BioMetals **16:** 321–329, 2003.

Mode of anti-fungal activity of 1,10-phenanthroline and its Cu(II), Mn(II) and Ag(I) complexes

Barry Coyle[1], Kevin Kavanagh[2], Malachy McCann[1], Michael Devereux[3] & Majella Geraghty[1]
[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*

Received 29 January 2002; accepted 2 May 2002; published on line September 2002

Key words: anti-fungal, *Candida*, fungicidal, fungistatic, metal complexes, 1,10-phenanthroline

Abstract

The mode of action of the anti-fungal compounds, 1,10-phenanthroline (phen), $[Cu(phen)_2(mal)] \cdot 2H_2O$, $[Mn(phen)_2(mal)] \cdot 2H_2O$ and $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ ($malH_2$ = malonic acid), was examined using the pathogenic yeast *Candida albicans*. The compounds have minimum inhibitory concentrations (MIC's) in the range 1.25–5.0 μg cm^{-3} and at a concentration of 10 μg cm^{-3} display some fungicidal activity. Yeast cells exposed to these drugs show a diminished ability to reduce 2,3,5-triphenyltetrazolium chloride (TTC), indicating a reduction in respiratory function. Treating exponential and stationary phase yeast cells with phen and the Cu(II) and Mn(II) complexes causes a dramatic increase in oxygen consumption. All of the drugs promote reductions in the levels of cytochromes b and c in the cells, whilst 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 whilst the Mn(II) complex induces an increase in the sterol concentration. The general conclusion is that the drugs damage mitochondrial function and uncouple respiration. That the drugs are not uniformly active suggests their bioactivity has a degree of metal-ion dependency. Phen and metal-phen complexes 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.

Introduction

Candida albicans is an opportunistic fungal pathogen capable of causing a range of superficial and systemic infections in the immuno-compromised host. The increasing incidence of fungal infections due to the advent of AIDS and immunosuppressive therapies has greatly stimulated the interest in the development of new anti-mycotic drugs for the treatment of infections caused by *C. albicans* and other *Candida* species (Pfaller *et al*. 1998; Denning *et al*. 1997; Lunel *et al*. 1999). In our laboratories we have synthesized an array of transition metal complexes and screened them for their ability to inhibit the growth of clinical isolates of *C. albicans* (Geraghty *et al.* 1999a, b, c; Devereux *et al*. 2000a, b; McCann *et al*. 2000), *Candida glabrata*, *Candida tropicalis* and *Candida krusei* (Geraghty *et al.* 2000) in RPMI medium at 37 °C. 1,10-Phenanthroline (phen) and a number of transition metal complexes incorporating this chelating ligand were found to be extremely active, *in vitro*, at 37 °C. Our recent efforts have focused on the mechanisms of the antimycotic effects and it has been shown that the potent copper(II) complex, $[Cu(phen)_2(mal)] \cdot 2H_2O$ ($malH_2$ = malonic acid), induces significant cellular oxidative stress decreased reduced:oxidized glutathione ratios (GSH:GSSG) and increased levels of lipid peroxides (McCann *et al*. 2000).

Existing therapies for systemic fungal infections rely upon the use of polyene and azole anti-fungal drugs. Resistance to these agents has been reported (Odds 1996; Klepser *et al*. 1997) and this reduces the efficacy of therapy and can ultimately lead to the death of the patient. Mechanisms that confer anti-fungal drug resistance in yeast include an increase in the expression of drug efflux pumps which remove the

drug from the cell before a toxic concentration can be reached (Wirsching *et al*. 2000; Moran *et al*. 1998), alterations in the target of the drug and variations in the ergosterol biosynthetic pathway (Odds 1996). Due to their different mode of anti-fungal activity metal-based drugs may represent a novel group of anti-fungal agents which may have potential applications either alone or in combination with existing agents and which may circumvent resistance to conventional drugs.

The present *in vitro* investigation concentrates on establishing the effects of phen and a selection of metal-phen complexes on the viability, mitochondrial function and cytochrome and ergosterol levels of *C. albicans* with a view to establishing their primary mode of action. The metal complexes chosen for the study all have the same ligands (phen and mal^{2-}) and differ only in the nature of the transition metal {Cu(II), Mn(II), Ag(I)}. This will allow conclusions to be drawn regarding how the mode of action of the complex is influenced by the nature of the metal ion. Although metal-free phen will also be administered to the cells it is assumed that this powerful chelating ligand will sequester a transition metal ion from either the medium or the cell and that any bioactivity is really attributable to the resultant metal-phen complex.

Methods and materials

Chemicals

Chemicals and growth media were obtained from commercial sources and used without further purification. $[Cu(phen)_2(mal)] \cdot 2H_2O$, $[Mn(phen)_2(mal)] \cdot 2H_2O$, $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ and $[Mn(phen)_2(CH_3CO_2)_2] \cdot 4H_2O$ were prepared as previously described (McCann *et al*. 2000).

Yeast isolate and culture conditions

C. albicans ATCC 10231 was obtained from the American Type Culture Collection (Manasas, 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 subcultured every 4-6 weeks. Cultures were grown to the stationary phase at 30 °C and 200 rpm in minimal medium (MM) (2% w/v agar, 2% w/v glucose, 0.5% w/v yeast nitrogen base (without amino acids or ammonium sulphate), 0.5% w/v ammonium sulphate). For media containing glycerol as the carbon source 2% w/v glycerol was substituted for the glucose in the above formulation. Media were sterilised by autoclaving at 121 °C and 100 kPa for 15 min. Alternatively, solutions that were susceptible to decomposition during autoclaving were sterilized by membrane filtration using 0.45 μm Millipore membrane filters.

Minimum inhibitory concentrations (MIC's)

RPMI-1640 broth medium was used for the anti-*Candida* Susceptibility testing. RPMI (5.15 g) was dissolved in cold distilled water (425 cm^3) in a 1L Duran bottle and the pH adjusted to 4.0 using a few drops of HCl (*ca.* 2 M). The resulting solution was autoclaved and then allowed to cool to approx. 50 °C. Morpholinepropanesulfonic acid (MOPS) (17.3 g) and L-glutamate (0.15 g) were dissolved together with stirring in distilled water (50 cm^3) and the resulting solution filter sterilized. The MOPS and L-glutamate solution was added to the warm RPMI and the pH of the mixture adjusted to 7.0 using sterile NaOH (6 M).

Prior to MIC testing, cells were grown on SDA at 37 °C for 24 h. Cell suspensions were prepared in sterile phosphate buffered saline (PBS, pH 7.2) and cells were counted microscopically following dilution with PBS. A microtitre plate was inoculated with cells at a density of 5×10^5 cells cm^{-3}. Solutions of the test drugs were prepared by dissolving the solid (0.02 g) in distilled water (10 cm^3) to yield a stock solution of concentration 2000 μg cm^{-3}. Doubling dilutions of these stock solutions were made to yield a series of test solutions ranging in concentrations from 20–1.25 μg cm^{-3}. The drug/cell mixtures were incubated at 37 °C for 24 h with continuous shaking and the assays were performed in triplicate. Plates were read using a Labsystems iEMS Reader MF (absorbance at $\lambda = 540$ nm) and data were statistically analysed.

Yeast cell viability

Yeast cells were grown in the presence of drug (10 μg cm^{-3}) to the late exponential phase (18–24 h) in MM at 30 °C in an orbital incubator. Through serial dilution, 2.5×10^3 cells cm^{-3} were obtained and resuspended in sterile PBS. From this culture 0.1 cm^3 was spread-plated onto SDA plates resulting in a final cell number of 250 per plate. Cells were incubated at 30 °C for 48 h and the number of colonies was ascertained and expressed as a percentage with respect to the control (100%).

Colonies were overlaid with molten agar (2% w/v agar, 0.5% w/v glucose) containing 2,3,5-

triphenyltetrazolium chloride (TTC, 0.05% w/v) and incubated at 30 °C for 2 h. Respiring colonies reduce TTC to a dark red colour while those with reduced respiratory function are unable to do this and remain white. Non-respiring (white) colonies were plated on MM containing either glucose or glycerol as the carbon source and then incubated at 30 °C for 48 h. The resulting colonies were counted and expressed as a percentage with respect to the control (100%). These colonies were overlaid once again with TTC, incubated and any colour changes noted.

Differential spectra of yeast cytochromes

Two separate experiments were conducted at 30 °C in MM in an orbital incubator using a drug concentration of 10 μg cm^{-3}. Firstly, drugs were added to yeast cells (cell density 1×10^5 cells cm^{-3}) in the lag/early exponential phase and incubated for 18–24 h. Cells were then harvested by centrifugation at 2100 $\times$ *g* for 5 min, washed three times with PBS (pH 7.2). Half of the sample (for oxidation) was re-suspended in 0.2% w/v sodium hypochlorite solution (5 cm^3). The cells were centrifuged and re-suspended in 50% v/v glycerol (10 cm^3). The other half of the sample (for reduction) was prepared by re-suspending cells in 50% v/v glycerol and adding a few crystals of sodium dithionite. The reduced-oxidised cytochrome difference spectrum (500–650 nm) was immediately measured using a Cary Win-UV Spectrophotometer. In the second series of experiments cells were grown to stationary phase (24 h) with approximate cell density 2×10^8 cells cm^{-3}. Drug was added and the mixture incubated for a further 24 h and the cells then harvested and treated as described above.

Respiratory measurements

Two separate experiments were conducted at 30 °C in MM in an orbital incubator (200 rpm) using a drug concentration of 10 μg cm^{-3}. Firstly, drugs were added to yeast cells (cell density of 1×10^5 cells cm^{-3}) in the lag/early exponential phase and incubated for 18–24 h. Cells were then harvested by centrifugation at 2100 $\times$ *g* for 5 min, washed with PBS and re-suspended in 0.05 M phosphate buffer (pH 7.2). In the second series of experiments cells were grown to stationary phase (24 h) with approximate cell density 2×10^8 cells cm^{-3}. Drug was added and the mixture incubated for a further 24 h and the cells then harvested as described above. Oxygen-uptake measurements were recorded using a Clark-type oxygen electrode at 30 °C. Cells (1×10^8) were introduced into the electrode chamber and the base respiration rate measured. Oxygen-uptake rates were calculated as μmol of O_2 consumed/30 sec /1×10^8 cells.

Ergosterol extraction and analysis

Ergosterol was extracted from yeast cells using the method of Arthington-Skaggs *et al*. 1999. Drug (concentration 10 μg cm^{-3}) was added to lag/early exponential phase cells (density 1×10^5 cells cm^{-3}) in MM and these were grown in an orbital incubator (200 rpm) for 24 h at 30 °C. The resulting cells were harvested by centrifugation, washed twice with PBS and re-suspended in a liquid comprising 20% w/w aqueous KOH and 60% v/v ethanol to give a final cell density of 1×10^9 cells cm^{-3}. The cells were placed in a water bath at 90 °C for 1 h. Heptane (3 cm^3) was added and the mixture vortexed for 2 min. The upper heptane layer containing the ergosterol was removed and dried over anhydrous Na_2SO_4. The sample was analysed using a Hewlett Packard 5890 Series II Gas Chromatograph (column: Chrompack WCOT fused silica; 25 m length; 0.25 mm diameter).

Results

MIC values for the drugs against *C. albicans* in RPMI medium at 37 °C are shown in Table 1. Metal-free phen had an MIC value in the range 1.25–2.5 μg cm^{-3} whereas the metal-phen complexes displayed slightly higher values (2.5–5.0 μg cm^{-3}). Cell viability studies carried out in MM at 30 °C (Table 2) indicate that at a concentration of 10 μg cm^{-3} all of the complexes displayed some fungicidal activity. Growth curves for the control and the drug-treated cells (using sub-MIC drug concentrations) show that treatment of *C. albicans* with the metal-phen complexes causes an extended lag phase (Figure 1). Such a phenomenon has previously been reported for *C. albicans* grown under oxygen radical stressed conditions (Hye-Jin *et al.* 1999).

In the viability experiments the colonies that developed following the growth of cells in medium supplemented with phen or metal phen complexes were overlaid with agar containing TTC in order to establish their respiratory status (Table 2). All of the colonies growing on the control plates reduced the dye to a red colour. However, after 24 h exposure to $[Mn(phen)_2(mal)] \cdot 2H_2O$ and $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ the resulting colonies produced a pink colour with TTC, indicating a diminution

Table 1. Growth of *C. albicans* in different concentrations of complexes in RPMI at 37 °C.

	Growth					
	20 μg cm^{-3}	10 μg cm^{-3}	5 μg cm^{-3}	2.5 μg cm^{-3}	1.25 μg cm^{-3}	0 μg cm^{-3}
phen	2%	3%	5%	11%	92%	100%
$[Cu(phen)_2(mal)] \cdot 2H_2O$	2%	4%	5%	78%	90%	100%
$[Mn(phen)_2(mal)] \cdot 2H_2O$	3%	5%	5%	78%	84%	100%
$[Ag_2(phen)_3(mal)] \cdot 2H_2O$	4%	4%	5%	80%	98%	100%

Table 2. Cell viability studies in MM at 30 °C and effects on TTC.

	Viability	TTC	Viability	TTC
Growth time (h)	24		48	
Control	100%	red	100%	red
phen	74%	red	69%	white
$[Cu(phen)_2(mal)] \cdot 2H_2O$	78%	red	70%	white
$[Mn(phen)_2(mal)] \cdot 2H_2O$	62%	pink	58%	white
$[Ag_2(phen)_3(mal)] \cdot 2H_2O$	80%	pink	65%	white

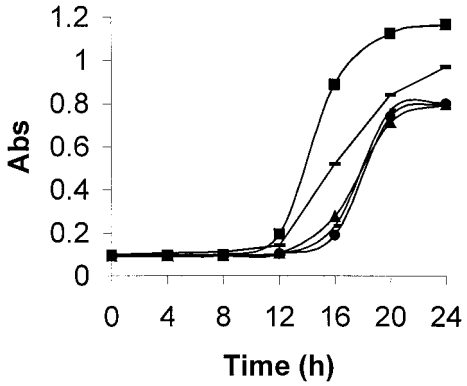

Fig. 1. Growth curves (absorbance at $\lambda = 540$ nm) for the control and the drug-treated cells RPMI over a 24 h period at 37 °C (sub-MIC drug concentrations: phen = 1.25 μg cm^{-3}; others 2.5 μg cm^{-3}). Control■, phen –, $[Cu(phen)_2(mal)] \cdot 2H_2$ ▲, $[Mn(phen)_2(mal)] \cdot 2H_2O$●, $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ –.

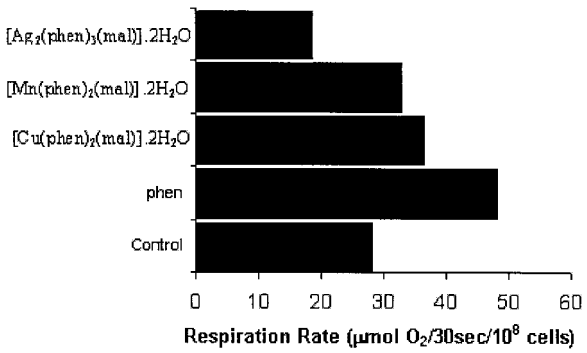

Fig. 2. Respiration rates for control and drug-treated cells in MM at 30 °C (drug concentration 10 μg cm^{-3}). Drugs were added to cells in the lag phase and the mixture incubated until they reached the late exponential growth phase. At this stage the respiration rate was recorded.

in respiratory function. After 48 h only the control colonies were capable of reducing TTC to a red colour, indicating that cells treated with either phen or a metal-phen complex experienced a significant reduction in respiratory function. Further growth tests on the controls and on colonies that were unable to reduce TTC revealed that whereas all of the colonies were able to grow on glucose as a carbon source many were incapable of growing on glycerol (Table 3). The inability to metabolise a non-fermentable carbon source such as glycerol is again a characteristic of respiratory deficient cells. These results indicate that exposure to either phen or a metal-phen complex creates a significant number of respiratory-deficient cells in the population and illustrates that these drugs interfere with mitochondrial function (Reeves *et al.* 1991).

Respiration rates

Two different oxygen-uptake experiments were carried out on the cells using drug concentrations of 10 μg cm^{-3}. Firstly, drug was added to cells in

Table 3. Viability of control and drug-treated cells at 30 °C in either glucose or glycerol as a carbon source (drug concentration 10 μg cm^{-3}).

	TTC	Viability in glucose	Viability in glycerol
Control	red	100%	93%
phen	white	100%	56%
$[Cu(phen)_2(mal)] \cdot 2H_2O$	white	100%	56%
$[Mn(phen)_2(mal)] \cdot 2H_2O$	white	100%	43%
$[Ag_2(phen)_3(mal)] \cdot 2H_2O$	white	100%	43%

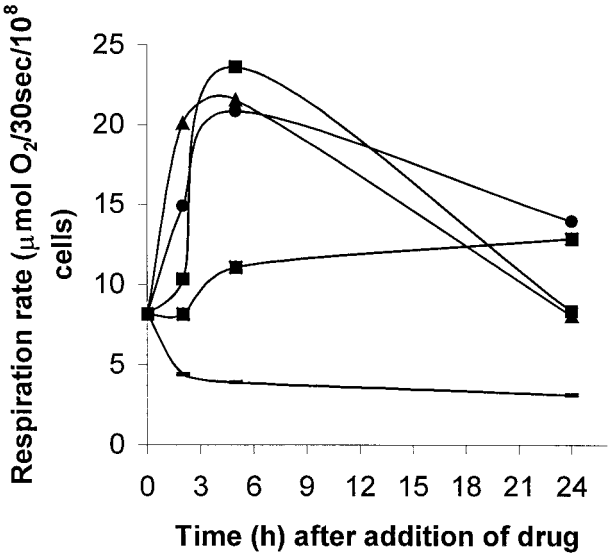

Fig. 3. Respiration rates for control and drug-treated cells in MM at 30 °C (drug concentration 10 μg cm^{-3}). Cells were grown to stationary phase and drug was then administered. Respiration rates were recorded at time intervals of 2, 5 and 24 h following the addition of drug. Control■, phen●, $[Cu(phen)_2(mal)] \cdot 2H_2O$▲, $[Mn(phen)_2(mal)] \cdot 2H_2O$■, $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ _.

the lag phase and the mixture incubated until they reached the late exponential phase, after which time the respiration rate was recorded. Cells treated with $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ at the lag phase had a lower oxygen-uptake rate than the control (Figure 2). In contrast, cells exposed to phen, $[Cu(phen)_2(mal)] \cdot 2H_2O$ and $[Mn(phen)_2(mal)] \cdot 2H_2O$ all showed higher uptake rates than the control, with phen producing the largest increase.

In the second series of experiments cells were grown to stationary phase and then drug was administered. Oxygen-uptake measurements were made at time intervals of 2, 5 and 24 h following the addition of drug (Figure 3). In comparison to the control again it was only the Ag(I) complex which prompted a reduction in oxygen-uptake over the 24 h period. All of the other drugs caused an increase in gas uptake over the first 5 h after administration compared to the control cells. However, whereas the control showed a slight increase in the uptake rate over the next 5–24 h time period all of the drug-treated systems had a net decrease in respiration rate over that period. Thus, with the exception of $[Ag_2(phen)_3(mal)] \cdot 2H_2O$, addition of drug to cells which are either in the lag/exponential or the stationary growth phase enhances oxygen-uptake by the cells.

Cytochrome analysis

The interaction of the complexes with cytochromes aa_3, b and c was monitored by differential absorption spectroscopy and results obtained using the lag/exponential phase *C. albicans* cultures are shown in (Figure 4). Whilst cells grown in the presence of the Cu(II) and Mn(II) complexes did show reduced cytochrome levels, particularly cytochrome aa_3, the effect on the actively respiring cells was most pronounced with the Ag(I) complex and metal-free phen. These latter drugs appear to have almost completely obliterated the cytochromes over the 24 h growth cycle. In contrast, when cells that have been allowed to grow for 24 h to stationary phase in a drug-free environment were subsequently exposed to drug for a period of 24 h no alteration in cytochrome profile occurred.

Ergosterol content

The relative ergosterol content of drug-treated and control cells was determined (Figure 5). *C. albicans* cells exposed to phen, $[Cu(phen)_2(mal)] \cdot 2H_2O$ and $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ showed diminished levels of ergosterol, with the Cu(II) complex inflicting the most deleterious effect. As sterol synthesis is dependent upon a fully functional cel-

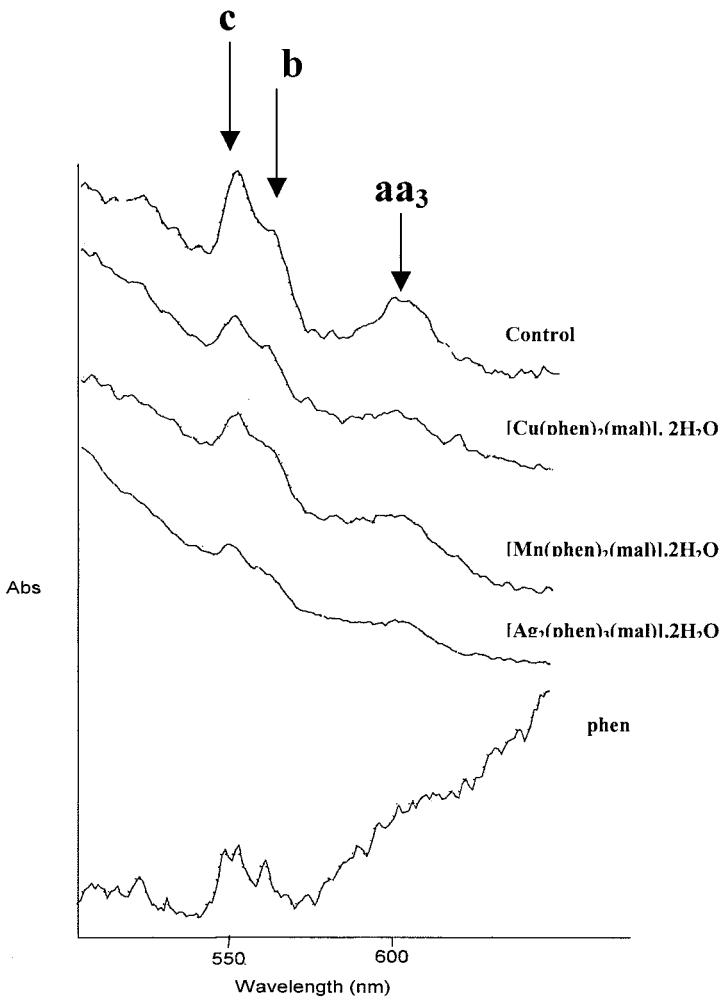

Fig. 4. Differential absorption spectra arising from the interaction of phen and the metal-phen complexes with cytochromes aa3 (λ_{max} 600–604 nm), b (λ_{max} 562–566 nm) and c (λ_{max} 550–554 nm). Drugs were added to lag/exponential phase cultures of *C. albicans*.

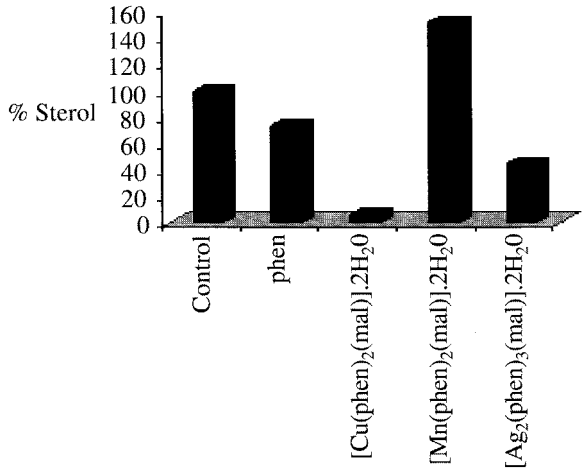

Fig. 5. Relative ergosterol contents of drug-treated and control cells (100%).

lular respiratory system the decrease in ergosterol levels observed with these drugs indicate a disabled mitochondria. In contrast, cells treated with $[Mn(phen)_2(mal)] \cdot 2H_2O$ showed an increase of $>50\%$ in ergosterol content compared to the control. Furthermore, tests carried out using the related Mn(II) complex, $[Mn(phen)_2(CH_3CO_2)_2] \cdot 4H_2O$, showed that this drug also prompted a similar increase in the level of ergosterol (57% increase).

Discussion

In RPMI at 37 °C metal-free phen, $[Cu(phen)_2(mal)] \cdot 2H_2O$, $[Mn(phen)_2(mal)] \cdot 2H_2O$ and $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ have a fungistatic effect on *C. albicans*

(ATCC 10231) and MIC values were in the range 2.5–5 μg cm^{-3}. Introducing phen and metal-phen drugs at a concentration of 10 μg cm^{-3} into lag phase cultures in MM at 30 °C slightly reduced cell viability. In addition, the resulting drug-treated cells were respiratory deficient (incapable of reducing TTC), indicating damage to the mitochondria. In many cases, these respiratory deficient cells were also unable to grow on glycerol as a carbon source, again demonstrating a loss of mitochondrial function. Previous kinetic studies (McCann *et al*. 2000), in which a clinical isolate of *C. albicans* was exposed to $[Mn(phen)_2(mal)] \cdot 2H_2O$, showed that the cells have to be metabolically active before the administered metal complex has an inhibitory effect. It has already been established (Geraghty *et al*. 2000) that, in general, simple salts such as $MnCl_2 \cdot 4H_2O$ and $CuCl_2 \cdot 2H_2O$, have limited activity against clinical isolates of *C. albicans*, indicating the importance of the bidentate phen ligand in promoting the anti-fungal effect of the metals.

An examination of the oxygen-uptake capabilities of cells cultured in the presence of the drugs revealed that, with the exception of the Ag(I) complex, there was a significant increase in gas uptake. This result indicates that although cells treated with phen and the Cu(II) and Mn(II) complexes are incapable of reducing TTC (respiratory deficient) and are unable to grow on glycerol they still display a marked ability to absorb oxygen. This apparent contradiction may be explained by the fact that cells are probably using the oxygen for a process other than respiration (e.g. generation of free radicals). Indeed, experiments using a clinical strain of *C. albicans* treated with $[Cu(phen)_2(mal)] \cdot 2H_2O$ (in RPMI at 37 °C) revealed that the complex caused significant cellular oxidative stress (decreased ratio of GSH:GSSG) and was also responsible for increasing significantly the level of lipid peroxides in the cells (McCann *et al*. 2000). Furthermore, it was found (Panimon *et al.* 1941) that whereas brain, kidney, liver and muscle brei oxygen consumption is accelerated by phen and its Fe(II) complex, $[Fe(phen)_3]^{2+}$, the related Co(II)-, Ni(II)- and Cu(II)-phen complexes produced no rate increase. No CO_2 was formed during the oxygen-uptake in the presence of phen and $[Fe(phen)_3]^{2+}$ and it was thought that the compounds were acting by catalysing the oxidation of either phospholipids or proteins. Other workers (Moussa *et al.* 1990) have also reported enhanced respiration when *C. albicans* is grown in the presence of either cisplatin or palladium complexes and with the effect being attributed to uncoupling of oxidative phosphorylation. These various findings support the claim that the extra oxygen taken up by the cells in the present experiments is not being utilized for normal respiration but is being used to induce oxidative stress in the organism. Evidently, the yeasts own antioxidant enzymes (e.g., the copper-zinc and manganese superoxide dismutases and the catalases) are being overwhelmed by the drug and are unable to offer any significant protection to the organism (Hye-Jin *et al.* 1999).

Cytochromes play a central role in cellular respiration, being integral parts of the electron transport chain of oxidative phosphorylation. Certain cytochromes (e.g., b and aa_3) are composed of mitochondrial and nuclear encoded material, so disruption of mitochondrial function results in a loss of cytochromes from the respiratory chain and this can be visualized in a differential spectrum (Whittaker & Danks 1978). The data presented here indicate that the three metal-phen complexes disrupt the synthesis of cytochromes b and c in the yeast cells. In addition, the Ag(I) complex also reduces the amount of cytochrome aa_3 produced in the cell. With uncomplexed phen cells exhibit lower levels of b and c. However, with the increased absorption beyond ca. 570 nm the effect on cytochrome aa_3 is not discernable.

The present complexes appear to be effective only against actively respiring cells, suggesting that they interfere with cytochrome synthesis but do not interact to any great extent with fully intact cytochromes. In the case of administering metal-free phen to cells in the lag/exponential phase it is likely that the powerfully chelating phen ligand is sequestering iron cations from the reservoir of uncomplexed cellular iron and thus making it unavailable for cytochrome synthesis. The reduced activity of $[Cu(phen)_2(mal)] \cdot 2H_2O$ and $[Mn(phen)_2(mal)] \cdot 2H_2O$, compared to phen itself, can be attributed to the chemical structures of the metal complexes. The X-ray crystal structures of closely related Cu(II) (McCann *et al*. 1995) and Mn(II) (Casey *et al*. 1995) dicarboxylate/phen complexes shows the phen ligands to be strongly chelated to the metal centers. As such, the equilibrium concentration of uncomplexed phen arising from the incorporation of the Cu(II) and Mn(II) complexes inside cells is expected to be relatively low, thus minimizing the extent of complexation of iron which was destined for cytochrome synthesis. Although the exact chemical structure of $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ is not known the more pronounced impact of this Ag(I) complex on cytochrome formation is most likely due to the weaker interaction of the phen ligand with the Ag(I) centre.

This would ensure a higher equilibrium concentration of the metal-free phen and which, subsequently, could be utilized for coordination to cellular iron.

Ergosterol is an essential component of the fungal cell membrane and provides stability and rigidity. This sterol is the target of the anti-fungal agent amphotericin B, which acts by binding ergosterol and forming a pore or aperture through which leakage of cell components can occur (Abu Salah 1996). In addition, the azole class of anti-fungal drugs function by targeting ergosterol biosynthesis thus leading to a weakened membrane and the synthesis of toxic intermediates which contribute to cell death (Klepser *et al*. 1997). The present drugs, with the exception of $[Mn(phen)_2(mal)] \cdot 2H_2O$, all reduce the levels of cellular ergosterol. As the biosynthesis of ergosterol is controlled by NADPH, which itself is generated in the mitochondria (Daum *et al*. 1998), it can be concluded that the increased oxygen-uptake seen with the present drugs is not leading to the production of more NADPH but more probably to the generation of toxic free radicals. The fact that the two Mn(II) complexes, $[Mn(phen)_2(mal)] \cdot 2H_2O$ and $[Mn(phen)_2(CH_3CO_2)_2] \cdot 4H_2O$, promote increased levels of ergosterol in the cell again suggests that the mode of action of the complexes depends upon the particular metal ion used {Mn(II), Cu(II) or Ag(I)}.

Conclusions

When tested against *C. albicans* phen, $[Cu(phen)_2(mal)] \cdot 2H_2O$, $[Mn(phen)_2(mal)] \cdot 2H_2O$ and $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ are strongly fungistatic at 1.25–5.0 μg cm^{-3} in RPMI and show some fungicidal activity in MM at 10 μg cm^{-3}. Under these conditions the drugs damage mitochondrial function and uncoupled respiration. With the exception of the Ag(I) complex, all of the drugs increase oxygen-uptake by the cells which is likely diverted to the formation of damaging oxygen free radicals. All of the drugs appear to inhibit cytochrome biosynthesis and, with the exception of $[Mn(phen)_2(mal)] \cdot 2H_2O$, also lead to a decrease in cellular ergosterol content.

Thus, phen and its Cu(II), Mn(II) and Ag(I) complexes represent a novel set of anti-fungal drugs with a mode of action quite different to that of the polyene and azole prescription drugs. That the drugs are not uniformly active indicates a degree of metal-ion dependency on their mode of action.

Acknowledgements

Barry Coyle acknowledges support from Enterprise Ireland.

References

Abu Salah KM. 1996 Amphotericin B: an update. *Brit J Biomed Sci* **53**, 122–133.

Arthington-Skaggs BA, Jradi H, Desai T, Morrison CJ. 1999 Quantitation of ergosterol content: novel method for determination of fluconazole susceptibility of *Candida albicans*. *J Clin Microbiol* **37**, 3332–3337.

Casey MT, McCann M, Devereux M, Curran M, McKee V. 1995 Synthesis and catalase activity of manganese(II) complexes of *cis*-5-norbornene-*endo*-2,3-dicarboxylic acid ($ndaH_2$): X-ray crystal structure of $[Mn(nda)(phen)_2] \cdot EtOH \cdot H_2O$ (phen = 1,10-phenanthroline). *Polyhedron* **14**, 2247–2253.

Daum G, Lees ND, Bard M, Dickson R. 1998 Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*. *Yeast* **14**, 1471–1510.

Dening DW, Evans EG, Kibbler CC. *et al*. 1997 Guidelines for the investigation of invasive fungal infections in haematological malignancy and solid organ transplantation. *Eur J Clin Microbiol Infect Dis* **16**, 424–436.

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 $[Mn(phen)_2(H_2O)_2](fum) \cdot 4H_2O$ ($fumH_2$ = 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 $[Mn(ph)(Phen)_2(H_2O)] \cdot 4H_2O$, $[Mn(Phen)_2(H_2O)_2]_2(Isoph)_2(Phen) \cdot 12H_2O$ and $[Mn(Isoph)(bipy)]_4 \cdot 2.75bipy\}_n$ (phH_2 = phthalic acid; Isoph = isophthalic acid; Phen = 1,10-phenanthroline; bipy = 2,2-bipyridine). *Metal-Based Drugs* **7**, 275–288.

Geraghty M, McCann M, Devereux M, Cronin JF, 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 $\{[Co(phen)_3](bdoa)_2\}_2 \cdot 24H_2O$ (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 $[Cu(norb)(phen)_2] \cdot 6.5H_2O$ ($salH_2$ = salicylic acid; $norbH_2$ = *cis*-5-norbornene-*endo*-2,3-dicarboxylic acid; bipy = 2,2′-bipyridine; phen = 1,10-phenanthroline). *Polyhedron* **18**, 2931–2939.

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 $[Co(phen)_3]oda \cdot 14H_2O$ and $[Co(phen)_3]nda \cdot 11.5H_2O$ (phen = 1,10-phenanthroline). *Inorg Chim Acta* **293**, 160–166.

Geraghty M, Cronim JF, Devereux M, McCann M. 2000 Synthesis and antimicrobial activity of copper(II) and manganese(II) α,ω-dicarboxylate complexes. *BioMetals* **13**, 1–8.

Hye-Jin K, Byoung-Kuk N, Moon-Bo K. *et al*. 1999 Protective effects of antioxidant enzymes of *Candida albicans* against oxidative killing by macrophages. *J Microbiol* 117–122.

Klepser ME, Ernst EJ, Pfaller MA. 1997 Update on anti-fungal resistance. *Trends Microbiol* **5**, 372–375.

Lunel FMV, Meis J, Voss A. 1999 Nosocomial fungal infections. Candidemia. *Diagn Microbiol Infect Dis* **34**, 213–220.

McCann M, Cronin FJ, Devereux M. *et al.* 1995 Copper(II) complexes of heptanedioic acid (hdaH2) and octanedioic acid ($odaH_2$): X-ray crystal structures of $[Cu(hda)(phen)_2] \cdot 11.73H_2O$ and $[Cu(oda)(phen)_2] \cdot 12H_2)$ (phen = 1,10-phenanthroline). *Polyhedron* **14**, 2379–2387.

McCann M, Geraghty M, Devereux M. *et al.* 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.

Moran GP, Sanglard D, Donnelly SM, Shanley D, Sullivan DJ, Colemen DC. 1998 Identification and expression of multi-drug transporters responsible for fluconazole resistance in *Candida dubliniensis. Antimicrob Agent Chemother* **42**, 1819–1830.

Moussa NM, Ghannoum MA, Whittaker PA, El-Ezaby MS, Quraman S. 1990 Effects of cisplatin and two novel palladium complexes on *Candida albicans*, *Microbios* **62**, 165–178.

Odds FC. 1996 Resistance of clinically important yeasts to antifungal agents. *Int J Antimicrob Agent* **6**, 145–147.

Panimon F, Horwitt MK, Gerard RW. 1941 Orthophenanthroline as accelerator of brain tissue oxygen consumption. *J Cell Comp Physiol* **17**, 17–29.

Pfaller MA, Jones RN, Messer SA, Edmond MB, Wenzel RP. 1998 National surveillance of nosocomial blood stream infection due to *Candida albicans*: frequency of occurrence and anti-fungal susceptibility in the SCOPE program. *Diagn Microbiol Infect Dis* **31**, 327–332.

Reeves E, Kavanagh K, Whitaker PA. 1991 Cupferron – a novel petite inducing agent in yeast. *Biomed Letts* **46**, 253–257.

Whittaker PA and Danks SM. 1978 *Mitochondria: Structure, Function and Assembly.* Integrated Themes in Biology. London: Longman.

Wirsching S, Michel S, Kohler G, Morschhauser J. 2000 Activation of the multiple drug resistance gene MDR1 in fluconazole resistant clinical *Candida albicans* strains is caused by mutations in a *trans*-regulatory factor. *J Bacteriol* **182**, 400–404.