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Use of *Galleria mellonella* larvae to evaluate the in vivo anti-fungal activity of $[Ag_2(mal)(phen)_3]$

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Abstract Larvae of the insect *Galleria mellonella* were employed to assess the in vivo antifungal efficacy of ($[Ag_2(mal)(phen)_3]$), $AgNO_3$ and 1,10-phenanthroline. Larvae pre-inoculated with these compounds were protected from a subsequent lethal infection by the yeast *Candida albicans* while larvae inoculated 1 and 4 h post-infection showed significantly increased survival ($P < 0.01$) compared to control larvae. Administration of these compounds resulted in an increase over 48 h in the density of insect haemocytes (immune cells) but there was no widespread activation of genes for antimicrobial peptides. This work demonstrates that *G. mellonella* larvae may be employed to ascertain the antifungal efficacy of silver(I) compounds and offers a rapid and effective means of assessing the in vivo activity of inorganic antimicrobial compounds.

Keywords Antifungal · *Candida albicans* · Silver(I) · Insect · *Galleria*

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Abbreviations

AMP	Antimicrobial peptide(s)
apim	1-(3-Aminopropyl) imidazole
$malH_2$	Malonic acid
phen	1,10-Phenanthroline

Introduction

The yeast *Candida albicans* is an opportunistic pathogen which is capable of inducing a wide range of superficial and systemic infections in AIDS patients (Fidel 2006), transplant recipients (Ascioglu et al. 2002), cancer patients (Ascioglu et al. 2002) and premature infants (Benjamin et al. 2004; Kaufman 2003). While conventional anti-fungal therapy relies upon the use of polyenes and azole drugs to inhibit the growth of the infecting yeast, rapid initiation of anti-fungal therapy is necessary to control systemic infection and has been shown to reduce mortality (Morell et al. 2005).

The anti-fungal activities of silver(I) and 1,10-phenanthroline have been known for many years and the silver(I) ion is the active agent in many healthcare products, such as silver-coated catheters (Rho et al. 2008; Khare et al. 2007), wound dressings (Totaro and Rambaldini 2008; Adams et al. 1999) and burn-treatment creams (Thomas and McCubbin 2003; Holder et al. 2003).

Evidence for the potent anti-*Candida* activity of silver(I) was provided by Desai and Herndon (1988)

while the efficacy of topically applied silver(I) against fungal pathogens of burned tissue has also been demonstrated (Wright et al. 1999). Investigations into the synthesis of silver(I)-containing complexes that incorporate either 1,10-phenanthroline, salicylic acid, malonic acid ($malH_2$) or 1-3(-aminopropyl) imidazole have generated silver(I) complexes with potent in vitro anti-*C. albicans* activity (Rowan et al. 2006; McCann et al. 2004; Coyle et al. 2003, 2004). In order to establish the in vivo anti-fungal activity of selected silver(I) compounds, it was decided to investigate the interaction of one of these compounds, $AgNO_3$ and 1,10-phenanthroline with *C. albicans* using the *Galleria mellonella* model of infection.

Larvae of *G. mellonella* (the greater wax moth) can be used to assess the pathogenicity of microbial isolates (Brennan et al. 2002) and the therapeutic potential of anti-microbial drugs and have yield results that are comparable to those obtained using mammalian models (Kavanagh and Reeves 2004). *G. mellonella* larvae have also been proven to be able to distinguish between pathogenic and non-pathogenic yeast isolates (Cotter et al. 2000) while a strong correlation between the virulence of *C. albicans* mutants in mice and in *G. mellonella* larvae has been demonstrated (Brennan et al. 2002). The insect immune response consists of a cellular component which is composed of haemocytes and a humoral element which involves the production of anti-microbial peptides (AMPs). The numbers of haemocytes circulating within the insect's body cavity increases in response to infection of *G. mellonella* by *C. albicans* (Bergin et al. 2003). Antimicrobial peptides are secreted into the haemolymph, diffuse to the site of infection and attack portions of the microbial cell wall (Ratcliffe 1985). The expression of the genes coding for these AMPs is increased in response to infection with *C. albicans* (Bergin et al. 2006). The aim of the work presented here was to examine the in vivo antifungal activity of a silver(I) complex ($[Ag_2(mal)(phen)_3]$), $AgNO_3$ and 1,10-phenanthroline and to ascertain their effect on the immune response of *G. mellonella* larvae.

Materials and methods

Culture conditions

Candida albicans ATCC 10231 was maintained on YEPD agar [2% (w/v) bacteriological peptone (Sigma), 2% (w/v) D-glucose (Sigma), 1% (w/v) yeast extract (Sigma) and 2% (w/v) agar (Sigma)]. *C. albicans* 10231 was cultured in liquid minimal medium (MM)[2% (w/v) D-glucose, 0.5% (w/v) ammonium sulphate and 0.17% (w/v) yeast nitrogen base without amino acids (Sigma)] at 30°C and 200 rpm overnight. Anti-fungal susceptibility testing was performed as described previously (Rowan et al. 2006). Susceptibility testing was carried out in triplicate on three independent occasions and results are presented as the mean ± standard deviation. The MIC_{80} values represent the concentration of a compound required to inhibit *C. albicans* cell growth by 80% in liquid culture.

Synthesis of silver(I) compounds

All chemicals were purchased from commercial sources and were used without any further purification. The $[Ag_2(mal)(phen)_3]$ compound was synthesized as described previously (McCann et al. 2000).

Inoculation of *G. mellonella* with *C. albicans*

Larvae of the sixth developmental stage of *Galleria mellonella* were obtained from the Meal Worm Company, (Sheffield, England). Larvae were maintained at 15°C in wood shavings, stored in the dark and used within 3 weeks of receipt. Larvae (330 ± 20 mg) were inoculated in triplicate with 5×10^5 *C. albicans* cells as described (Cotter et al. 2000). All compounds were diluted in sterile PBS to produce concentrations corresponding to twice their MIC_{80} values (McCann et al. 2000). As negative controls, un-infected larvae were inoculated with 20 µl of each compound and the toxicity of the individual drug solutions was also monitored. Un-infected larvae were also inoculated with 20 µl of sterile PBS and the toxicity of 20 µl PBS was also monitored.

Determination of haemocyte density in *G. mellonella* larvae

Haemocyte density was assessed by bleeding three larvae per treatment into a pre-chilled test tube containing 4 mg phenylthiourea (Sigma–Aldrich) to prevent melanisation. Haemocytes were diluted in

PBS containing 0.37% (v/v) mercaptoethanol (Sigma) and their density was ascertained by haemocytometer count.

RNA extraction and RT-PCR analysis

Galleria larvae were inoculated with *C. albicans* and exposed to the selected silver(I) compounds for the indicated times. RNA was extracted as described previously (Bergin et al. 2006). cDNA synthesis was performed using isolated RNA (1 μg) with the Superscript III first-strand synthesis system. RT-PCR analysis of the expression of the genes coding for the anti-microbial peptides gallerimycin (*GLM*), galiomicin (*GIM*), transferrin (*TFN*) and inducible metallo-proteinase inhibitor (*IMPI*) was performed as described (Bergin et al. 2006).

Results

Anti-fungal activity of selected compounds in vivo using *G. mellonella*

Larvae were inoculated with 5×10^5 *C. albicans* cells and followed up 1 h later with either $[Ag_2(mal)(phen)_3]$, $AgNO_3$ or 1,10-phenanthroline. The most effective compound at providing protection over the first 24 h was $[Ag_2(mal)(phen)_3]$ followed by 1,10-phenanthroline and by the silver(I) salt $AgNO_3$ (Fig. 1a). However, after 72 h, the most effective compounds were 1,10-phenantroline followed by $AgNO_3$ (Fig. 1a). Un-infected larvae were also inoculated with 20 μl of the compound solutions and 100% survival was observed after 72 h (data not presented).

In order to mimic a clinical response to infection caused by *C. albicans*, larvae were inoculated with *C. albicans* and subsequently administered the selected anti-fungal compounds after 1 and 4 h (Fig. 1b). The increased efficiency of a double dose of $AgNO_3$ was observed after 72 h of infection when the survival of these larvae was significantly increased compared to the survival of untreated infected larvae ($P < 0.01$). Double dosing of larvae with $[Ag_2(mal)(phen)_3]$ increased the survival of infected larvae compared to the survival of control larvae at 48 h. The 1,10 phenanthroline also increased larval survival after 48 and 72 h.

Larvae were also pre-dosed with each compound and followed up 1 h later by inoculation with *C. albicans*. The results (Fig. 1c) demonstrated that the survival of infected larvae prophylactically treated with 1,10-phenanthroline prior to infection with *C. albicans* was significantly increased (Fig. 1c). Larvae treated with $[Ag_2(mal)(phen)_3]$ demonstrated an increase in survival after 24 h (Fig. 1c) with an increase in survival was also evident after 72 h in larvae that had been treated with 1-10-phenanthroline and $AgNO_3$ (Fig. 1c).

Prior exposure of *Galleria mellonella* to selected compounds increases the number of circulating hemocytes

In order to determine the efficacy of the compounds in *G. mellonella* larvae, it was essential to establish the effect that compounds had on the insect immune response. Larvae were inoculated with the test compounds and the density of circulating haemocytes was ascertained at 1 and 4 h. After 1 h of exposure of the larvae to 1,10-phenanthroline, the haemocyte density had decreased, however, after 4 h the number of haemocytes had significantly increased (Fig. 2). In addition, the $AgNO_3$ also increased the haemocyte density following 4 h exposure (Fig. 2). Larvae exposed to $[Ag_2(mal)(phen)_3]$ demonstrated an increase in haemocyte density after 4 h (Fig. 2). These results demonstrated that administration of the compounds results in an increase in the haemocyte density of treated larvae. Hemocytes have an important role in the insect immune response to infection (Bergin et al. 2003) so this elevated number may work in combination with the antifungal properties of the compounds to arrest and kill *C. albicans* and thus prevent larval death (Fig. 2).

Prior exposure of *Galleria mellonella* to selected drugs increases the expression of genes coding for selected anti-microbial peptides

The expression of the genes that code for the defensin molecule galiomicin, a cysteine rich anti-fungal peptide gallerimycin, the iron binding protein transferrin and the inducible metallo-proteinase inhibitor was investigated following exposure of the larvae to the compounds in order to establish whether the test compounds primed this element of the insect immune

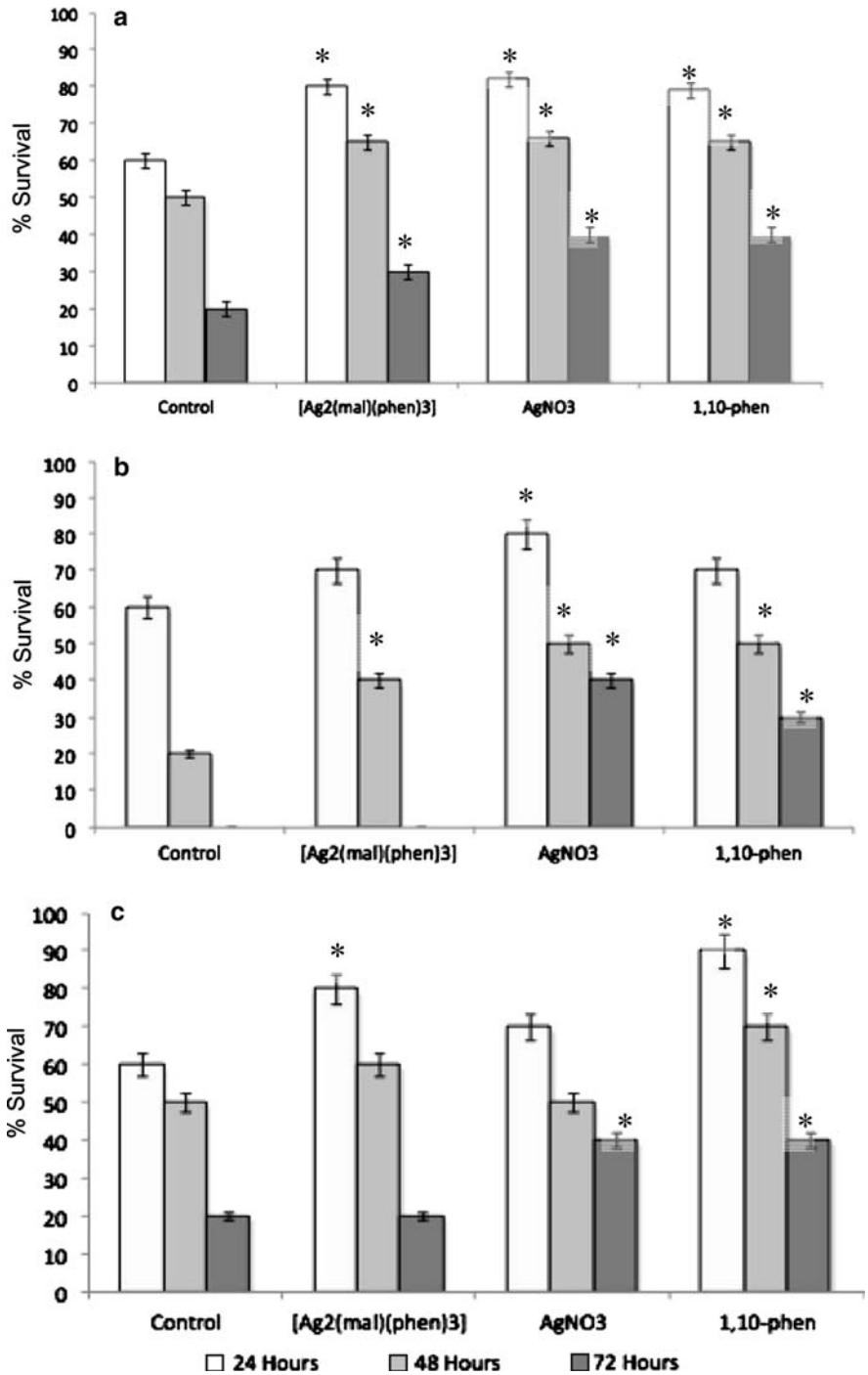

Fig. 1 Percentage survival of *G. mellonella* larvae inoculated with 5×10^5 *C. albicans* 10231 cells and followed by inoculation with compounds after **a** 1 h or **b** 1 and 4 h or **c** 1 h prior to inoculation. Larvae were treated with 20 μl of drug solutions with concentrations corresponding to twice their in vitro anti-*C. albicans* MIC_{80} values ($AgNO_3 = 3.6$ μM, $[Ag_2(mal)(phen)_3] = 4$ μM and 1,10-phenanthroline = 6 μM). Protection was deemed to be significant at $P < 0.01$(*) relative to the sterile water control

response. The gene coding for *GLM* demonstrated the most significant increase in expression in response to the compounds (Fig. 3). Exposure of larvae to $[Ag_2(mal)(phen)_3]$ and 1,10-phenanthroline increased the expression of *GLM* (Fig. 3). The expression of the *GIM* gene was found not to be significantly increased ($P < 0.01$) in response to the compounds. Interestingly, the expression of the *TFN* gene was found to be significantly reduced ($P < 0.01$) within larvae exposed to $AgNO_3$ while the expression of this gene

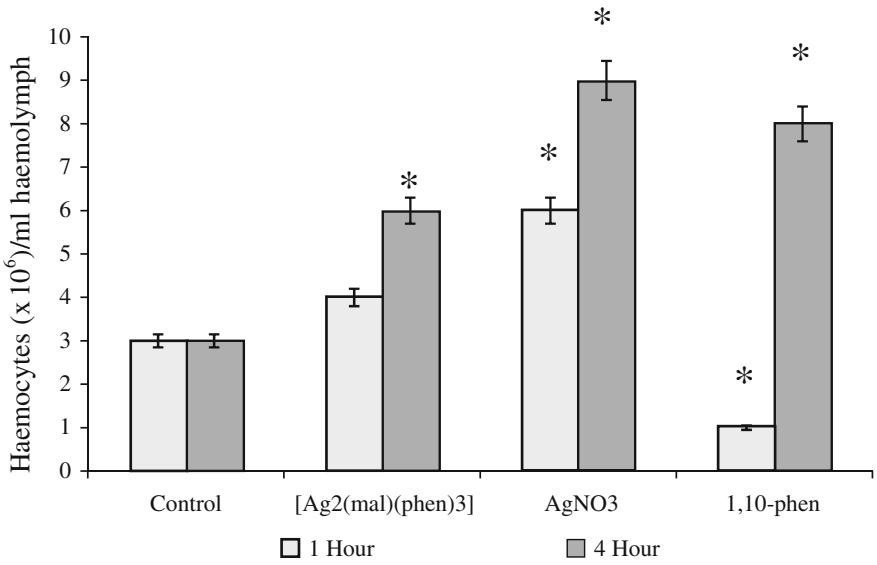

Fig. 2 Effect of compounds upon the *G. mellonella* haemocyte density after 1 and 4 h. *Galleria* were exposed to the compounds at concentrations as in Fig. 1

was not found to be changed in response to exposure to $[Ag_2(mal)(phen)_3]$ or 1,10-phenanthroline (Fig. 3). The expression of the *IMPI* within *G. mellonella* was not found to be significantly altered ($P < 0.01$) in any of the larvae (Fig. 3). These results demonstrate that of the four anti-microbial peptide genes investigated, only the expression of the gene coding for gallerimycin was significantly increased following administration of the compounds.

Discussion

Due to the strong similarities between the insect immune response and the innate immune response of mammals the use of insects as a model for studying the interaction of microbial pathogens with the immune response has increased (Kavanagh and Reeves 2004). Insect models of microbial infections have been employed to investigate the anti-microbial properties of drugs (Mahajan-Miklos et al. 1999; Bernall and Kimbrell 2000; Lionakis and Kontoyiannis 2005) and offer many advantages over the use of mammals (Kavanagh and Reeves 2004). *G. mellonella* has also been used to evaluate the anti-fungal activity of amphotericin B, flucytosine and fluconazole in an insect model of *C. neoformans* infection (Mylonakis et al. 2005) while silkworms have also been used to investigate the activity of commonly used antibiotics including the antifungal fluconazole (Hamamoto et al. 2004).

The results presented here demonstrate that administration of $[Ag_2(mal)(phen)_3]$, 1,10 phenanthroline or $AgNO_3$ to *G. mellonella* larvae 1 h prior to, or 1 or 4 h subsequent to infection with a lethal dose of *C. albicans* can significantly increase the survival of larvae (Fig. 1). Administration of the compounds did lead to an alteration in the haemocyte density of treated larvae (Fig. 2). These results demonstrate an increase in haemocyte numbers which could contribute to the ability of the insect to kill *C. albicans* and this may function in combination with the antifungal properties of the compounds. Administration of the test compounds resulted in the increased expression of the gene for one AMP (*gallerimycin*) which indicates that there is not widespread activation of this arm of the insect immune response.

The data presented here demonstrate that larvae of *G. mellonella* may be used to assess the in vivo antifungal activity of $[Ag_2(mal)(phen)_3]$, $AgNO_3$ and 1,10-phenanthroline. The in vitro activity of these compounds was demonstrated previously (Rowan et al. 2006) and this is the first demonstration of the use of insects for evaluating the in vivo antifungal activity of these compounds. The use of insects for determining the in vivo activity of antimicrobial compounds is now well established (Hamamoto et al. 2004) and this work demonstrates that the *G. mellonella* system can be used for assessing the antimicrobial properties of inorganic compounds. The use of *G. mellonella* offers many advantages over the use of mice for this type of screening including reduced cost, speed of results

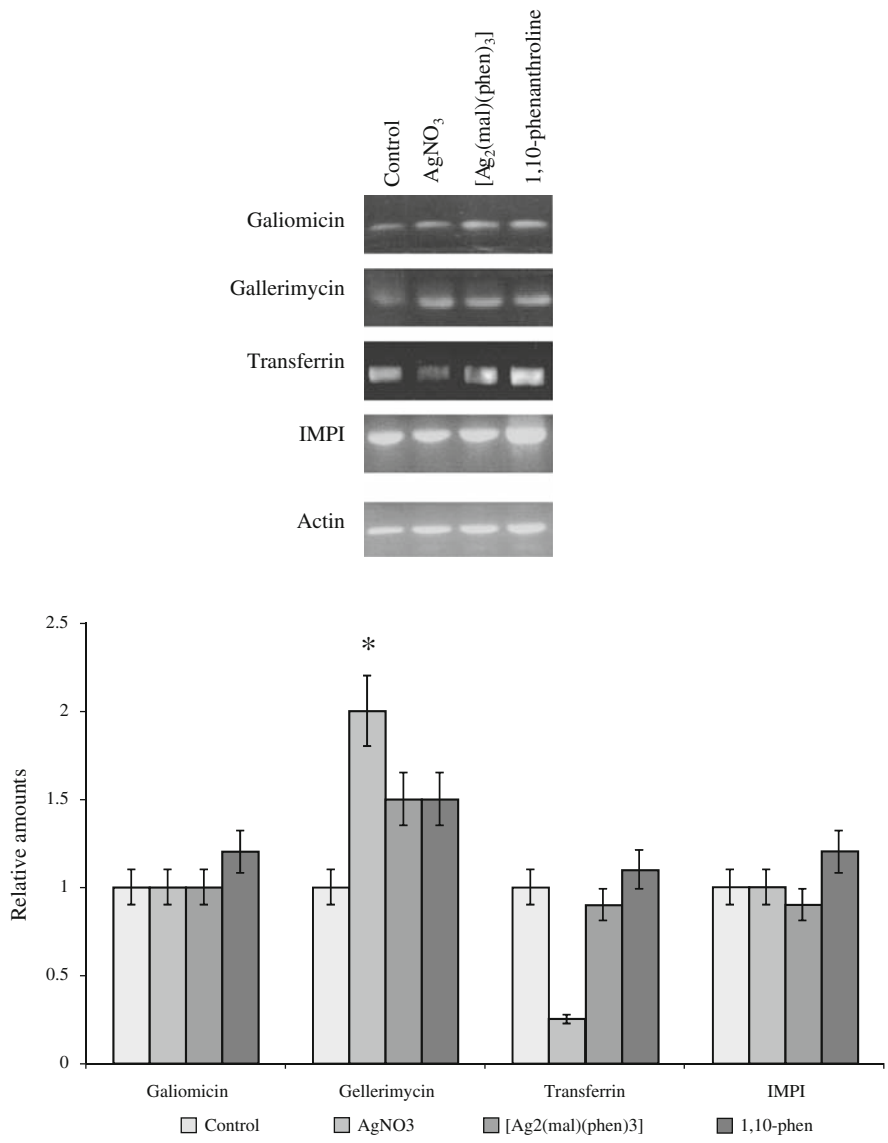

Fig. 3 Effect of compounds upon the expression of genes coding for AMPs in *G. mellonella* larvae. Larvae were inoculated as in Fig. 1 and incubated at 30°C for 4 h. RNA was extracted and RT-PCR was performed as described

and the absence of ethical and legal considerations (Kavanagh and Reeves 2004).

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