at 37 °C for 48 h. Untreated cells show the typical morphology of adherent epithelial cells (Fig. 4A). Exposure to $[Cu(phen)_2(mal)].2H_2O$ for 24 h produced cells showing evidence of membrane 'blebbing' (Fig. 4b)— a feature of cell death by apoptosis (Verhaegen, 1998). After 48 h incubation a number of cells had fragmented to give clusters of small sub-cellular packets (Fig. 4c)— tentatively identified as apoptotic bodies, a feature of the latter stages of apoptosis (Verhaegen, 1998).

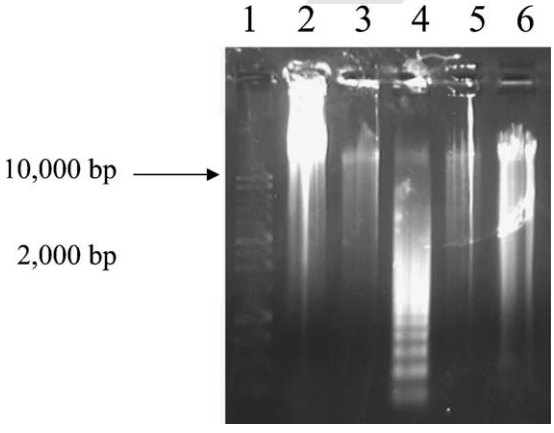

Fig. 3. DNA banding pattern from HEp-2 cells exposed to phen and metal–phen complexes for 24 h. Lane 1: molecular weight standards, Lane 2: control, Lane 3: phen, Lane 4: $[Cu(phen)_2(mal)].2H_2O$, lane 5: $[Mn(phen)_2(mal)].2H_2O$, Lane 6: $[Ag_2(phen)_3(mal)].2H_2O$.

4. Discussion

The in vitro antibacterial action of phen has been demonstrated on several species of bacteria (Dwyer et al., 1969; Feeney et al., 1957). Whereas metal–phen complexes can be bacteristatic (Dwyer et al., 1969) and bactericidal (Butler et al., 1969) towards many Gram-positive bacteria they are relatively ineffective against Gram-negative organisms. In addition, dilute aqueous solutions of phen and its Cu(II) and Mn(II) complexes were highly toxic to clinical isolates of *Candida* species (Geraghty et al., 2000; Geraghty et al., 1998).

Earlier in vitro experiments in our laboratories have shown that phen and a number of transition metal complexes incorporating this chelating ligand are extremely active anti-fungal drugs (Geraghty et al., 1999a,b,c; Devereux et al., 2000a,b; McCann et al., 2000; Geraghty et al., 2000). The compounds have minimum inhibitory concentrations in the range 1.25–5.0 μg/ml and, at a concentration of 10 μg/ml, display some fungicidal activity. Treating exponential and stationary phase yeast cells with phen and the Cu(II) and Mn(II) complexes induces a dramatic increase in oxygen consumption. All of the drugs cause reductions in the levels of cytochromes b and c in the cells, while the Ag(I) complex also lowers the amount of cytochrome aa_3. Cells treated with phen and the Cu(II) and Ag(I) species show reduced levels of ergosterol while the Mn(II) complex induces an increase in the sterol content. Extensive studies with $[Cu(phen)_2(mal)].2H_2O$ indicated that this drug induces significant cellular oxidative stress (decreased reduced:oxidized glutathione ratios (GSH:GSSG) and increased levels of lipid peroxides). Furthermore, as the drugs were not uniformly active this suggested that their bioactivity has a degree of metal-ion dependency. The drugs disrupt mitochondrial function, uncouple respiration and promote oxidative stress in the organism (Coyle et al., 2003). As such, phen and the metal–phen complexes may represent a novel set of highly active anti-fungal agents whose mode of action is significantly different to that of the polyene and azole prescription drugs.

The work presented here is a progression of the above mechanistic studies and examines the effects of the metal–phen complexes on the integrity of DNA and cellular morphology of *C. albicans* and cultured mammalian cells. Exposing *C. albicans* to $[Ag_2(phen)_3(mal)].2H_2O$ or $[Mn(phen)_2(mal)].2H_2O$ leads to non-specific DNA cleavage. In the case of the Mn(II) complex this may be as a result of oxidative damage to the cells caused by elevated levels of oxygen uptake (Coyle et al., 2003). The Cu(II) complex and metal-free phen seem to have little effect on fungal DNA. This latter finding is surprising since phen and copper(I)–phen complexes are known to cleave DNA with the same preferences as micrococcal nuclease (Jessee et al., 1982; Chen & Sigman, 1986). It is possible that either the concentration used was not optimal to induce fungal cell DNA degradation or that the cells were capable of repairing the damage over the timeframe of the experiment. In addition, it is possible that the oxidative stress induced by these compounds can inhibit Caspase activity which is sensitive to the redox balance within the cell (Green & Kroemer, 1998).

Electron-micrographic examination of fungal cells exposed to phen and the metal–phen complexes reveals severe disruption of internal cellular structures (Fig. 2a–e). In particular, nuclear disruption is evident following exposure to $[Ag_2(phen)_3(mal)].2H_2O$ $[Mn(phen)_2(mal)].2H_2O$ and $[Cu(phen)_2(mal)].2H_2O$. This is consistent with the cleavage of fungal DNA evident in Fig. 1. Nuclear fragmentation is characteristics of apoptosis as is nuclear 'crescent' formation evident in many cells (Cohen, 1993). Apoptosis in fungal cells follows many of the same steps evident in animal cells including fragmentation of the nucleus, degradation of DNA and disruption of internal organelles (Roze & Linz, 1998). Discrete apoptotic bodies are not formed.

Exposure of mammalian cells to $[Cu(phen)_2(mal)].2H_2O$ at a concentration of 0.003 μg/ml results in a DNA fragmentation pattern which is characteristic of cells dying by apoptosis (Cohen, 1993; Verhaegen, 1998). In this mode of cell death, the injured cell plays an active role in its own demise and one part of the process is the cleavage of nuclear DNA into specific sized fragments by an endonuclease giving rise to a 'ladder' pattern of fragments upon gel electrophoresis (Cohen, 1993; Cotter and Al-Rubeai, 1995). Copper–phen complexes have previously been shown to induce apoptosis in a range of cell lines (Zhou et al., 2002 a,b; De Vizcaya-Ruiz et al., 2002). Exposure of cultured mammalian cells to metal-free phen, $[Ag_2(phen)_3(mal)].2H_2O$ or $[Mn(phen)_2(mal)].2H_2O$ did

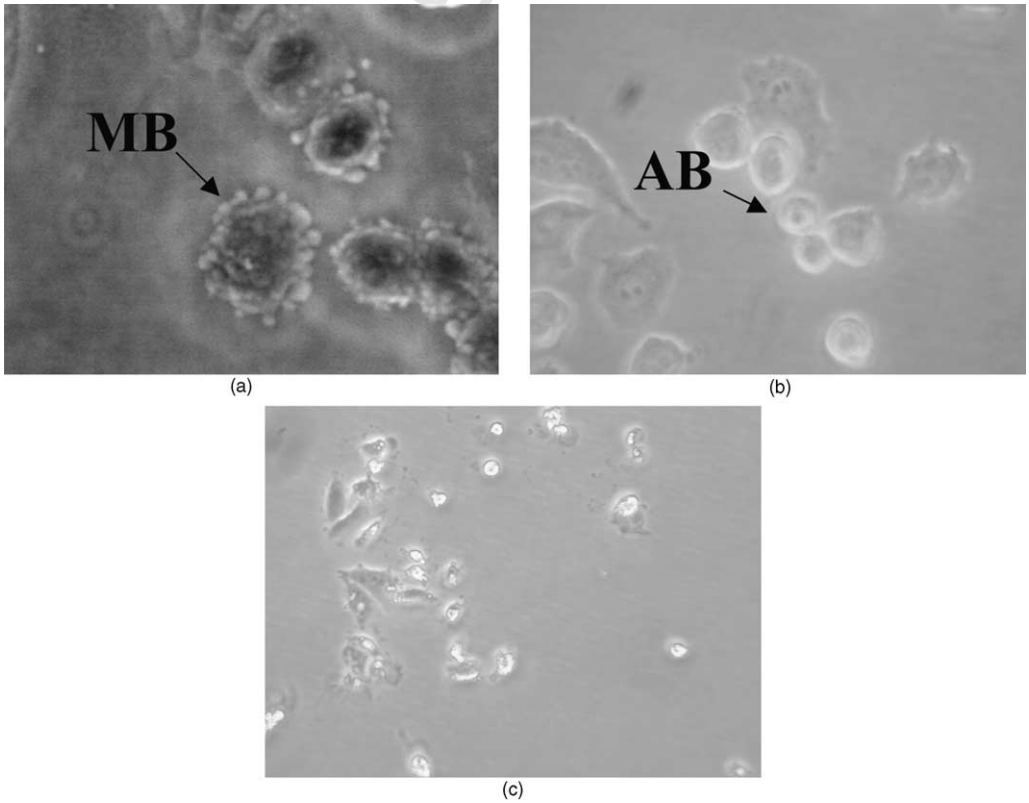

Fig. 4. Micrographs of DLKP cells cultured in the presence of $[Cu(phen)_2(mal)].2H_2O$. (a) Cells showing membrane 'blebbing' after 24 h exposure, (b) Apoptotic bodies after 48 h exposure, (c) Dead and detached cells after 96h exposure. (MB: membrane blebbing; AB: Apoptotic bodies). (Original magnification: (a) and (b) ×400, (c) ×100).

not give rise to a specific DNA fragmentation ladder pattern but extensive non-specific DNA fragmentation is visible. From this it is possible to conclude that $[Cu(phen)_2(mal)].2H_2O$ activates mammalian cell death by apoptosis and that the other drugs induce non-specific cleavage of DNA possibly due to the nuclease-like activity of the phen ligand (Jessee et al., 1982; Chen and Sigman, 1986).

Examination of cultured mammalian cells exposed to $[Cu(phen)_2(mal)].2H_2O$ for 24 h show cells undergoing membrane 'blebbing'. After 48 h a number of cells were observed to have undergone fragmentation to yield structures tentatively identified as 'apoptotic bodies' (Fig. 4). These findings and the DNA degradation pattern observed previously with this drug (Fig. 3) are consistent with the induction of cell death by apoptosis (Verhaegen, 1998). A similar finding was made by Vizcaya-Ruiz et al. (2000) who demonstrated the ability of copper based anti-cancer drugs to induce apoptosis in human ovarian carcinoma cells. In that case the induction of apoptosis was monitored by changes in cell morphology, activation of caspases and the degradation of DNA to give a ladder pattern of fragments. Copper–1,10-phenanthroline complexes have been shown to induce G1-phase specific apoptosis in a liver carcinoma cell line (Zhou et al., 2002a,b), further supporting the view that the $[Cu(phen)_2(mal)].2H_2O$ employed in this work is capable of activating cell death by apoptosis. Copper chelators have been shown to accumulate copper within thymocytes which can trigger oxidative-stress induced apoptosis (Nobel et al., 1995).

In conclusion, the metal-phen complexes examined here have a potent anti-fungal effect being capable of inhibiting growth of *C. albicans* by 95% at a concentration of 5 μg/ml (Coyle et al., 2003). Yeast and mammalian cells exposed to these complexes at a concentration of 10 μg/ml show DNA cleavage. In the case of mammalian cells $[Cu(phen)_2(mal)].2H_2O$ induces a DNA fragmentation pattern indicative of apoptosis. TEM examination of yeast cells reveals gross distortion of cellular structures and nuclear fragmentation. This work indicates that, in addition to its effect on mitochondrial function and oxygen uptake (Coyle et al., 2003), $[Cu(phen)_2(mal)].2H_2O$ also plays a role in inducing cell death by apoptosis in yeast and mammalian cells. The mitochondrion plays a central role in governing the induction of apoptosis (Green & Kroemer, 1998) and the drugs examined here, particularly $[Cu(phen)_2(mal)].2H_2O$, interfere with mitochondrial function (Coyle et al., 2003) and integrity which may be sufficient to push the cell towards apoptotic cell death. Whether or not apoptosis is a direct effect of exposure to the metal based drugs or is related to an effect on the mitochondrion induced by them (increased oxygen uptake, disruption of cytochrome synthesis (Coyle et al., 2003) is currently being investigated.

The conventional polyene and azole anti-fungal drugs target ergosterol in the fungal cell membrane or inhibit ergosterol biosynthesis, respectively. We have demonstrated that the metal–phen complexes examined here have a distinct mode of action and may represent a novel group of anti-fungal agents to be used alone or in combination with existing anti-fungal drugs. In addition, metal-based drugs may offer the possibility of over-coming the emerging problem of resistance to conventional anti-fungal drugs (Van den Bossche et al., 1998; Kontoyiannis & Lewis, 2002).

Uncited references

Brandt, 1954; McNaught and Owen, 1949 and Turian, 1951 are not cited in the text.

References

Abu-Salah, K.M., 1996. Amphotericin B: an update. British Journal of Biomedical Science 53, 122–133.

Butler, H.M., Hurse, A., Thursky, E., Shulman, A., 1969. Bactericidal action of selected phenanthroline chelates and related compounds. Australian Journal of Experimental Biological and Medical Science 47, 541–552.

Brandt, W.W., Dwyer, F.P., Gyarfas, E.C., 1954. Chelate complexes of 1,10-phenanthroline and related compounds. Chemical Reviews 54, 959–1017.

Chen, C.H., Sigman, D.S., 1986. Nuclease activity of 1,10 phenanthroline-copper: sequence specific targeting. Proceedings of the National Academy of Science 83, 7147–7151.

Cohen, J.J., 1993. Overview: mechanisms of apoptosis. Immunology Today 14, 126–130.

Cotter, T.G., Al-Rubeai, M., 1995. Cell death (apoptosis) in cell culture systems. Trends in Biotechnology 13, 150–155.

Coyle, B., Kavanagh, K., McCann, M., Devereux, M., Geraghty, M., 2003. Mode of anti-fungal activity of 1,10-phenanthroline and its Cu(II), Mn(II) and Ag(I) complexes. BioMetals. 16, 321–329.

De Pauw, B.E., 1997. Practical modalities for prevention of fungal infections in cancer patients. European Journal of Clinical Microbiology and Infectious Disease 16, 32–41.

De Vizcaya-Ruiz, A., Riverro-Muller, A., Ruiz-Ramirez, L., Kass, G.N., Kelland, L.R., Orr, R.M., Dobrota, M., 2000. Induction of apoptosis by a novel copper-based anticancer compound, Casiopenia II, in L1210 murine leukaemia and CH1 human ovarian carcinoma cell. Toxicology in vitro 14, 1–5.

Devereux, M., McCann, M., Leon, V., Geraghty, M., McKee, V., Wikaira, J., 2000a. Synthesis and fungitoxic activity of manganese(II) complexes of fumaric acid: X-ray crystal structures of $[Mn(fum)(bipy)(H_2O)]$ and $[Mn(phen)_2(H_2O)_2](fum).4H_2O$ ($fumH_2$ = fumaric acid; bipy = 2,2′-bipyridine; phen = 1,10-phenanthroline). Polyhedron 19, 1205–1211.

Devereux, M., McCann, M., Leon, V., Geraghty, M., McKee, V., Wikaira, J., 2000b. Synthesis and biological activity of manganese(II) complexes of phthalic and isophthalic acid: X-ray crystal structures of $[Mn(ph)(Phen)_2(H_2O)].4H_2O$, $[Mn(Phen)_2(H_2O)_2](Isoph)_2(Phen).12H_2O$ and $\{[Mn(Isoph)(bipy)]_4.2.75bipy\}_n$ (phH_2 = phthalic acid; Isoph = isophthalic acid; Phen = 1,10-phenanthroline; bipy = 2,2-bipyridine). Metal-Based Drugs 7, 275–288.

Dwyer, F.P., Reid, I.K., Shulman, A., Laycock, G.M., Dixon, S., 1969. The biological actions of 1,10-phenanthroline and 2,2′-bipyr-

idine hydrochlorides, quaternary salts and metal chelates and related compounds. 1. Bacteriostatic action on selected gram-positive, gram-negative and acid-fast bacteria. Australian Journal of Experimental and Biological Medical Science 47, 203–218.

Feeney, R.E., Petersen, I.M., Sahinkaya, H., 1957. "Liesegang-like" rings of growth and inhibition of bacteria in agar caused by metal ions and chelating agents. Journal of Bacteriology 73, 284–290.

Geraghty, M., McCann, M., Devereux, M., Cronin, J.F., Curran, M., McKee, V., 1999a. Synthesis and anti-*Candida* activity of cobalt(II) complexes of benzene-1,2-dioxyacetic acid ($bdoaH_2$). X-ray crystal structures of $[Co(bdoa)(H_2O)_3]3.5H_2O$ and $\{[Co(phen)_3](bdoa)_2\}_2.24H_2O$ (phen = 1,10-phenanthroline). Metal-Based Drugs 6, 41–48.

Geraghty, M., Sheridan, V., McCann, M., Devereux, M., 1999b. Synthesis and anti-*Candida* activity of copper(II) and manganese(II) carboxylate complexes: X-ray crystal structures of $[Cu(sal)(bipy)]\cdot C_2H_5OH\cdot H_2O$ and $[Cu(norb)(phen)_2]6.5H_2O\cdot(salH_2$ = salicylic acid; $norbH_2$ = *cis*-5-norbornene-*endo*-2,3-dicarboxylic acid; bipy = 2,2′-bipyridine; phen = 1,10-phenanthroline). Polyhedron 18, 2931–2939.

Geraghty, M., McCann, M., Devereux, M., McKee, V., 1999c. Synthesis and anti-*Candida* activity of cobalt(II) complexes of octanedioic acid ($odaH_2$) and nonanedioic acid ($ndaH_2$): X-ray crystal structures of $[Co(phen)_3]oda.14H_2O$ and $[Co(phen)_3]nda.11.5H_2O$ (phen = 1,10-phenanthroline). Inorganic Chimica Acta 293, 160–166.

Geraghty, M., Cronin, J.F., Devereux, M., McCann, M., 2000. Synthesis and anti-microbial activity of copper(II) and manganese(II) α,ω-dicarboxylate complexes. BioMetals 13, 1–8.

Geraghty, M., Cronin, J.F., Devereux, M., McCann, M., 1998. Activity of Copper(II) and Manganese(II) Carboxylate Complexes: X-Ray Crystal Structures of $[Cu(sal)(bipy)]\cdot C_2H_5OH\cdot H_2O$ and $[Cu(norb)(phen)_2]\cdot 6.5H_2O$ ($salH_2$ = salicylic acid; $norbH_2$ = *cis*-5-norbornene-*endo*-2,3-dicarboxylic acid; bipy = 2,2′-bipyridine; phen = 1,10-phenanthroline). Polyhedron 18, 2931–2939.

Green, D., Kroemer, G., 1998. The central executioners of apoptosis: caspases or mitochondria? Trends in Cell Biology 8, 267–271.

Jessee, B., Gargiulo, G., Razvi, F., Worcel, A., 1982. Analogous cleavage of DNA by micrococcal nuclease and a 1,10-phenanthroline-cuprous complex. Nucleic Acid Research 10, 5823–5833.

Lunel, F.M., Meis, F.G., Voss, A., 1999. Nosocomial fungal infections: Candidemia. Diagnostic Microbiology and Infectious Diseases 34, 213–220.

Kontoyiannis, D.P., Lewis, R.E., 2002. Antifungal drug resistance in pathogenic fungi. Lancet 359, 1135–1143.

Martin, A., Clynes, M., 1993. Comparison of 5 microtitre colormetric assays for in vitro cytotoxicity testing and cell proliferation assays. Cytotechnology 11, 49–58.

McCann, M., Geraghty, M., Devereux, M., O'Shea, D., Mason, J., O'Sullivan, L., 2000. Insights into the mode of action of the anti-*Candida* activity of 1,10-phenanthroline and its metal chelates. Metal-Based Drugs 7, 185–193.

MacLeod, R.A., 1952. The toxicity of o-phenanthroline for lactic acid bacteria. Journal of Biological Chemistry 197, 751–761.

McNaught, M.L. and Owen, E.C. (1949). Metals and rumen bacteria. In: Int. Congr. Biochem., Abstr. of Communs., 1st Congr., Cambridge, UK, pp. 340–341.

Nobel, C., Kimland, M., Lind, B., Orrenius, S., Slater, A.F., 1995. Dithiocarbamates induce apoptosis in thymocytes by raising the intracellular level of redox-active copper. Journal of Biological Chemistry 270, 26202–26208.

Pfaller, M.A., Jones, R.N., Messer, S.A., Edmond, M.B., Wenzel, R.P., 1998. National surveillance of nosocomial blood stream infection due to Candida albicans: frequency of occurrence and anti-fungal susceptibility in the SCOPE programme. Diagnostic Microbial Infectious Disease 31, 327–332.

Roze, L.V., Linz, J.E., 1998. Lovastatin triggers an apoptosis-like death process in the fungus Mucor recemosus. Fungal Genetics Biology 25, 119–133.

Turian, G., 1951. Tuberculostatic action of o-phenanthroline. Schweiz. Z. allgem. Pathol. U. Bakteriol 14, 338–344.

Van den Bossche, H., Dromer, F., Improvissi, I., Lozane-Chiu, M., Rex, J.H., Sanglard, D., 1998. Anti-fungal drug resistance in pathogenic fungi. Medical Mycology 36 (Supp. 1), 119–128.

Verhaegen, S., 1998. Microscopical study of cell death via apoptosis. Microscopy Analysis 1, 5–7.

Zhou, H., Zheng, C., Zou, G., Tao, D., Gong, J., 2002a. G1-phase specific apoptosis in liver carcinoma cell line induced by copper-1,10-phenanthroline. International Journal of Biochemistry and Cell Biology 34, 678–684.

Zhou, H., Liu, Y., Zheng, C., Gong, J., Liang, Y., Wang, C., Zou, G., 2002b. Microcalorimetric studies of the synergistic effects of copper-1,10-phenanthroline combined with hyperthermia on a liver hepatoma cell line Bel-7402. Thermochimica Acta 7149, 1–9.