

Caspofungin primes the immune response of the larvae of *Galleria mellonella* and induces a non-specific antimicrobial response

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The echinocandins (e.g. caspofungin) function by inhibiting the synthesis of 1,3- β -glucan in the fungal cell wall. While the potent antifungal activity of caspofungin has been well characterized in mammals, this study investigated the *in vivo* antifungal effect of caspofungin using larvae of the insect *Galleria mellonella*. Caspofungin was successful in increasing the survival of larvae that were inoculated with *Candida albicans* 1 h before the drug was administered, particularly when a concentration of 0.19 $\mu\text{g ml}^{-1}$ was used. Pre-injecting larvae with caspofungin also increased their survival when they were inoculated with either *Staphylococcus aureus* or *C. albicans*. Caspofungin administration resulted in an increase in the number of circulating immune cells (haemocytes), an increase in the expression of the genes encoding *IMPI* and *transferrin*, and an increase in the expression of a number of proteins (identified by liquid chromatography–mass spectrometry) some of which have immune functions. This work indicates that administration of caspofungin can increase the survival of infected *G. mellonella* larvae, and this is due to the antifungal properties of caspofungin and also to the ability of caspofungin to prime the insect's immune response.

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INTRODUCTION

Interest in using insects as alternative models to mammals for the *in vivo* screening of novel antimicrobial drugs (Hamamoto *et al.*, 2004, 2009) or for assessing the virulence of microbial pathogens (Brennan *et al.*, 2002; Lionakis & Kontoyiannis, 2005) has been growing for a number of years, since the immune response of insects shares a high level of homology with the innate immune response of mammals (Kavanagh & Reeves, 2004). Insect models of microbial infections have been employed to investigate the antimicrobial properties of drugs (Lionakis & Kontoyiannis, 2005; Johny *et al.*, 2007) and offer many advantages over the use of mammals (Kavanagh & Reeves, 2004). Larvae of the Greater Wax Moth, *Galleria mellonella*, have been used to evaluate the antifungal activity of amphotericin B, flucytosine and fluconazole following infection with *Cryptococcus neoformans* (Mylonakis *et al.*, 2005), while silkworms have also been used to investigate the activity of commonly used antimicrobials including the antifungal fluconazole (Hamamoto *et al.*, 2004).

Larvae of *G. mellonella* are inexpensive to purchase, results can be obtained within 48 h and a large number of insects

can be inoculated in a short period of time (Cotter *et al.*, 2000). *G. mellonella* larvae have been used to assess the pathogenicity of mutants of *Pseudomonas aeruginosa* and a correlation has been established between their virulence in *G. mellonella* and in mice (Dunphy *et al.*, 1986; Jander *et al.*, 2000). Similarly a correlation between the virulence of *Candida albicans* mutants in *G. mellonella* and mice has been demonstrated (Brennan *et al.*, 2002), and the larval model has been used to allow the determination of the virulence of relatively 'weak' fungal pathogens (Bergin *et al.*, 2003). In addition, *G. mellonella* larvae have been established as a good model for studying the virulence of *Cryptococcus neoformans* (Mylonakis *et al.*, 2005).

The insect immune system bears a number of structural and functional similarities to the innate immune response of mammals (Zhao & Kanost, 1996; Rock *et al.*, 1998; Wittwer *et al.*, 1999). The cuticle of the insect serves a function analogous to the skin in mammals and provides a barrier to infection. The haemolymph of insects is contained within the body cavity (haemocoel) and serves a function comparable to blood, where it transports nutrients, waste products and signal molecules (Matha & Acek, 1984). Haemolymph contains circulating immune cells (haemocytes) that are capable of immobilizing and killing invading microbes (Lavine & Strand, 2002). The humoral element of the insect immune response consists of the processes of melanization, haemolymph clotting,

Abbreviations: 2D, two dimensional; AMP, antimicrobial peptide; JH, juvenile hormone; JHBP, juvenile hormone binding protein; LC–MS, liquid chromatography–mass spectrometry; NUI, National University of Ireland.

antimicrobial peptide (AMP) synthesis and wound healing (Kavanagh & Reeves, 2004). AMPs are released from haemocytes, other cells and organs into the haemolymph, where they attack components of the bacterial and fungal cell membrane and wall (Ratcliffe, 1985).

The echinocandins are a relatively new group of antifungal agents and caspofungin was the first of this group to be licensed for use (Kartsonis *et al.*, 2003). Echinocandins show potent activity against the yeast *C. albicans* and are unique in their mode of action by targeting the biosynthesis of the fungal cell wall. In particular, echinocandins inhibit the synthesis of 1,3- β -glucan, the major polymer of the fungal cell wall (Eggimann *et al.*, 2003). Since caspofungin targets the synthesis of the cell wall, a structure not found in mammalian cells, minimal toxicities are observed in humans, and excellent *in vivo* activity has been observed in patients (Petraitiene *et al.*, 2002).

This study examined the *in vivo* activity of caspofungin in larvae of the insect *G. mellonella*. The objective of this work was to determine whether the survival of larvae administered caspofungin was due to the antifungal activity of the drug alone or to the induction of a protective immune response by the drug in the treated larvae.

METHODS

Assessment of the effect of caspofungin on the viability of *G. mellonella* inoculated with *C. albicans* or *Staphylococcus aureus*. Sixth instar larvae of *G. mellonella* (the greater wax moth, which belongs to order Lepidoptera, family Pyralidae) were obtained from The Mealworm Company (Sheffield, England) and stored in wood shavings in the dark at 15 °C. Larvae chosen for experiments weighed between 0.2 and 0.4 g. Ten healthy larvae were placed in sterile 9 cm Petri dishes containing a sheet of Whatman filter paper.

C. albicans strain MEN (a gift from Professor D. Kerridge, University of Cambridge, Cambridge, England) was grown to early stationary phase ($1-2 \times 10^8$ ml⁻¹) in YEPD liquid [2%, w/v, glucose; (Sigma-Aldrich), 2%, w/v, bacteriological peptone (Oxoid) and 1%, w/v, yeast extract (Scharlau Chemie)] in an orbital shaker (200 r.p.m.) at 30 °C. Cells were harvested and washed in PBS (Sigma-Aldrich), and resuspended in PBS at cell densities of either 5×10^5 or 1×10^6 cells per 20 μ l. Larvae were inoculated through the last left pro-leg into the haemocoel with a volume of 20 μ l using a Myjector syringe (Terumo Europe) and placed at 30 °C in the dark. All experiments included a control that consisted of larvae injected with sterile PBS (20 μ l) and/or untouched larvae.

For the assessment of the *in vivo* antifungal activity of caspofungin, larvae of *G. mellonella* were inoculated with *C. albicans* as described, and 1 h post-inoculation were further injected with a 20 μ l volume of caspofungin at concentrations of 0.19, 0.095 or 0.0475 μ g ml⁻¹ (Merck) or PBS, and placed at 30 °C for 24, 48 and 72 h. The concentrations of caspofungin used here relate to the MIC₉₀, 1/2 MIC₉₀ and 1/4 MIC₉₀ values for *C. albicans*, respectively (Kelly *et al.*, 2009).

Alternatively, larvae were injected with PBS or caspofungin (as above), placed at 30 °C for 24 h and subsequently inoculated with a 20 μ l volume of either *C. albicans* (5×10^5 cells) or *S. aureus* [clinical isolate grown in nutrient broth (Oxoid) at 37 °C and 200 r.p.m. to

early stationary phase]. A culture of *S. aureus* with an OD₆₀₀ value of 1 was diluted 1 in 4 in PBS for the inoculation. Infected larvae were placed at 30 °C and survival was monitored over 72 h.

Determination of the haemocyte density of *G. mellonella*.

G. mellonella larvae were injected with PBS or caspofungin as above, placed at 30 °C, and the haemocyte density was determined after 4 and 24 h. Haemocyte density was ascertained by collecting the haemolymph in a pre-chilled 1 ml tube containing a few grains of 1-phenyl-3-(2-thiazolyl)-2-thiourea (Sigma-Aldrich) to prevent melanization. Haemolymph was diluted 1 in 10 in cold PBS containing 0.37% (v/v) 2-mercaptoethanol (Sigma-Aldrich) to reduce clotting and melanization. Haemocytes were counted on a haemocytometer and the density (ml haemolymph)⁻¹ was calculated.

RNA extraction from *G. mellonella*. Larvae were inoculated with PBS or caspofungin (0.19, 0.095 or 0.0475 μ g ml⁻¹) and incubated at 30 °C for 4 h. Three larvae per treatment were selected, and crushed using liquid nitrogen and a sterile pestle and mortar. To this, 3 ml TRI-reagent (Sigma-Aldrich) was added, mixed and left at room temperature for 15 min. The mixture was placed in sterile 1.5 ml tubes and centrifuged at 12 000 g for 10 min at 4 °C (Eppendorf centrifuge 5417R). The pellets were discarded and the supernatants collected. Chloroform (200 μ l) (molecular grade; Sigma-Aldrich) was added and mixed vigorously by vortexing. The solution was allowed to stand at room temperature for 10 min and centrifuged at 12 000 g for 10 min at 4 °C. The top layer was collected, placed in a tube and 500 μ l 2-propanol (molecular grade; Sigma-Aldrich) was added. The tube was inverted several times, left to stand for 10 min and centrifuged at 12 000 g for 10 min at 4 °C. The supernatant was discarded and the resulting pellet was washed in 75% (v/v) ethanol (50 μ l molecular grade; Sigma-Aldrich) and centrifuged at 12 000 g for 10 min at 4 °C. The supernatant was removed completely, and the resulting pellet was air-dried and resuspended in RNase-free water, dispensed into aliquots and stored at -70 °C or used immediately.

cDNA synthesis and PCR analysis from *G. mellonella* RNA.

Extracted RNA was DNase treated (DNaseI; Sigma-Aldrich) prior to cDNA synthesis. cDNA synthesis was performed using a Superscript III first-strand synthesis system for RT-PCR kit from Invitrogen according to the manufacturer's instructions. Primers and cycle conditions were as used by Bergin *et al.* (2006). The housekeeping gene *ACT1* (*actin*) was used as a control. PCR product (4 μ l) was visualized on a 1% (w/v) agarose gel and images were analysed by densitometry to determine the relative fold increase/decrease by using the array analysis tool in the ImageQuantL software.

Effect of caspofungin on the haemolymph protein profile assessed by two-dimensional (2D) gel electrophoretic separation and liquid chromatography-mass spectrometry (LC-MS).

Haemolymph was collected from larvae that had been injected 24 h previously with either PBS or caspofungin (0.19 μ g ml⁻¹), and the protein (300 μ g) was separated by 2D gel electrophoresis as described by Bergin *et al.* (2006). PBS-injected larvae were used as a control. Gel results were visualized using colloidal Coomassie staining. Gel images (in triplicate) were analysed by Progenesis SameSpots software (Nonlinear Dynamics) in order to assess the fold change between the control and caspofungin-injected larvae. Protein spots that displayed altered intensities between control and drug-treatment samples were excised, washed and trypsin digested as described by Shevchenko *et al.* (2006). Samples were analysed using a 6340 Ion Trap LC-MS spectrometer (Agilent Technologies) with BSA as external standards. The mass lists generated were BLAST searched using the Mascot MS/MS ion search program available at <http://www.matrixscience.com>.

Statistical analysis. All experiments were performed on three independent occasions. The results presented are given as the mean \pm SE. A 2-tailed, 2-sample equal variance (homoscedastic) Student's *t*-test was performed and samples with a *P* value \leq 0.05 were deemed significant.

RESULTS

Analysis of the effect of caspofungin on the survival of *G. mellonella* infected with *C. albicans* prior to administration of the drug

To establish the effectiveness of caspofungin in an insect model, larvae of *G. mellonella* were inoculated with *C. albicans* (5×10^5 cells per insect). After 1 h, larvae were injected with caspofungin (0.19, 0.095 or 0.0475 $\mu\text{g ml}^{-1}$; 20 μl) or PBS (20 μl) and incubated for up to 72 h at 30 °C. Larvae that were injected with the given concentrations of caspofungin showed no ill-effects and there was no decrease in survival over 72 h (data not presented). Fig. 1 illustrates the effect of caspofungin on the survival of *G. mellonella* after inoculation with *C. albicans*. A statistically significant increase in survival at all time points was detected for 0.19 $\mu\text{g caspofungin ml}^{-1}$ treatment compared to the control. The survival rate increased from $66.6 \pm 8.8\%$ in the control to $93.3 \pm 3.3\%$ after 24 h with this concentration of caspofungin. After 72 h, survival in the control larvae was $3.3 \pm 3.3\%$ compared to $56.6 \pm 12.0\%$ in larvae that had received caspofungin. Smaller increases in survival were observed using 0.095 and 0.0475 $\mu\text{g caspofungin ml}^{-1}$.

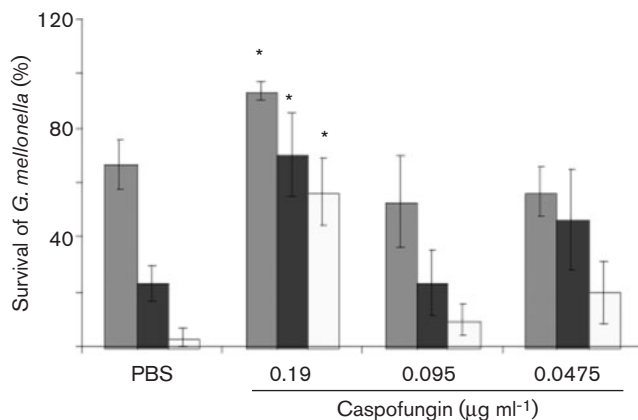


Fig. 1. Effect of caspofungin on the survival of *G. mellonella* inoculated with *C. albicans*. *G. mellonella* were inoculated with *C. albicans* (5×10^5 cells per larva) and subsequently (1 h) injected with PBS (20 μl) or caspofungin (20 μl ; 0.19, 0.095 or 0.0475 $\mu\text{g ml}^{-1}$). Percentage survival was scored at 24, 48 and 72 h. The asterisks indicate a *P* value \leq 0.05 relative to the PBS-injected control. Light grey bars, 24 h; dark grey bars, 48 h; white bars, 72 h.

Analysis of the effect of caspofungin on the survival of *G. mellonella* infected with *C. albicans* or *S. aureus* after administration of the drug

Larvae of *G. mellonella* were injected with PBS or caspofungin, placed at 30 °C for 24 h and inoculated with 20 μl of either *C. albicans* (5×10^5 cells) or *S. aureus* (a culture with an OD_{600} value of 1 was diluted 1 in 4 in PBS). Fig. 2(a) illustrates the effect of pre-injecting larvae with caspofungin 24 h before inoculating with *C. albicans*. A statistically significant increase in survival was observed after 48 h with all concentrations of caspofungin compared to the control. After 48 h, survival increased from $43.3 \pm 3.3\%$ in the control to $80 \pm 5.7\%$ in 0.019 $\mu\text{g caspofungin ml}^{-1}$ injected larvae. A significant increase in survival was also observed after 72 h when 0.19 and 0.0475 $\mu\text{g caspofungin ml}^{-1}$ was administered.

Although caspofungin is a well established antifungal agent, we endeavoured to examine whether the immunomodulatory effect observed above was active against a

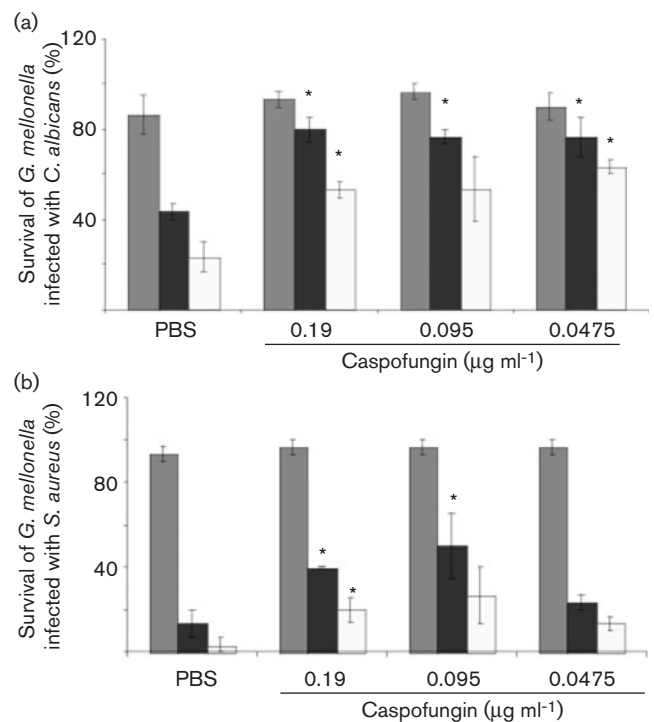


Fig. 2. Effect of caspofungin on the survival of *G. mellonella* inoculated with *S. aureus* or *C. albicans*. (a) *G. mellonella* were injected with caspofungin 24 h before injection with *C. albicans* (5×10^5 cells per larva). Larvae were placed at 30 °C and survival was monitored over 72 h. (b) *G. mellonella* were injected with caspofungin 24 h before injection with 20 μl *S. aureus* (a culture with an OD_{600} value of 1 was diluted 1 in 4 in PBS for use in the inoculation). Larvae were placed at 30 °C and survival was monitored over 72 h. The asterisks indicate a *P* value \leq 0.05 relative to the PBS-injected control. Light grey bars, 24 h; dark grey bars, 48 h; white bars, 72 h.

bacterial infection. Fig. 2(b) illustrates the effect of pre-injecting larvae with caspofungin 24 h before infecting with *S. aureus*. It is evident that pre-injecting larvae with caspofungin resulted in a significant increase in survival of larvae compared to the control. For example, after 48 h larval survival was $13.3 \pm 6.6\%$ in the control and $50 \pm 15.3\%$ when larvae were administered $0.095 \mu\text{g}$ caspofungin ml^{-1} . Indeed, after 72 h, survival of larvae increased from $3.3 \pm 3.3\%$ in the control to $26.6 \pm 13.3\%$ in those larvae injected with the same concentration of caspofungin. These data illustrate that pre-injecting larvae with caspofungin provides protection to subsequent fungal (*C. albicans*) or bacterial (*S. aureus*) infection, indicating that survival is not due to the antifungal activity of caspofungin alone.

Analysis of the effect of caspofungin on haemocyte densities in *G. mellonella*

Administration of a foreign agent to an insect has the potential to evoke an antimicrobial immune response independent of whether the original agent has any inherent antimicrobial properties (Bergin *et al.*, 2006; Mowlds *et al.*, 2010). We investigated whether caspofungin altered the haemocyte density since this is an indication of larval susceptibility to infection (Bergin *et al.*, 2003). Larvae were injected with caspofungin (0.19 , 0.095 and $0.0475 \mu\text{g ml}^{-1}$) or PBS, and after 4 and 24 h, haemocytes were extracted and counted using a haemocytometer. The result (Fig. 3) indicates that after 24 h incubation, caspofungin-treated larvae demonstrated an increase in haemocyte density. The haemocyte density was $1.7 \pm 0.1 \times 10^6$ haemocytes $(\text{ml haemolymph})^{-1}$ in the control, $2.8 \pm 0.3 \times 10^6$ haemocytes $(\text{ml haemolymph})^{-1}$ in larvae that had received a dose of $0.19 \mu\text{g}$ caspofungin ml^{-1} , $3.1 \pm 0.4 \times 10^6$ haemocytes $(\text{ml haemolymph})^{-1}$ in larvae that received a dose of $0.095 \mu\text{g}$ caspofungin ml^{-1} and $2.8 \pm 0.5 \times 10^6$ haemocytes $(\text{ml haemolymph})^{-1}$ in larvae that received $0.0475 \mu\text{g}$ caspofungin ml^{-1} . These results suggest that administration of caspofungin to larvae induced an elevation in haemocyte density, which could contribute to the overall antimicrobial activity within the haemolymph.

Analysis of the effect of caspofungin on the expression of antimicrobial genes in *G. mellonella*

It has been demonstrated previously that survival of larvae of *G. mellonella* following inoculation with *C. albicans* is mediated by an increase in the expression of AMP and immune protein-encoding genes (Bergin *et al.*, 2006). The expression of the genes that encode *transferrin* (iron-binding protein; Yoshiga *et al.*, 1997), *galiomicin* (defensin; Lee *et al.*, 2004), *IMPI* (inducible metallo-proteinase inhibitor; Clermont *et al.*, 2004) and *gallerimycin* (cysteine-rich antifungal peptide; Schuhmann *et al.*, 2003) in *G. mellonella* in response to caspofungin administration was investigated. Expression of the *IMPI*-encoding gene increased to $206.1 \pm$

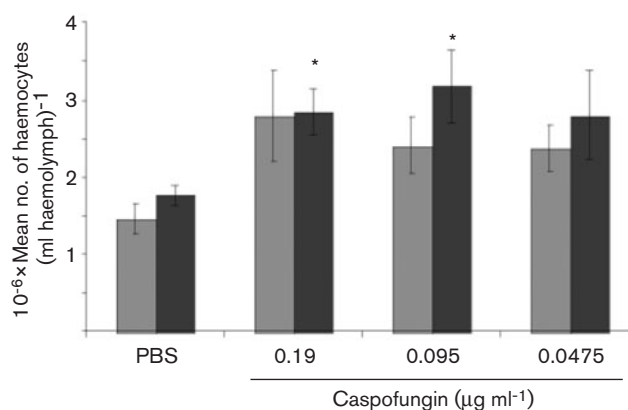


Fig. 3. Effect of caspofungin on the haemocyte density in *G. mellonella*. Haemocyte density in larvae of *G. mellonella* injected with PBS or caspofungin (0.19 , 0.095 or $0.0475 \mu\text{g ml}^{-1}$) was assessed after 4 and 24 h. The asterisks indicate a P value ≤ 0.05 relative to the PBS-injected control. Light grey bars, 4 h; dark grey bars, 24 h.

26.1% relative to the control when $0.095 \mu\text{g}$ caspofungin ml^{-1} was used (Fig. 4). In the case of the *transferrin*-encoding gene, the greatest increase in expression was noted at a concentration of $0.19 \mu\text{g}$ caspofungin ml^{-1} , which gave a relative value of $208.6 \pm 6.7\%$. Smaller increases were observed at the other concentrations of drug used (133.3 ± 19.9 and $128.7 \pm 4.2\%$ at 0.095 and $0.0475 \mu\text{g ml}^{-1}$, respectively). Expression of the *galiomicin*-encoding gene showed small increases over the different concentrations resulting in increases to 122.1 ± 3.6 , 130.4 ± 10.4 and $119.0 \pm 16.0\%$ at 0.19 , 0.095 and $0.0475 \mu\text{g}$ caspofungin ml^{-1} , respectively. The *gallerimycin*-encoding gene demonstrated the smallest alteration in expression following exposure to the drug.

Assessment of the effect of caspofungin on the expression of larval proteins

Larvae were injected with $0.019 \mu\text{g}$ caspofungin ml^{-1} and after 24 h, haemolymph protein was extracted and resolved by 2D electrophoresis in order to determine changes in the protein profile. Spots of interest were excised from 2D gels (Fig. 5) and trypsin-digested prior to identification by LC-MS (Table 1). A number of spots showing homology to proteins with immune function or involved in regulating metabolism were found to be altered in expression. The 32 kDa ferritin subunit I and II (spots 3 and 4) were shown to have increased by 2 and 1.6-fold, respectively, following caspofungin administration. Ferritin is important in iron binding, and storage and maintaining cellular iron homeostasis, along with playing a role in the immune response (Levy *et al.*, 2004). Recent research has demonstrated increased expression of this protein in larvae in response to mercury (Choi *et al.*, 2006). Spot 6 showed homology to prophenoloxidase,

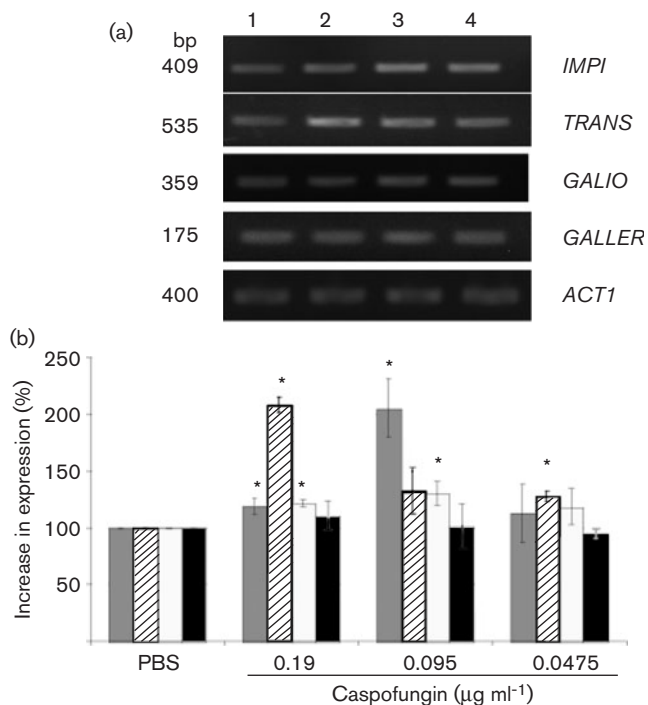


Fig. 4. Relative expression of four genes involved in the immune response of *G. mellonella* and a housekeeping gene. (a) Visualization of PCR products on a 1% (w/v) agarose gel. RT-PCR was performed as described in Methods. Lane: 1, PBS; 2, 0.19 µg caspofungin ml⁻¹; 3, 0.095 µg caspofungin ml⁻¹; 4, 0.0475 µg caspofungin ml⁻¹. (b) Densitometric quantification of the PCR products normalized to the values of the *ACT1* control. The asterisks indicate a *P* value ≤ 0.05 relative to the PBS-injected control. Light grey bars, *IMPI*-encoding gene; hatched bars, *transferrin*-encoding gene; white bars, *galiomicin*-encoding gene; black bars, *gallerimycin*-encoding gene.

which is a protein involved in melanin synthesis, a key defence against pathogens (Kavanagh & Reeves, 2004). Prophenoloxidase is found in the haemocyte as the inactive form of phenoloxidase, which catalyses the formation of melanin. Administration of caspofungin to *G. mellonella* resulted in a 1.9-fold increase in expression. Spot 8 showed homology to juvenile hormone binding protein (JHBP), which is an important regulator of growth, development and reproduction in insects. JHBP carries juvenile hormone (JH) to the target tissues and protects it from digestion by non-specific esterases (Sanburg *et al.*, 1975; Kramer & Childs, 1977). Here it was shown that this protein was increased by 1.2-fold upon caspofungin treatment of larvae. Spot 7 showed homology to arginine kinase, which is a kinase involved in the transfer of a phosphate from ATP, and the protein was observed to have increased by 2.2-fold when larvae were injected with caspofungin. These results demonstrate that upon administration of caspofungin there is an increase in the expression of some proteins with antimicrobial functions or with metabolic activity.

DISCUSSION

Conventional assays to examine the virulence of various microbial pathogens have traditionally relied upon the use of vertebrate species. In recent years it has been demonstrated that insects may be employed to evaluate the virulence of fungi (Cotter *et al.*, 2000; Reeves *et al.*, 2004; Lionakis & Kontoyiannis, 2005; Mylonakis *et al.*, 2005), bacteria (Mansfield *et al.*, 2003) and parasites (Boulanger *et al.*, 2001), and may be employed for the *in vivo* screening of antimicrobial drugs (Hamamoto *et al.*, 2004, 2009; Johny *et al.*, 2007). The immune response of insects is similar to the innate immune response of mammals (Kavanagh & Reeves, 2004), and contains structural and passive barriers, as well as cellular and humoral responses mediated by haemocytes and the fat body within the haemolymph. As a consequence of these similarities there is a strong correlation between the virulence of various pathogens when tested in insects and in mice (Brennan *et al.*, 2002; Jander *et al.*, 2000).

Caspofungin demonstrates potent *in vivo* antifungal activity (Petraitiene *et al.*, 2002) and an excellent safety profile with patients suffering few adverse effects (Maertens *et al.*, 2004; Walsh *et al.*, 2004). In this work it was demonstrated that injection of larvae with caspofungin following inoculation with *C. albicans* resulted in statistically significant increases in survival. Similarly, it was demonstrated that injection of caspofungin before infecting with a fungal or bacterial pathogen significantly increases the survival rate. Interestingly, administration of caspofungin can provide protection against both a fungal (*C. albicans*) and a bacterial (*S. aureus*) infection indicating that, in addition to the inherent antifungal activity of the drug, caspofungin administration also induces a non-specific antimicrobial response in larvae.

Haemocytes are similar to the phagocytes of mammals (Bergin *et al.*, 2005) and the majority circulate freely in the haemolymph, but a significant number can be found associated with internal organs such as the fat body, trachea or digestive system (Ratcliffe, 1985). There are six types of haemocytes, and some are involved in phagocytosis and encapsulation, which are important in the insect cellular defence against microbes (Tojo *et al.*, 2000). The administration of caspofungin to *G. mellonella* larvae caused an increase in haemocyte density after 4 h and this increase was also evident after 24 h, which may be due to the release of haemocytes from the associated organs and/or the proliferation of haemocytes. The ability of caspofungin-treated larvae to survive a fungal or bacterial infection could be due, in part, to the elevation in the haemocyte density evident in such larvae.

G. mellonella can synthesize a number of AMPs that are released into the haemolymph where they can attack invading microbes (Kavanagh & Reeves, 2004). It was observed that the expression of the gene encoding *transferrin* was upregulated approximately twofold when larvae were injected with 0.19 µg caspofungin ml⁻¹. Interestingly, the

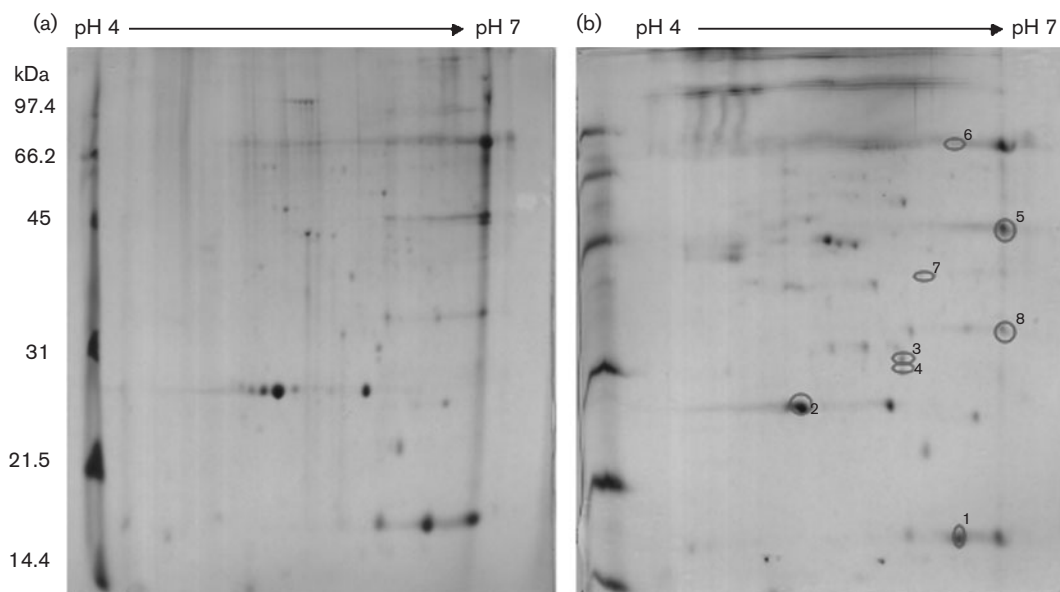


Fig. 5. 2D gels of *G. mellonella* haemolymph protein showing spots chosen for identification by LC–MS. (a) Haemolymph protein separated from PBS-injected larvae. (b) Haemolymph protein separated from ($0.19 \mu\text{g ml}^{-1}$) caspofungin-injected larvae. Spots 1–8 were excised for identification by LC–MS. The gel images are representative images of experiments performed in triplicate.

Table 1. *G. mellonella* protein spots identified by LC–MS plus fold changes in expression in response to caspofungin

Spots were removed, trypsin-digested and analysed by LC–MS as described in Methods. Images were analysed with Progenesis SameSpots software to assess the fold change in expression.

Protein	Spot no.	Fold change upon injection of $0.19 \mu\text{g caspofungin ml}^{-1}$ (compared to control)	Mascot score	Coverage (%)	UniProtKB accession no.	Function
Apolipoporphin III	1	–	459	44	P80703	Antimicrobial; antibiotic; induced by bacterial infection; immune response; transport of lipids
27 kDa haemolymph protein	2	+1.2	552	49	P83632	Secreted; signal; extracellular region
32 kDa ferritin subunit I	3	+2	329	20	Q8WSA5	Ferric iron binding and transport; oxidoreductase activity
32 kDa ferritin subunit II	4	+1.6	314	20		
Transferrin precursor	5	–1.2	609	21	Q6UQ29	Cellular iron ion homeostasis and transport; extracellular region; ferric iron binding
Prophenoloxidase subunit II	6	+1.9	393	10	Q6UEH6	Transport; oxygen transporter activity; oxidoreductase activity
Arginine kinase	7	+2.2	201	19	B3TG12	Transferase, kinase activity
JHBP	8	+1.2	182	20	Q8ITP4	JH regulates embryogenesis and stimulates reproductive maturation in the adult; JHBP protects JH molecules from hydrolysis by non-specific esterases

protein transferrin precursor (spot 5 in Fig. 5) was observed to have a fold change of -1.2 when larvae were injected with caspofungin. A possible reason for this difference may be due to the time at which the larvae were sampled after administration of the drug (gene expression after 4 h, protein analysis after 24 h). The levels of *transferrin* in *G. mellonella* may initially rise upon caspofungin injection, but may decrease after several hours. Expression of *IMPI* was also increased approximately twofold following caspofungin treatment ($0.095 \mu\text{g ml}^{-1}$). Smaller increases were observed for *galiomicin* and *gallerimycin* following administration of the agent. These results suggest that while caspofungin may not induce the expression of a wide range of AMPs it may activate a selected subset of proteins involved in the immune response (i.e. *transferrin* and *IMPI*). This phenomenon has been observed previously where physical stress induced the expression of a selected number of AMPs in *G. mellonella* (Mowlds *et al.*, 2008). Analysis of the proteome of larvae that had received caspofungin 24 h previously revealed the increased expression of a number of peptides that show homology to proteins that exhibit immune properties (e.g. prophenoloxidase, ferritin), and those involved with growth and development (arginine kinase, JHBP).

An increase in AMP or immune protein gene expression and haemocyte density in *G. mellonella* following caspofungin treatment may indicate a novel means of protection against infection. It appears that not only can caspofungin kill fungal cells *in vivo*, but also it may enhance the insect's natural defences against infection. The injection of non-self material into the haemocoel of *G. mellonella* larvae can prime the immune response against a subsequent microbial challenge although the original material may have no inherent antimicrobial properties (Bergin *et al.*, 2006; Mowlds *et al.*, 2010). Administration of mercury to larvae also resulted in an increase in the expression of ferritin, which has a role in the insect's immune response (Choi *et al.*, 2006). The ability of caspofungin to stimulate the larval immune response is an added benefit in that it constitutes a second line of attack against the invading pathogen.

While the utility of employing insects to assess the virulence of microbial pathogens or examine the *in vivo* properties of antimicrobial agents is now well established, researchers should be aware that, in addition to the inherent antimicrobial activity of the test agent, administration of the agent to larvae can trigger an increase in the insect's immune response. Controls to allow discrimination between the antimicrobial properties of a novel agent and the alteration in the immune response of insects induced by the agent should be included in all experiments to ensure that the efficacy of the antimicrobial agent, rather than its immune priming abilities, is measured.

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REFERENCES

- Bergin, D., Brennan, M. & Kavanagh, K. (2003). Fluctuations in haemocyte density and microbial load may be used as indicators of fungal pathogenicity in larvae of *Galleria mellonella*. *Microbes Infect* **5**, 1389–1395.
- Bergin, D., Reeves, E. P., Renwick, J., Wientjes, F. B. & Kavanagh, K. (2005). Superoxide production in *Galleria mellonella* hemocytes: identification of proteins homologous to the NADPH oxidase complex of human neutrophils. *Infect Immun* **73**, 4161–4170.
- Bergin, D., Murphy, L., Keenan, J., Clynes, M. & Kavanagh, K. (2006). Pre-exposure to yeast protects larvae of *Galleria mellonella* from a subsequent lethal infection by *Candida albicans* and is mediated by the increased expression of antimicrobial peptides. *Microbes Infect* **8**, 2105–2112.
- Boulanger, N., Ehret-Sabatier, L., Brun, R., Zachary, D., Bulet, P. & Imler, J. L. (2001). Immune response of *Drosophila melanogaster* to infection with the flagellate parasite *Crithidia* spp. *Insect Biochem Mol Biol* **31**, 129–137.
- Brennan, M., Thomas, D. Y., Whiteway, M. & Kavanagh, K. (2002). Correlation between virulence of *Candida albicans* mutants in mice and *Galleria mellonella* larvae. *FEMS Immunol Med Microbiol* **34**, 153–157.
- Choi, C. W., Seo, D. H., Yuk, J. E., Park, J. B., Hwang, S. Y., Koh, S. K. & Yun, C. Y. (2006). Upregulation of hemolymph and tissue ferritin by dietary HgCl_2 in the wax moth *Galleria mellonella*. *Entomol Res* **36**, 185–190.
- Clermont, A., Wedde, M., Seitz, V., Podsiadlowski, L., Lenze, D., Hummel, M. & Vilcinskis, A. (2004). Cloning and expression of an inhibitor of microbial metalloproteinases from insects contributing to innate immunity. *Biochem J* **382**, 315–322.
- Cotter, G., Doyle, S. & Kavanagh, K. (2000). Development of an insect model for the *in vivo* pathogenicity testing of yeasts. *FEMS Immunol Med Microbiol* **27**, 163–169.
- Dunphy, G. B., Morton, D. B., Kropinski, A. & Chadwick, J. M. (1986). Pathogenicity of lipopolysaccharide mutants of *Pseudomonas aeruginosa* for larvae of *Galleria mellonella*: bacterial properties associated with virulence. *J Invertebr Pathol* **47**, 48–55.
- Eggimann, P., Garbino, J. & Pittet, D. (2003). Management of *Candida* species infections in critically ill patients. *Lancet Infect Dis* **3**, 772–785.
- Hamamoto, H., Kurokawa, K., Kaito, C., Kamura, K., Razanajatovo, I. M., Kusuhara, H., Santa, T. & Sekimizu, K. (2004). Quantitative evaluation of the therapeutic effects of antibiotics using silkworms infected with human pathogenic microorganisms. *Antimicrob Agents Chemother* **48**, 774–779.
- Hamamoto, H., Tonoike, A., Narushima, K., Horie, R. & Sekimizu, K. (2009). Silkworm as a model animal to evaluate drug candidate toxicity and metabolism. *Comp Biochem Physiol C Toxicol Pharmacol* **149**, 334–339.
- Jander, G., Rahme, L. & Ausbel, F. (2000). Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. *J Bacteriol* **182**, 3843–3845.
- Johny, S., Lange, C. E., Solter, L. F., Merisko, A. & Whitman, D. W. (2007). New insect system for testing antibiotics. *J Parasitol* **93**, 1505–1511.
- Kartsonis, N. A., Nielsen, J. & Douglas, C. M. (2003). Caspofungin: the first in a new class of antifungal agents. *Drug Resist Updat* **6**, 197–218.
- Kavanagh, K. & Reeves, E. P. (2004). Exploiting the potential of insects for *in vivo* pathogenicity testing of microbial pathogens. *FEMS Microbiol Rev* **28**, 101–112.

- Kelly, J., Rowan, R., McCann, M. & Kavanagh, K. (2009).** Exposure to caspofungin activates Cap and Hog pathways in *Candida albicans*. *Med Mycol* **47**, 697–706.
- Kramer, K. J. & Childs, C. N. (1977).** Interaction of juvenile hormone with carrier proteins and hydrolases from insect haemolymph. *Insect Biochem* **7**, 397–403.
- Lavine, M. D. & Strand, M. R. (2002).** Insect haemocytes and their role in immunity. *Insect Biochem Mol Biol* **32**, 1295–1309.
- Lee, Y. S., Yun, E. K., Jang, W. S., Kim, I., Lee, J. H., Park, S. Y., Ryu, K. S., Seo, S. J., Kim, C. H. & Lee, I. H. (2004).** Purification, cDNA cloning and expression of an insect defensin from the great wax moth, *Galleria mellonella*. *Insect Mol Biol* **13**, 65–72.
- Levy, F., Bulet, P. & Ehret-Sabatier, L. (2004).** Proteomic analysis of the systemic immune response of *Drosophila*. *Mol Cell Proteomics* **3**, 156–166.
- Lionakis, M. S. & Kontoyiannis, D. P. (2005).** Fruit flies as a minihost model for studying drug activity and virulence in *Aspergillus*. *Med Mycol* **43**, S111–S114.
- Maertens, J., Raad, I., Petrikkos, G., Boogaerts, M., Selleslag, D., Petersen, F. B., Sable, C. A., Kartsonis, N. A., Ngai, A. & other authors (2004).** Efficacy and safety of caspofungin for treatment of invasive aspergillosis in patients refractory to or intolerant of conventional antifungal therapy. *Clin Infect Dis* **39**, 1563–1571.
- Mansfield, B. E., Dionne, M. S., Schneider, D. S. & Freitag, N. E. (2003).** Exploration of host-pathogen interactions using *Listeria monocytogenes* and *Drosophila melanogaster*. *Cell Microbiol* **5**, 901–911.
- Matha, V. & Acek, Z. (1984).** Changes in haemocyte counts in *Galleria mellonella* (L.) (Lepidoptera: Galleriidae) larvae infected with *Steinernema* sp. (Nematoda: Steinernematidae). *Nematologica* **30**, 86–89.
- Mowlds, P., Barron, A. & Kavanagh, K. (2008).** Physical stress primes the immune response of *Galleria mellonella* larvae to infection by *Candida albicans*. *Microbes Infect* **10**, 628–634.
- Mowlds, P., Coates, C., Renwick, J. & Kavanagh, K. (2010).** Dose-dependent cellular and humoral responses in *Galleria mellonella* larvae following β -glucan inoculation. *Microbes Infect* **12**, 146–153.
- Mylonakis, E., Moreno, R., El Khoury, J. B., Idnurm, A., Heitman, J., Calderwood, S. B., Ausubel, F. M. & Diener, A. (2005).** *Galleria mellonella* as a model system to study *Cryptococcus neoformans* pathogenesis. *Infect Immun* **73**, 3842–3850.
- Petraitiene, R., Petraitis, V., Groll, A. H., Sein, T., Schaufele, R. L., Francesconi, A., Bacher, J., Avila, N. A. & Walsh, T. J. (2002).** Antifungal efficacy of caspofungin (MK-0991) in experimental pulmonary aspergillosis in persistently neutropenic rabbits: pharmacokinetics, drug disposition, and relationship to galactomannan antigenemia. *Antimicrob Agents Chemother* **46**, 12–23.
- Ratcliffe, N. A. (1985).** Invertebrate immunity – a primer for the non-specialist. *Immunol Lett* **10**, 253–270.
- Reeves, E. P., Messina, C. G. M., Doyle, S. & Kavanagh, K. (2004).** Correlation between gliotoxin production and virulence of *Aspergillus fumigatus* in *Galleria mellonella*. *Mycopathologia* **158**, 73–79.
- Rock, F. L., Hardiman, G., Timans, J. C., Kastelein, R. A. & Bazan, J. F. (1998).** A family of human receptors structurally related to *Drosophila* Toll. *Proc Natl Acad Sci U S A* **95**, 588–593.
- Sanburg, L. L., Kramer, K. J., Kezdy, F. J., Law, J. H. & Oberlander, H. (1975).** Role of juvenile hormone esterases and carrier proteins in insect development. *Nature* **253**, 266–267.
- Schuhmann, B., Seitz, V., Vilcinskas, A. & Podsiadlowski, L. (2003).** Cloning and expression of gallerimycin, an antifungal peptide expressed in immune response of greater wax moth larvae, *Galleria mellonella*. *Arch Insect Biochem Physiol* **53**, 125–133.
- Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V. & Mann, M. (2006).** In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* **1**, 2856–2860.
- Tojo, S., Naganuma, F., Arakawa, K. & Yokoo, S. (2000).** Involvement of both granular cells and plasmatocytes in phagocytic reactions in the greater wax moth *Galleria mellonella*. *J Insect Physiol* **46**, 1129–1135.
- Walsh, T. J., Teppler, H., Donowitz, G. R., Maertens, J. A., Baden, L. R., Dmoszynska, A., Cornely, O. A., Bourque, M. R., Lupinacci, R. J. & other authors (2004).** Caspofungin versus liposomal amphotericin B for empirical antifungal therapy in patients with persistent fever and neutropenia. *N Engl J Med* **351**, 1391–1402.
- Wittwer, D., Franchini, A., Ottaviani, E. & Wiesner, A. (1999).** Presence of IL-1 and TNF like molecules in *Galleria mellonella* (Lepidoptera) haemocytes and in an insect cell line from *Estimene acraea* (Lepidoptera). *Cytokine* **11**, 637–642.
- Yoshiga, T., Hernandez, V. P., Fallon, A. M. & Law, J. H. (1997).** Mosquito transferrin, an acute-phase protein that is up-regulated upon infection. *Proc Natl Acad Sci U S A* **94**, 12337–12342.
- Zhao, L. & Kanost, M. R. (1996).** In search of a function for hemolin, a haemolymph protein from the immunoglobulin superfamily. *J Insect Physiol* **42**, 73–79.