



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

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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 MIC₈₀ 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.

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1. Introduction

Coumarin is 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 A₁ 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 A₁ 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).

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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) bacto-peptone (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 (Cre-

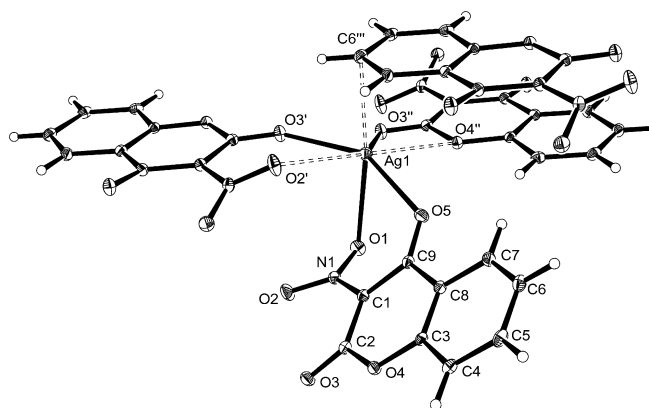


Fig. 1. Crystal structure for [Ag(phen)₂(hnc)].

aven 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)] together 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).

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 a 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). MIC₈₀ 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 3000g for 5 min, washed twice with PBS and divided into two

136 equal volumes. One half of the sample was oxidised by sus-
 137 pending in 0.2% (w/v) sodium hypochlorite solution fol-
 138 lowed by harvesting by centrifugation and then
 139 resuspended in 50% (v/v) glycerol. The remaining half sam-
 140 ple was resuspended in 50% (v/v) glycerol and reduced by
 141 adding a few crystals of sodium-dithionite. The reduced
 142 and oxidised cytochrome differential spectrum was immedi-
 143 ately recorded using a double beam UV–Visible spectro-
 144 photometer at 500–650 nm (Cary IE Varian).

145 2.6. Sterol extraction and quantification

146 Yeast cells were grown in the presence of half MIC₈₀ of
 147 test agent until cells reached the late exponential phase
 148 (approximately 1×10^7 /ml, and typically for 18–24 h) in
 149 Antibiotic Medium 3 at 30 °C in an orbital incubator. Yeast
 150 cells (2×10^9 cells) were harvested and washed with PBS.
 151 Cells were resuspended in 1.5 ml of a solution containing
 152 20% (w/v) potassium hydroxide and 60% (v/v) ethanol and
 153 placed in a shaking water bath at 90 °C for 1.5–2 h. Heptane
 154 was added to this solution and vortexed for 10 s. The upper
 155 layer containing sterols was removed according to the
 156 method of Arthington-Skaggs et al. (2000).

157 Sterol analysis was determined by using a double beam
 158 UV–Visible spectrophotometer (Cary IE Varian) over the
 159 range 240–320 nm. An ergosterol standard curve was con-
 160 structed over the range 100–0.25 µg/ml. Sterol concentra-
 161 tions were also determined using a gas chromatographic
 162 system (Hewlett Packard 5890, Series 11) with a flame ioni-
 163 sation detector and a Chromopack capillary column (Chro-
 164 mopack International BV, Middleburg, The Netherlands)
 165 operated isothermally at 300 °C. Injector and detector tem-
 166 peratures were 320 °C and the carrier gas was N₂.

167 2.7. Evaluation of membrane leakage

168 Stationary phase cells (1×10^{10} in total) were harvested
 169 by centrifugation, washed with PBS and resuspended in
 170 10 ml PBS. Test agents were added to this suspension at a
 171 final concentration of half MIC₈₀. Samples of cell suspen-
 172 sion were recovered after 4 h and assayed for the presence
 173 of amino acids using the ninhydrin method (Reeves et al.,
 174 2004). The filtrate was passed through a 0.45 µm syringe
 175 filter (Sartorius, AG Goettingen, Germany) and free amino
 176 acid was measured as described. The amino acid concentra-
 177 tion was expressed in terms of aspartic acid and glutamic
 178 acid which were used as standards. Ninhydrin (Sigma Ald-
 179 rich) solution (200 µl of 0.35 g/100 ml ethanol) was added to
 180 each sample (1 ml) and the mixture heated to 95 °C for
 181 4 min. After cooling to room temperature in an ice bath, the
 182 OD at 570 nm was recorded on a UV–Visible spectropho-
 183 tometer (Beckmann, DU 640).

184 3. Electron microscopy

185 Yeast cells were grown to the stationary phase in the
 186 presence of half-MIC₈₀ levels of each test agent. Primary

187 fixation of yeast cells was carried out in a 3% (v/v) solution
 188 of glutaraldehyde in 0.1 M phosphate buffer for 2 h. Sec-
 189 ondary fixation was achieved in 0.2% (w/v) osmium tetrox-
 190 ide in 0.1 M phosphate buffer for 1 h. Samples were
 191 dehydrated in graded alcohol solutions of 10, 30, 50, 75, 95,
 192 and 100% (v/v) for 15 min. Samples were embedded in Agar
 193 100 resin (Agar Scientific Ltd., UK) and viewed with a Hit-
 194achi H-7000 transmission electron microscope operating at
 195 100 kV accelerating voltage.

4. Extraction of DNA from *C. albicans*

197 Yeast cells were grown in the presence of test agent at a
 198 concentration equivalent to half MIC₈₀ in Antibiotic
 199 Medium 3 at 30 °C and 200 rpm, using an orbital shaker.
 200 DNA was extracted from cells (4×10^9) as described earlier
 201 (Coyle et al., 2004). The integrity of extracted DNA was
 202 determined by agarose gel electrophoresis as described
 203 (Coyle et al., 2004). Samples were loaded onto an agarose
 204 gel and electrophoresed at 80 V for 1 h. DNA bands were
 205 visualised by irradiation at 300 nm and photographed using
 206 a Pharmacia 3D imaging system.

5. Statistical analysis

208 All experiments were performed on three independent
 209 occasions and results are the mean ± SEM. Statistical anal-
 210 ysis was performed using the non-parametric Mann–Whit-
 211 ney test at a 95% confidence interval.

6. Results

6.1. Effects of coumarin derivatives on fungal cells

214 A series of novel coumarin ligands and their silver(I)
 215 complexes (Table 1) were screened for their anti-fungal
 216 activity. The MIC₈₀ of each compounds was determined
 217 over a concentration range of 500–0.25 µM. Results indi-
 218 cate that both the number and position of functional
 219 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 $p < 0.05$.

nucleus, greatly affected the fungistatic capacity of the coumarin derivatives. This is evident from the MIC₈₀ 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 MIC₈₀ 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 MIC₈₀ 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 MIC₈₀ 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/10⁸ cells) and [Ag(phen)₂(hnc)] (42.47 μmol oxygen/10⁸ cells). These results clearly indicate that

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/10⁸ 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.

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.

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 aa₃ (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

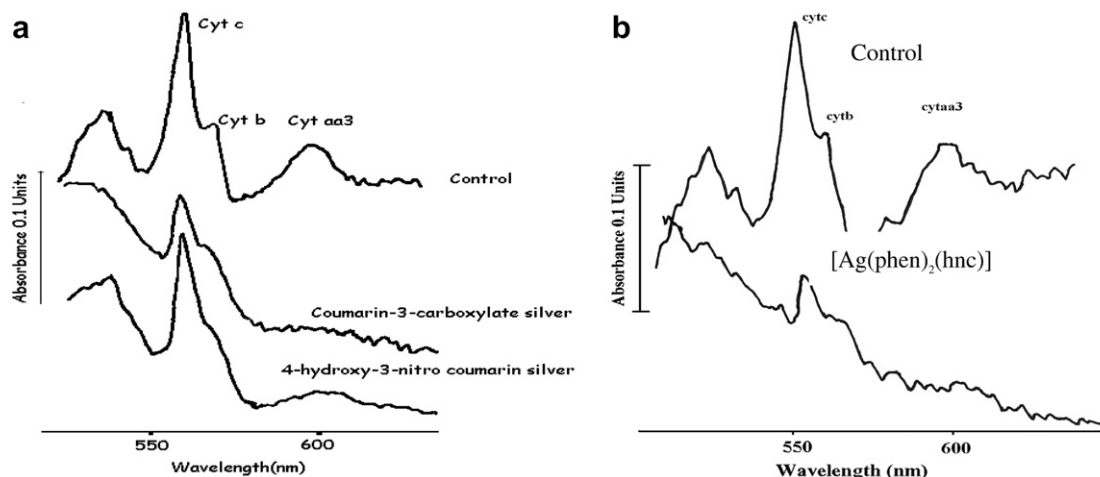


Fig. 2. Cytochrome profile of *C. albicans* following exposure to various silver–coumarin complexes. Fungal cells were treated with coumarin derivatives at concentrations equivalent to half their MIC₈₀ value for 12 h.

275 cytochrome aa₃ peak was severely disrupted in cultures
276 treated with half MIC₈₀ of both [Ag(Cca)] and [Ag(hnc)].
277 The peaks associated with all three cytochromes (aa₃, b,
278 and c) were reduced when cells were treated with
279 [Ag(phen)₂(hnc)], as shown in Fig. 2b. This result suggests
280 that when silver and phenanthroline were added to couma-
281 rin, a reduction in cytochrome synthesis was achieved.
282 However, cytochrome peaks were not disrupted when cells
283 were treated with [Ag(hmnc)], [Ag(8-OHCca)], [Ag(7-
284 OHCca)] and [Ag(6-OHCca)] (data not shown).

285 Disruption of the mitochondrial cytochrome content of
286 a cell has the potential to reduce its respiratory efficacy, and
287 this is evident when cells were treated with several of the
288 novel silver-based coumarin derivatives. [Ag(Cca)] and
289 [Ag(hnc)] reduced cytochrome aa₃, which is an important
290 component of the mitochondrial electron transfer chain.
291 This may have caused respiratory deficiency in *C. albicans*.
292 [Ag(phen)₂(hnc)] produced the greatest reduction in all
293 three cytochrome peaks, which may also explain the reduction
294 (50%) in oxygen consumption compared to control
295 cells.

296 6.4. Determination of ergosterol content of *C. albicans* cells

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

308 In order to elucidate the mechanism of action of the
309 most potent coumarin–silver agents including CcaH,
310 [Ag(Cca)], [Ag(hnc)], [Ag(hmnc)], [Ag(8-OHCca)], [Ag(7-
311 OHCca)], [Ag(6-OHCca)] and [Ag(phen)₂(hnc)], it was
312 decided to investigate their effects on ergosterol synthesis.
313 Concentrations of test agent used here were equivalent to
314 half MIC₈₀ and ergosterol was quantified spectrophotomet-
315 rically by scanning over the wavelength range of 240–
316 330 nm using a dual beam spectrophotometer. Results
317 indicate that treatment with [Ag(Cca)], [Ag(hnc)] and
318 [Ag(7-OHCca)] leads to a reduction in ergosterol content
319 (Table 3). Also, the greatest reduction was seen following
320 treatment with [Ag(phen)₂(hnc)], indicating that this is the
321 most potent of all of the derivatives studied.

322 6.5. Assessment of membrane leakage in coumarin–silver 323 complex-treated cells

324 Reduced levels of ergosterol can adversely affect mem-
325 brane integrity and lead to increased membrane permeabil-
326 ity. In order to ascertain the effect of depleted ergosterol on
327 membrane integrity, leakage of amino acids across the

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 MIC₈₀ 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.

328 plasma membrane was measured when cells were exposed 328
329 to the coumarin compounds for a period of 4 h. The results 329
330 (Fig. 3) indicate that exposure of stationary phase cells to 330
331 [CcaH] lead to the release of 15 µg amino acids per 10¹⁰ 331
332 cells. Exposure of cells to each of the silver–coumarin deri- 332
333 vatives significantly increased this leakage, with the greatest 333
334 seen following incubation with [Ag(hnc)]. These results 334
335 indicate that the addition of silver to coumarin serves to 335
336 increase the leakage of amino acids from *C. albicans*, a phe- 336
337 nomenon which may be due to the depletion of the ergos- 337
338 terol levels which renders the membrane unstable. Leakage 338
339 of amino acids together with other small molecular weight 339
340 compounds from the cell would serve to damage the cell's 340

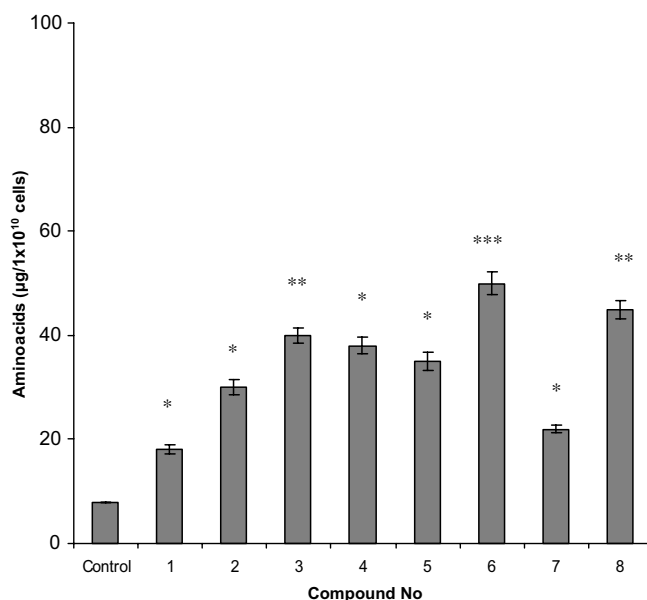
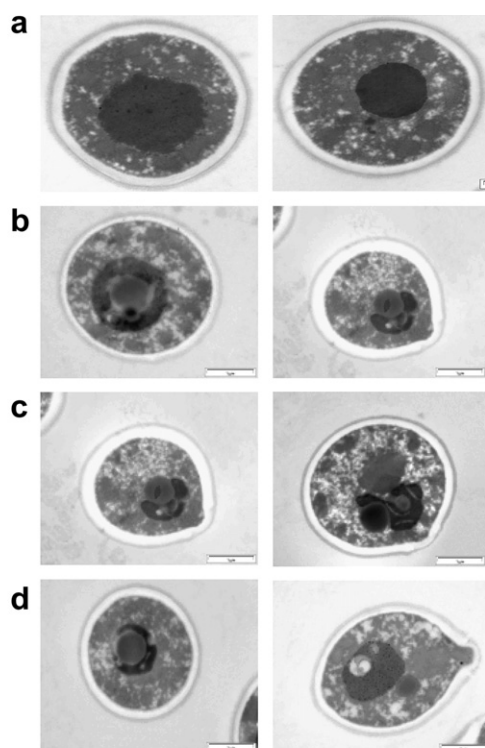


Fig. 3. Amino acids release from *C. albicans*. Membrane leakage was quantified following treatment of stationary phase cells with test agent for 4 h and measuring the escape of amino acids as described. Assay was performed on three independent occasions and results are the mean ± SE. **p* < 0.05, ***p*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

341 ability to respire and grow and thus may ultimately con-
342 tribute to their death.

343 6.6. Electronmicrographic analysis of cell morphology

344 Exposure of *C. albicans* cells to metal-phenanthroline
345 complexes induces many of the morphological characteris-
346 tics of apoptosis (programmed cell death) (Coyle et al.,
347 2004; McCann et al., 2004). In particular, there was evi-
348 dence of ruptured cell walls, withdrawal of the cytoplasm
349 from within the cell wall and the presence of large distended
350 nuclei. In some cases distinct nuclear fragments were also
351 apparent (Coyle et al., 2004). In the work presented here
352 TEM was used to examine the internal morphology of cells
353 grown in the presence of each of the test compounds. Con-
354 trol cells showed normal cellular morphology, including a
355 distinct cell wall, an intact nucleus and numerous membra-
356 nous organelles (Fig. 4a). In contrast, cells exposed to CcaH
357 showed evidence of nuclear crescent formation and loss of
358 membranous organelles. In addition, cells exposed to
359 [Ag(Cca)] or [Ag(hnc)] also showed evidence of nuclear
360 crescent formation but furthermore demonstrated nuclear
361 fragmentation, features which are consistent with the
362 induction of apoptotic cell death (Fig. 4b–d). In parallel
363 with this analysis DNA was extracted from cells exposed to
364 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 phe-
nanthroline ligand, serves to significantly increase their
anti-fungal potency. Most of the silver(I)–coumarin deriva-
tives appear to reduce the respiration rate of *C. albicans*,
possibly by disrupting the synthesis of cytochromes in the
mitochondrion. Disruption of the mitochondrial cyto-
chrome content of a cell has the potential to reduce its
respiratory efficacy, while restoration of respiratory func-
tion 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)-
(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; Gera-
ghy and Kavanagh, 2003). Synthesis of ergosterol is depen-
dent 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 ergos-
terol 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 deriva-
tives demonstrated increased membrane leakage, as evident
by the increased leakage of amino acids. A similar effect has
been shown previously with the fungus *Aspergillus fumiga-
tus* 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 activi-
ty of coumarin–silver(I) complexes is mediated by the dis-
ruption 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 mam-
malian 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 spec-
ulated 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

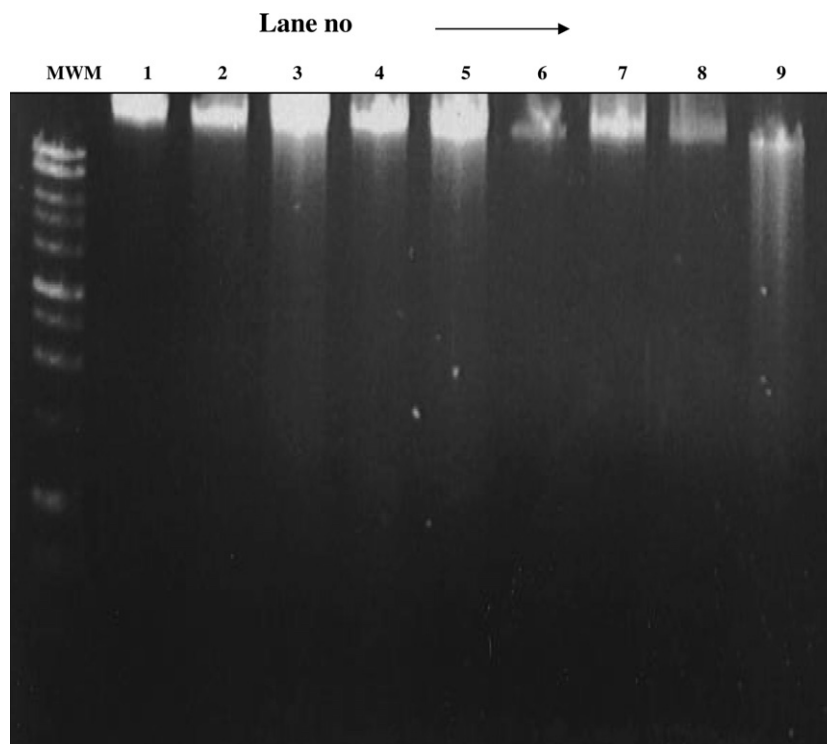


Fig. 5. DNA fragmentation patterns following exposure to various silver–coumarin complexes. Cells were treated with the following coumarin derivatives as described: Lane 1: control cells, 2: [Ag(Cca0)], 3: [Ag(8-OHCca)], 4: [Ag(7-OHCca)], 5: [Ag(6-OHCca)], 6: [Ag(hmnc)], 7: [Ag(hnc)], 8: [Ag(phen)2(hnc)] and 9: AgNO₃ 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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