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Mechanism of action of coumarin and silver(I)–coumarin complexes against the pathogenic yeast *Candida albicans*

Bhumika Thati [a,b], Andy Noble [b], Raymond Rowan [a,c], Bernadette S. Creaven [b], Maureen Walsh [b], Malachy McCann [c], Denise Egan [b], Kevin Kavanagh [a,*]

[a] *Medical Mycology Unit, National Institute for Cellular Biotechnology, Department of Biology, National University of Ireland Maynooth, Co. Kildare, Ireland*
[b] *Pharma R&D Team, Institute of Technology Tallaght, Dublin 24, Ireland*
[c] *Department of Chemistry, National University of Ireland Maynooth, Co. Kildare, Ireland*

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Abstract

The anti-fungal activity and mode of action of a range of silver(I)–coumarin complexes was examined. The most potent silver(I)–coumarin complexes, namely 7-hydroxycoumarin-3-carboxylatosilver(I), 6-hydroxycoumarin-3-carboxylatosilver(I) and 4-oxy-3-nitrocoumarinbis(1,10-phenanthroline)silver(I), had MIC_{80} values of between 69.1 and 4.6 μM against the pathogenic yeast *Candida albicans*. These compounds also reduced respiration, lowered the ergosterol content of cells and increased the trans-membrane leakage of amino acids. A number of the complexes disrupted cytochrome synthesis in the cell and induced the appearance of morphological features consistent with cell death by apoptosis. These compounds appear to act by disrupting the synthesis of cytochromes which directly affects the cell's ability to respire. A reduction in respiration leads to a depletion in ergosterol biosynthesis and a consequent disruption of the integrity of the cell membrane. Disruption of cytochrome biosynthesis may induce the onset of apoptosis which has been shown previously to be triggered by alteration in the location of cytochrome *c*. Silver(I)–coumarin complexes demonstrate good anti-fungal activity and manifest a mode of action distinct to that of the conventional azole and polyene drugs thus raising the possibility of their use when resistance to conventional drug has emerged or in combination with such drugs.

Keywords: Anti-fungal; *Candida albicans*; Coumarin; Phenanthroline; Silver(I); Yeast

1. Introduction

Coumarin is a a benzopyrone and a naturally occurring constituent of many plants and essential oils, including tonka beans, sweet clover, woodruff, oil of cassia and lavender. Antibiotics containing the coumarin nucleus, such as novobiocin, clorobiocin, and coumermycin A_1 produced by a number of the *Streptomyces* species, were identified over forty years ago. The use of these antibiotics has been limited due to their poor water solubility, low activity against Gram-negative bacteria and the rapid emergence of resistance (Lewis et al., 1996; Laurin et al., 1999). However, renewed interest in these antibiotics has arisen following the discovery that they are potent catalytic inhibitors of DNA gyrase. Additionally, these antibiotics have been shown to be active against Gram-positive bacteria, especially against methicillin-resistant *Staphylococcus aureus* (MRSA) (Laurin et al., 1999). Further derivatisation of novobiocin, clorobiocin, and coumermycin A_1 has allowed for the production of novel coumarin antibiotics displaying excellent inhibition of DNA supercoiling by DNA gyrase B and good antibacterial activity against vancomycin, teicoplanin and novobiocin resistant *Enterococci* species (Laurin et al., 1999).

* Corresponding author. Tel.: +353 1 708 3859; fax: +353 1 708 3845.
E-mail address: kevin.kavanagh@NUIM.ie (K. Kavanagh).

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Candida albicans is pathogenic yeast which is consistently the most frequently isolated etiological agent of candidosis in humans (Coleman et al., 1998). Candidosis is the commonest invasive fungal infection in patients with malignant haematological disease and in bone marrow transplant recipients (Warnock, 1998). Nosocomial infections due to opportunistic fungal pathogens are a common cause of mortality among hospitalised patients (Micheal, 1995). The development of azole-based anti-fungal drugs has revolutionized the treatment of many fungal infections, but therapy may still necessitate application of the highly toxic drug amphotericin B or a combination of drugs.

Plant extracts containing coumarin derivatives demonstrate anti-fungal activity (Tiew et al., 2003) and some synthetic coumarin derivatives are also active against the yeast *C. albicans* (Zaha and Hazem, 2002). The presence of phenolic, hydroxy and carboxylic acid groups on the coumarin nucleus has been considered necessary for antimicrobial activity (Kawase et al., 2001). Coumarin derivatives are able to coordinate a transition metal ion via the oxygen of the carbonyl group on the lactone ring (Irena et al., 2001) which raises the possibility that coordinating metals to coumarin may potentiate its anti-microbial toxicity.

The aim of the work presented here was to investigate the anti-fungal activity of some coumarin derivatives and also the silver(I) complexes of these derivatives.

2. Materials and methods

2.1. Culture conditions

C. albicans ATCC 10231 (obtained from the American Type Culture Collection, Maryland, USA) was maintained on YEPD agar [2% (w/v) glucose (Sigma–Aldrich Chemical Co Ltd. Dublin, Ireland), 2% (w/v) bactopeptone (Difco Laboratories, Detroit, USA), 1% (w/v) yeast extract (Oxoid Ltd., Basingstoke, England), 2% (w/v) agar] plates, sub-cultured every 6–8 weeks and stored at 4 °C. Fresh cultures were grown at 30 °C in YEPD broth (as above but without agar). All cultures were grown to the stationary phase (approximately 1×10^8 cells/ml) overnight in 50 ml Antibiotic Medium 3 (Oxoid Ltd.) broth at 30 °C and 200 rpm.

2.2. Synthesis of coumarin derivatives

Coumarin-3-carboxylic acid (CcaH), 4-hydroxy-3-nitrocoumarin (hncH) and 7-hydroxy-4-methyl-8-nitrocoumarin (hmnc), were purchased from Sigma–Aldrich Co. (Dorset, UK) and used without further purification. The synthesis of the ligands, 6-hydroxycoumarin-3-carboxylic acid (6-OHCcaH), 7-hydroxycoumarin-3-carboxylic acid (7-OHCcaH), 8-hydroxycoumarin-3-carboxylic acid (8-OHCcaH), and the silver(I) complexes, coumarin-3-carboxylatosilver(I) [Ag(Cca)], 6-hydroxycoumarin-3-carboxylatosilver(I) [Ag(6-OHCca)], 7-hydroxycoumarin-3-carboxylatosilver(I) [Ag(7-OHCca)], and 8-hydroxycoumarin-3-carboxylatosilver(I) [Ag(8-OHCca)], have been described previously (Creaven et al., 2006). The synthesis of the silver(I) complexes of hncH and hmncH, namely 4-oxy-3-nitrocoumarinsilver(I) [Ag(hnc)] and 4-methyl-8-nitro-7-oxycoumain silver(I) [Ag(hmnc)] togetehr with the 1,10-phenanthroline (phen) complex, 4-oxy-3-nitrocoumarinbis(1,10-phenanthroline) silver(I) [Ag(phen)$_2$(hnc)] have been described (Creaven et al., 2005). The crystal structure of [Ag(phen)$_2$(hnc)] is displayed in Fig. 1. The structure of all other complexes and ligands are displayed in (Creaven et al., 2005 and 2006).

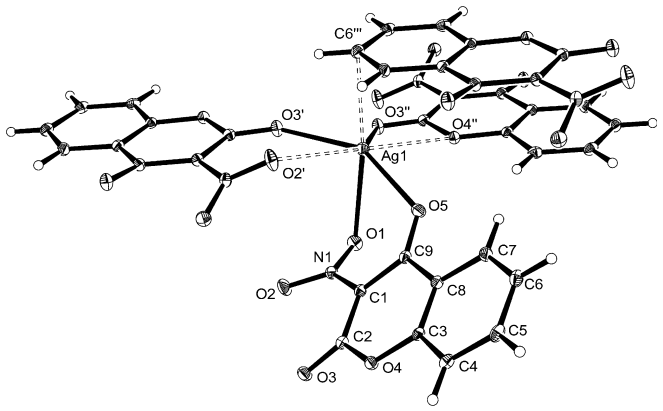

Fig. 1. Crystal structure for [Ag(phen)$_2$(hnc)].

2.3. Anti-fungal susceptibility test

Yeast cells were grown overnight to the stationary phase in 50 ml Antibiotic Medium 3 (Oxoid) at 30 °C and 200 rpm. Cell density was determined using an haemocytometer and adjusted to 1×10^6 cells/ml, by dilution with Antibiotic Medium 3. Cells were added to each well of a 96-well plate containing the test compound, diluted in medium using serial dilutions from 100 to 0.25 μg/ml. Plates were incubated at 30 °C for 24 h, and the optical density (OD) was determined at 450 nm using a MRX spectrophotometer (Dynex Technology). MIC_{80} was determined to be the lowest concentration of drug required to reduce cell growth by 80% relative to the control.

2.4. Measurement of oxygen uptake

Stationary phase cells (approximately 1.5×10^8 cells/ml) grown in YEPD broth at 30 °C overnight were harvested, washed with 0.025 M phosphate buffered saline (PBS, pH 7.2) and resuspended in 0.025 M phosphate buffer (pH 7.2) at a cell density of 5×10^8/ml. Oxygen uptake measurements were made at 30 °C using a Clark-type oxygen electrode. Oxygen uptake rates were calculated as μmoles of oxygen consumed/60 s/10^8 cells.

2.5. Cytochrome analysis

Yeast cultures were supplemented with specific concentrations of each drug and incubated for 18–24 h at 30 °C. Cells (2×10^{10}) were harvested by centrifugation at 3000*g* for 5 min, washed twice with PBS and divided into two

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equal volumes. One half of the sample was oxidised by suspending in 0.2% (w/v) sodium hypochlorite solution followed by harvesting by centrifugation and then resuspended in 50% (v/v) glycerol. The remaining half sample was resuspended in 50% (v/v) glycerol and reduced by adding a few crystals of sodium-dithionate. The reduced and oxidised cytochrome differential spectrum was immediately recorded using a double beam UV–Visible spectrophotometer at 500–650 nm (Cary IE Varian).

2.6. Sterol extraction and quantification

Yeast cells were grown in the presence of half MIC_{80} of test agent until cells reached the late exponential phase (approximately 1×10^7/ml, and typically for 18–24 h) in Antibiotic Medium 3 at 30 °C in an orbital incubator. Yeast cells (2×10^9 cells) were harvested and washed with PBS. Cells were resuspended in 1.5 ml of a solution containing 20% (w/v) potassium hydroxide and 60% (v/v) ethanol and placed in a shaking water bath at 90 °C for 1.5–2 h. Heptane was added to this solution and vortexed for 10 s. The upper layer containing sterols was removed according to the method of Arthington-Skaggs et al. (2000).

Sterol analysis was determined by using a double beam UV–Visible spectrophotometer (Cary IE Varian) over the range 240–320 nm. An ergosterol standard curve was constructed over the range 100–0.25 μg/ml. Sterol concentrations were also determined using a gas chromatographic system (Hewlett Packard 5890, Series 11) with a flame ionisation detector and a Chromopack capillary column (Chromopack International BV, Middleburg, The Netherlands) operated isothermally at 300 °C. Injector and detector temperatures were 320 °C and the carrier gas was N_2.

2.7. Evaluation of membrane leakage

Stationary phase cells (1×10^{10} in total) were harvested by centrifugation, washed with PBS and resuspended in 10 ml PBS. Test agents were added to this suspension at a final concentration of half MIC_{80}. Samples of cell suspension were recovered after 4 h and assayed for the presence of amino acids using the ninhydrin method (Reeves et al., 2004). The filtrate was passed through a 0.45 μM syringe filter (Sartorius, AG Goettingen, Germany) and free amino acid was measured as described. The amino acid concentration was expressed in terms of aspartic acid and glutamic acid which were used as standards. Ninhydrin (Sigma Aldrich) solution (200 μl of 0.35 g/100 ml ethanol) was added to each sample (1 ml) and the mixture heated to 95 °C for 4 min. After cooling to room temperature in an ice bath, the OD at 570 nm was recorded on a UV–Visible spectrophotometer (Beckmann, DU 640).

3. Electron microscopy

Yeast cells were grown to the stationary phase in the presence of half-MIC_{80} levels of each test agent. Primary fixation of yeast cells was carried out in a 3% (v/v) solution of glutaraldehyde in 0.1 M phosphate buffer for 2 h. Secondary fixation was achieved in 0.2% (w/v) osmium tetroxide in 0.1 M phosphate buffer for 1 h. Samples were dehydrated in graded alcohol solutions of 10, 30, 50, 75, 95, and 100% (v/v) for 15 min. Samples were embedded in Agar 100 resin (Agar Scientific Ltd., UK) and viewed with a Hitachi H-7000 transmission electron microscope operating at 100 kV accelerating voltage.

4. Extraction of DNA from *C. albicans*

Yeast cells were grown in the presence of test agent at a concentration equivalent to half MIC_{80}, in Antibiotic Medium 3 at 30 °C and 200 rpm, using an orbital shaker. DNA was extracted from cells (4×10^9) as described earlier (Coyle et al., 2004). The integrity of extracted DNA was determined by agarose gel electrophoresis as described (Coyle et al., 2004). Samples were loaded onto an agarose gel and electrophoresed at 80 V for 1 h. DNA bands were visualised by irradiation at 300 nm and photographed using a Pharmaciae 3D imaging system.

5. Statistical analysis

All experiments were performed on three independent occasions and results are the mean ± SEM. Statistical analysis was performed using the non-parametric Mann–Whitney test at a 95% confidence interval.

6. Results

6.1. Effects of coumarin derivatives on fungal cells

A series of novel coumarin ligands and their silver(I) complexes (Table 1) were screened for their anti-fungal activity. The MIC_{80} of each compounds was determined over a concentration range of 500–0.25 μM. Results indicate that both the number and position of functional groups along with the presence of silver on the coumarin

Table 1
Anti-fungal activity of coumarin derivatives

Compound no.	Compounds screened	MIC_{80} (μM) ± SEM
1	CcaH	332.00 ± 1.75
2	[Ag(Cca)]	163.40 ± 2.44
3	[Ag(8-OHCca)]	270.00 ± 1.46
4	[Ag(7-OHCca)]	69.30 ± 0.95
5	[Ag(6-OH-Cca)]	34.10 ± 0.58
6	[Ag(hmnc)]	246.00 ± 2.24
7	[Ag(hnc)]	222.00 ± 1.82
8	$[Ag(phen)_2(hnc)]$	4.60 ± 0.75

Anti-fungal activity of coumarin derivatives screened against *C. albicans* following continuous incubation in the presence of drug for 24 h, using micro-dilution assay.
Results are mean of three independent experiments ± SEM. All values were statistically different when compared to the control (compound 1) at $p < 0.05$.

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nucleus, greatly affected the fungistatic capacity of the coumarin derivatives. This is evident from the MIC_{80} values presented in Table 1 and include: [Ag(8-OHCca)] (270 μM), [Ag(hmnc)] (246 μM), [Ag(hnc)] (222 μM), [Ag(Cca)] (163 μM), [Ag(7-OHCca)] (69.30 μM) and [Ag(6-OHCca)] (34.10 μM). Phenanthroline has previously been shown to be a potent anti-fungal agent against *C. albicans* (Coyle et al., 2003). In the this study, the 1,10-phenanthroline was combined with coumarin–silver complex to yield $[Ag(phen)_2(hnc)]$. This compound was the most potent anti-fungal agent screened, with an MIC_{80} of 4.60 μM (Table 1).

Aromatic nitration and hydroxylation of coumarin lead to the production of derivatives with improved anti-fungal activity. This is particularly true in the case of [Ag(6-OHCca)], which had an MIC_{80} value of 34.1 μM. Therefore, oxygenation particularly at the sixth position with a carboxylic group at third position leads to the production of the most active silver–coumarin derivative. However, when this coumarin derivative was complexed with phenanthroline to form $[Ag(phen)_2(hnc)]$, the MIC_{80} value was seen to decrease further from a value of 332 μM to 4.6 μM (Table 1).

6.2. Effect of coumarin derivatives on cellular respiration

Previous studies have shown that fungal respiration is affected when cells are exposed to metal-based drugs (Coyle et al., 2003; Eshwika et al., 2004; McCann et al., 2004). Therefore, the respiration rate of cells exposed to each of the different coumarin–silver complexes was determined. The results presented in Table 2 clearly demonstrate that cells pre-grown in the presence of coumarin-3-carboxylic acid and the various silver(I)–coumarin complexes show reduced consumption of oxygen. Those coumarin derivatives which caused the greatest decrease in oxygen consumption were [Ag(Cca)] (48.24 μmol), [Ag(6-OHCca)] (47.61 μmol oxygen/10^8 cells) and $[Ag(phen)_2(hnc)]$ (42.47 μmol oxygen/10^8 cells). These results clearly indicate that cells pre-grown in the presence of the coumarin derivatives, demonstrated a 20–50% reduction in oxygen consumption, relative to the control (Table 2). Additionally, [Ag(6-OHCca)] and $[Ag(phen)_2(hnc)]$ were previously shown to be the most active anti-fungal agents (Table 1), and the results presented here indicate that they have a significant impact on cellular respiration.

Table 2
Oxygen consumption by *C. albicans* cells exposed to complexes

Compound no.	Compounds screened	Consumption of oxygen (μmol) ± SEM
1	CcaH	75.45 ± 2.57
2	[Ag(Cca)]	48.24 ± 1.46
3	[Ag(8-OHCca)]	62.94 ± 2.94
4	[Ag(7-OHCca)]	56.02 ± 2.86
5	[Ag(6-OH-Cca)]	47.61 ± 0.85
6	[Ag(hmnc)]	68.32 ± 1.86
7	[Ag(hnc)]	72.59 ± 1.24
8	$[Ag(phen)_2(hnc)]$	42.47 ± 1.78
	Control	82.38 ± 2.21

Oxygen consumption was measured using a Clark-type oxygen electrode and expressed as μmol oxygen consumed/10^8 cells/min. All values are the mean of three independent determinations ± SE. All values were statistically different when compared to the control at $p < 0.05$.

6.3. Cytochromes profiles of coumarin–silver complex-treated C. albicans cells

Previous work has indicated that impairment of cytochrome synthesis and/or function leads to a reduction in respiration rates in cells following exposure to metal-based drugs (Coyle et al., 2003; Geraghty and Kavanagh, 2003). The cytochrome profile of control cells indicates the presence of cytochromes aa_3 (602 nm), b (564 nm) and c (550–554 nm) (Fig. 2a and b). However, cytochrome spectroscopic profiles were altered when cells were exposed to selected silver(I)–coumarin derivatives at concentration equivalent to half their MIC 80 values. In particular, the

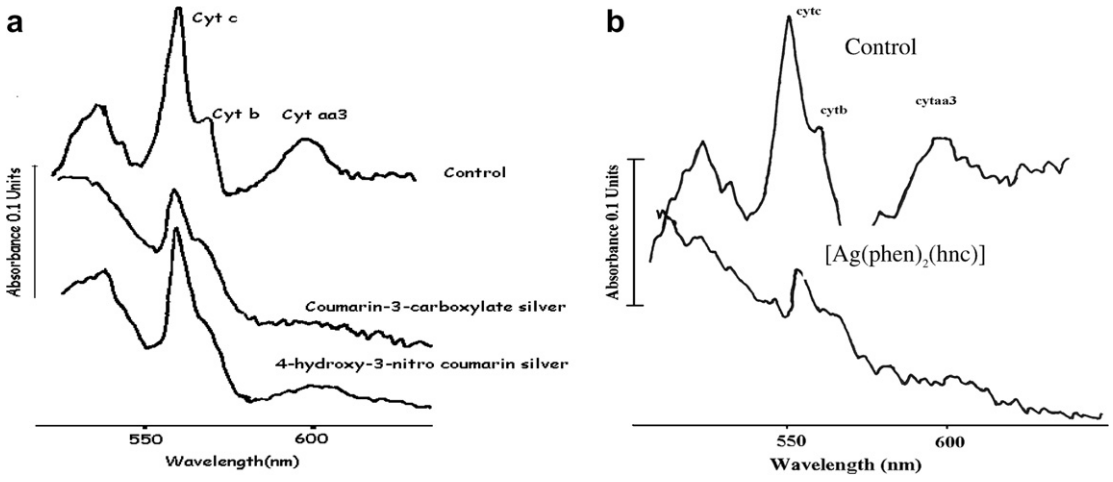

Fig. 2. Cytochrome profile of *C. albicans* following exposure to various silver–coumarin complexes. Fungal cells were treated with coumarin derivatives at concentrations equivalent to half their MIC_{80} value for 12 h.

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cytochrome aa_3 peak was severely disrupted in cultures treated with half MIC_{80} of both [Ag(Cca)] and [Ag(hnc)]. The peaks associated with all three cytochromes (aa_3, b, and c) were reduced when cells were treated with [Ag(phen)$_2$(hnc)], as shown in Fig. 2b. This result suggests that when silver and phenanthroline were added to coumarin, a reduction in cytochrome synthesis was achieved. However, cytochrome peaks were not disrupted when cells were treated with [Ag(hmnc)], [Ag(8-OHCca)], [Ag(7-OHCca)] and [Ag(6-OHCca)] (data not shown).

Disruption of the mitochondrial cytochrome content of a cell has the potential to reduce its respiratory efficacy, and this is evident when cells were treated with several of the novel silver-based coumarin derivatives. [Ag(Cca)] and [Ag(hnc)] reduced cytochrome aa_3, which is an important component of the mitochondrial electron transfer chain. This may have caused respiratory deficiency in *C. albicans*. [Ag(phen)$_2$(hnc)] produced the greatest reduction in all three cytochrome peaks, which may also explain the reduction (50%) in oxygen consumption compared to control cells.

6.4. Determination of ergosterol content of C. albicans cells

Fungal cells require oxygen in order to synthesise the membrane sterol ergosterol, and so either a reduction in respiratory efficiency or an inability to respire leads to reduced levels of this sterol (Parks and Casey, 1995). Reduction in the ergosterol content in *C. albicans* has been identified previously as a mechanism for increased growth in the presence of amphotericin B (Kelly et al., 1997; Geraghty and Kavanagh, 2003). The requirement for a functional mitochondrion in ergosterol biosynthesis is well characterised and arises from the provision of NADPH for squalene dimerisation (Parks and Casey, 1995).

In order to elucidate the mechanism of action of the most potent coumarin–silver agents including CcaH, [Ag(Cca)], [Ag(hnc)], [Ag(hmnc)], [Ag(8-OHCca)], [Ag(7-OHCca)], [Ag(6-OHCca)] and [Ag(phen)$_2$(hnc)], it was decided to investigate their effects on ergosterol synthesis. Concentrations of test agent used here were equivalent to half MIC_{80} and ergosterol was quantified spectrophotometrically by scanning over the wavelength range of 240–330 nm using a dual beam spectrophotometer. Results indicate that treatment with [Ag(Cca)], [Ag(hnc)] and [Ag(7-OHCca)] leads to a reduction in ergosterol content (Table 3). Also, the greatest reduction was seen following treatment with [Ag(phen)$_2$(hnc)], indicating that this is the most potent of all of the derivatives studied.

Table 3
Ergosterol content of *C. albicans* cells

Compound no.	Compounds screened	Ergosterol content (μg/ml) ± SEM
1	CcaH	45 ± 2.45
2	[Ag(Cca)]	30 ± 1.58
3	[Ag(8-OHCca)]	50 ± 1.46$^+$
4	[Ag(7-OHCca)]	40 ± 1.53
5	[Ag(6-OH-Cca)]	20 ± 0.75
6	[Ag(hmnc)]	60 ± 2.24$^+$
7	[Ag(hnc)]	32 ± 1.82
8	[Ag(phen)$_2$(hnc)]	15 ± 0.75
	Control	50 ± 1.45

Fungal cells were treated with coumarin derivatives at a concentration equivalent to half their MIC_{80} value for 12 h. Ergosterol was extracted and quantified according to the method of Arthington-Skaggs et al. (2000). Results are the mean of three independent determinations ± SE. All values were statistically different when compared to the control (compound 1) at $p < 0.05$ except the values for compound 3 and 6 which were not deemed statistically significant.

6.5. Assessment of membrane leakage in coumarin–silver complex-treated cells

Reduced levels of ergosterol can adversely affect membrane integrity and lead to increased membrane permeability. In order to ascertain the effect of depleted ergosterol on membrane integrity, leakage of amino acids across the plasma membrane was measured when cells were exposed to the coumarin compounds for a period of 4 h. The results (Fig. 3) indicate that exposure of stationary phase cells to [CcaH)] lead to the release of 15 μg amino acids per 10^{10} cells. Exposure of cells to each of the silver–coumarin derivatives significantly increased this leakage, with the greatest seen following incubation with [Ag(hnc)]. These results indicate that the addition of silver to coumarin serves to increase the leakage of amino acids from *C. albicans*, a phenomenon which may be due to the depletion of the ergosterol levels which renders the membrane unstable. Leakage of amino acids together with other small molecular weight compounds from the cell would serve to damage the cell's

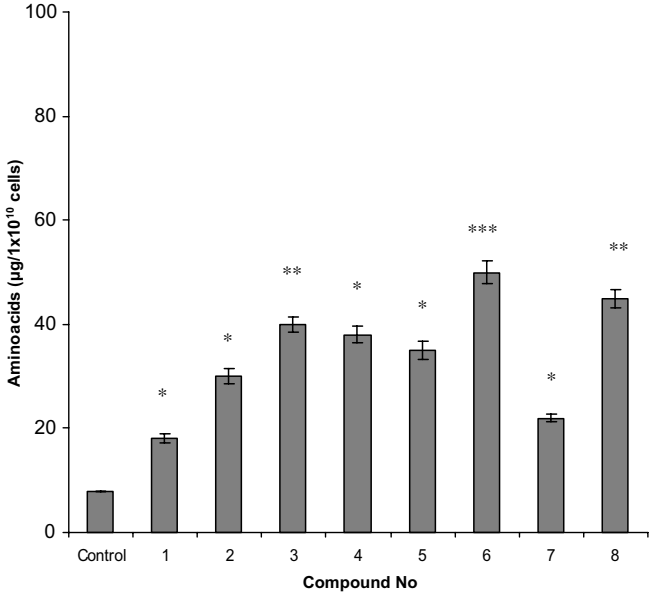

Fig. 3. Amino acids release from *C. albicans*. Membrane leakage was quantified following treatment of stationary phase cells with test agent for 4 h and measuring the escape of amino acids as described. Assay was performed on three independent occasions and results are the mean ± SE. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.005$ with respect to the control. Compound number refer to the complexes listed in Table 1.

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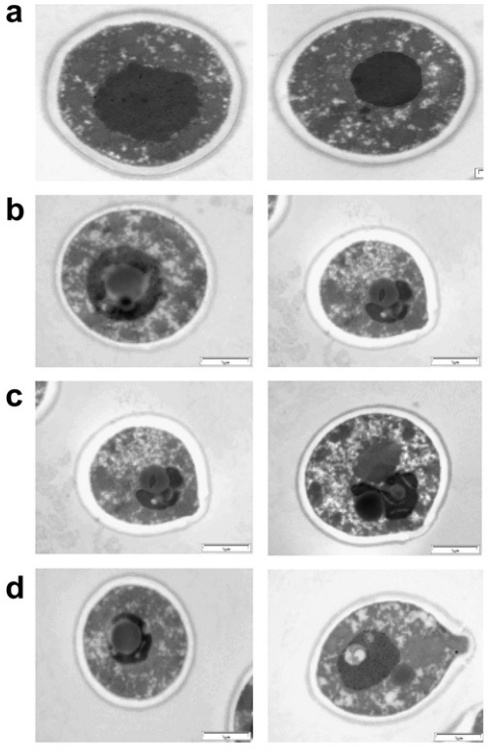
ability to respire and grow and thus may ultimately contribute to their death.

6.6. Electronmicrographic analysis of cell morphology

Exposure of *C. albicans* cells to metal-phenanthroline complexes induces many of the morphological characteristics of apoptosis (programmed cell death) (Coyle et al., 2004; McCann et al., 2004). In particular, there was evidence of ruptured cell walls, withdrawal of the cytoplasm from within the cell wall and the presence of large distended nuclei. In some cases distinct nuclear fragments were also apparent (Coyle et al., 2004). In the work presented here TEM was used to examine the internal morphology of cells grown in the presence of each of the test compounds. Control cells showed normal cellular morphology, including a distinct cell wall, an intact nucleus and numerous membranous organelles (Fig. 4a). In contrast, cells exposed to CcaH showed evidence of nuclear crescent formation and loss of membranous organelles. In addition, cells exposed to [Ag(Cca)] or [Ag(hnc)] also showed evidence of nuclear crescent formation but furthermore demonstrated nuclear fragmentation, features which are consistent with the induction of apoptotic cell death (Fig. 4b–d). In parallel with this analysis DNA was extracted from cells exposed to test agents and separated by agarose gel electrophoresis.



Panel a: *C. albicans:* Control.
Panel b: *C. albicans* treated with coumarin-3-carboxylic-acid
Panel c: *C. albicans* treated with coumarin-3-carboxylate silver
Panel d: *C. albicans* treated with 4-Hydroxy-3-nitro-coumarin-silver

Fig. 4. Electronmicrographs of *C. albicans* cells exposed to coumarin and coumarin–silver derivatives.

The results indicate extensive degradation of DNA from those cells exposed to [Ag(8-OHCca)], [Ag(7-OHCca)] and [Ag(6-OHCca)] (Fig. 5) – a feature consistent with the apoptotic induced fragmentation of DNA in yeast (Coyle et al., 2004).

7. Discussion

The work presented here indicates that while CcaH and [Ag(Cca)] demonstrate anti-fungal activity, derivatising these agents with the inclusion of a hydroxy, nitro or phenanthroline ligand, serves to significantly increase their anti-fungal potency. Most of the silver(I)–coumarin derivatives appear to reduce the respiration rate of *C. albicans*, possibly by disrupting the synthesis of cytochromes in the mitochondrion. Disruption of the mitochondrial cytochrome content of a cell has the potential to reduce its respiratory efficacy, while restoration of respiratory function following transfer of mitochondria by protoplast fusion has the capacity to restore, partially or completely, respiratory status (Ferenczy and Maraz, 1977). In our study, all three cytochromes were reduced by $[Ag(phen)_2$-(hnc)] which may explain why this complex caused the greatest diminution in oxygen consumption by *C. albicans*. Reduced respiration has previously been shown to lead to a reduction in the synthesis of ergosterol, a sterol essential for maintaining membrane integrity (Kelly et al., 1997; Geraghty and Kavanagh, 2003). Synthesis of ergosterol is dependent upon a functional mitochondrion to provide NADPH for one of the steps in its biosynthesis. Previous work has demonstrated that cells which have reduced levels of ergosterol are more tolerant of the anti-fungal effects of agents such as amphotericin B which acts by binding ergosterol and forming pores in the cell membrane (Geraghty and Kavanagh, 2003). Cells treated with the coumarin derivatives demonstrated increased membrane leakage, as evident by the increased leakage of amino acids. A similar effect has been shown previously with the fungus *Aspergillus fumigatus* when treated with either amphotericin B or DMSO. In both cases, increased membrane permeability was observed (Reeves et al., 2004). Additionally, cells exposed to specific silver–coumarin derivatives demonstrated non-specific cleavage of DNA and many of the morphological features of programmed cell death.

The current work demonstrates that the anti-fungal activity of coumarin–silver(I) complexes is mediated by the disruption of respiration which leads to increased membrane leakage due to the depleted synthesis of ergosterol. A further effect is the appearance of morphological features consistent with the induction of the apoptotic death pathway. In mammalian cells, the key event in the induction of apoptosis is loss of cytochrome c from the mitochondrion which triggers the apoptotic cascade (Green and Kroemer, 1998). It is speculated that the critical event in the anti-fungal activity of the compounds studied here, is the disruption of cytochrome synthesis. This would lead to the inhibition of respiration, reduced ergosterol biosynthesis and the increased membrane

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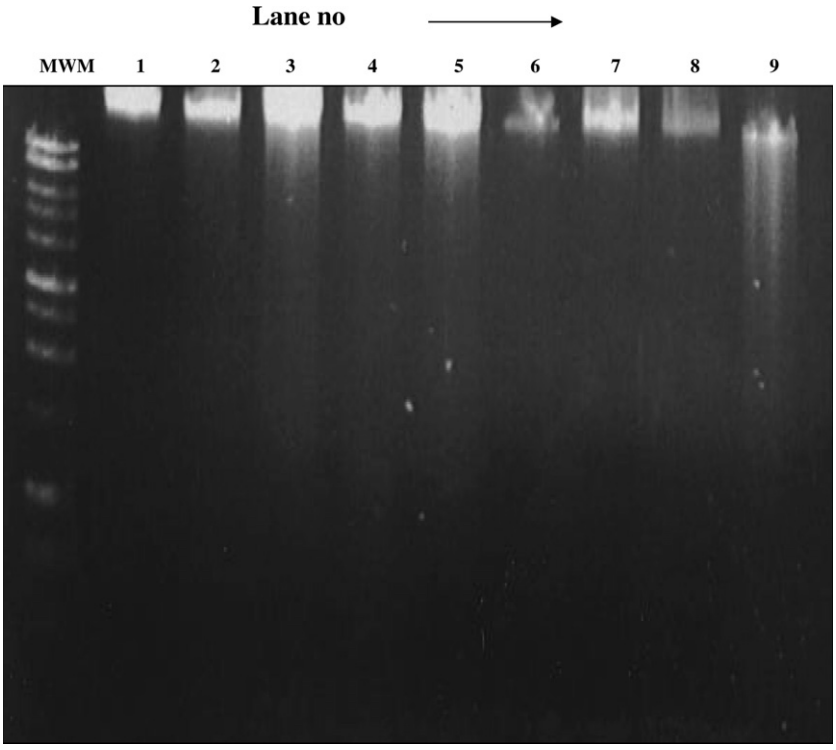

Fig. 5. DNA fragmentation patterns following exposure to various silver–coumarin complexes. Cells were treated with the following coumarin derivatives as described: Lane 1: control cells, 2: [Ag(Cca0], 3: [Ag(8-OHCca)], 4: [Ag(7-OHCca)], 5: [Ag(6-OHCca)], 6: [Ag(hmnc)], 7: [Ag(hnc)], 8: [Ag(phen)2(hnc)] and 9: $AgNO_3$ as the positive control. Molecular weight marker (MWM) were used to indicate the size of the DNA fragments (Promega 10kb marker).

permeability. In addition, loss of cytochrome c (or depletion of its levels within the cell) may trigger an apoptotic response in the cell leading to DNA cleavage and the appearance of features (eg. crescent formation, nuclear fragmentation) consistent with this mode of cell death.

While coumarins have well characterised anti-neoplastic properties (Thornes et al., 1982; Thornes 1983; Ebbinghaus et al., 1997; Finn et al., 2002, 2004, 2005) the results presented here demonstrate that coumarin-3-carboxylic acid and the various silver(I)–coumarin complexes exhibit strong anti-fungal activities which are mediated through the disruption of respiratory function and the induction of apoptosis. In recent years, there has been an increase in the number of fungal isolates manifesting resistance to conventional anti-fungal agents (Kontoyiannis and Lewis, 2002). In light of this, there has been an intensive search for new drugs designed either to circumvent resistance or target alternative cellular targets to the existing range of drugs. While polyene anti-fungals bind ergosterol in the fungal cell membrane and azoles inhibit ergosterol biosynthesis, the compounds described here demonstrate a different mode of action and consequently may have potential applications in the treatment of infections caused by fungi that are resistant to conventional drugs. Due to the problems of drug resistance among pathogenic fungi, it is possible that the agents described here could be employed either alone or in combination with existing agents in order to treat specific infections (Antonella et al., 2003).

Conflict of interest

The authors have no conflicts of interest to declare.

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