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Assessment of in vivo antimicrobial activity of the carbene silver(I) acetate derivative SBC3 using *Galleria mellonella* larvae

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Abstract The antimicrobial drug candidate 1,3-dibenzyl-4,5-diphenyl-imidazol-2-ylidene silver(I) acetate (SBC3) was evaluated for its ability to function in vivo using larvae of *Galleria mellonella*. A SBC3 concentration of 25 μg/ml inhibited the growth of *Staphylococcus aureus* by 71.2 % and *Candida albicans* by 86.2 % in vitro. Larvae inoculated with 20 μl of SBC3 solution showed no ill effects up to a concentration of 250 μg/ml but administration of 500 μg/ml resulted in a 40 % reduction in larval survival and administration of a dose of 1,000 μg/ml resulted in total larval death at 24 h. Larvae inoculated with *S. aureus* or *C. albicans* and subsequently administered SBC3 showed increased survival. Administration of SBC3 to larvae did not boost the insect immune response as indicated by lack of an increase in the density of circulating haemocytes (immune cells). The abundance of a number of proteins involved in the insect immune response was reduced in larvae that received 20 μl SBC3 solution of 100 μg/ml. This is the first demonstration of the in vivo activity of SBC3 against *S. aureus* and *C. albicans* and demonstrates that SBC3 does not stimulate a non-specific immune response in larvae.

Keywords Antimicrobial drug · *Candida albicans* · Carbene-silver complex · *Galleria mellonella* · In vivo toxicity · *Staphylococcus aureus*

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Introduction

Extensive use and misuse of the available antibiotic arsenal and the shortage of new drugs reaching the market have made antimicrobial resistance a major threat to healthcare worldwide (Maple et al. 1989). The continuous development of novel resistance-disrupting antimicrobial drugs should therefore be a prime activity of industry and academia (Rex 2014). There is significant unexplored space for antimicrobial silver-based drugs (Mijnendonckx et al. 2013; Oehninger et al. 2013) and it has been suggested that carbene-silver acetates derived from methylated caffeine may have the stability and antibiotic activity to become drug candidates (Kascatan-Nebioglu et al. 2006; Hindi et al. 2008). This idea led to the development of more lipophilic benzyl-substituted imidazole- and benzimidazole-derived carbene-silver complexes showing activity against Gram-positive and Gram-negative bacteria (Hackenberg and Tacke 2014; Patil and Tacke 2011). So far, the most promising derivative is 1,3-dibenzyl-4,5-diphenyl-

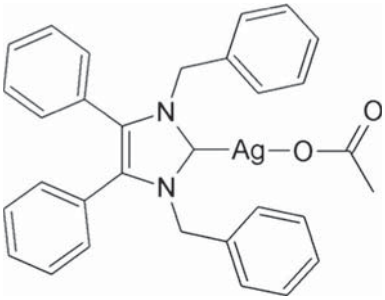

Fig. 1 Molecular structure of SBC3

imidazol-2-ylidene silver(I) acetate (SBC3) (Patil et al. 2011) (Fig. 1). SBC3 shows MIC values ranging from 20 to 3.13 μg/ml against Methicillin-sensitive and -resistant *Staphylococcus aureus* as well as *Salmonella*, *Escherichia coli* and *Pseudomonas aeruginosa* (Sharkey et al. 2012).

Evaluating the in vivo efficacy and stability of novel antimicrobial compounds is possible using insects. The insect immune system shows many similarities to the innate immune system of mammals (Kavanagh and Reeves 2004; Browne et al. 2013) and, as a consequence, insects have been used as models to measure the virulence of microbial pathogens (Fuchs and Mylonakis 2006) and to evaluate the potency of antimicrobial drugs (Hamamoto et al. 2004; Lionakis et al. 2005; Lionakis and Kontoyiannis 2005; Rowan et al. 2009) and give results consistent with those that can be obtained using mammals (Jander et al. 2000; Brennan et al. 2002). A number of insect species can be employed for evaluating the in vivo activity of novel antimicrobial drugs (Kavanagh and Fallon 2010) and larvae of the Greater Wax Moth, *Galleria mellonella* are now widely used in this capacity (Kelly and Kavanagh 2011; Rowan et al. 2009; Desbois and Coote 2012).

This paper describes the antimicrobial effects of SBC3 against the bacterium *S. aureus* and the yeast *Candida albicans* in vitro and demonstrates the in vivo activity and toxicity of SBC3 against these two pathogens in *G. mellonella* larvae.

Materials and methods

Synthesis of SBC3

SBC3 (1,3-dibenzyl-4,5-diphenyl-imidazol-2-ylidene silver(I) acetate) was synthesised as published (Patil et al. 2011) and dissolved in a minimal amount of DMSO (dimethylsulfoxide) before being diluted with water-based media as described.

Inoculation of *G. mellonella* larvae

Sixth instar larvae of *G. mellonella* (Lepidoptera: Pyralidae, the Greater Wax Moth) (Mealworm Company, Sheffield, England) were stored in the dark at 15 °C (Cotter et al. 2000; Hornsey and Wareham 2011). Larvae of the same age and weighing 0.3 g were inoculated with 20 μl of PBS containing 1×10^6 *C. albicans* cells or 4×10^7 *S. aureus* cells through the last pro-leg using a Myjector U100 insulin syringe (Terumo Europe, Leuven, Belgium).

Microbial cell culture

Staphylococcus aureus was cultured to the stationary phase ($OD_{600nm} = 2$) overnight in nutrient broth at 37 °C and 200 rpm. *C. albicans* was cultured to the stationary phase (approx. 1×10^8/ml) overnight in YEPD broth (2 % (w/v) glucose, 2 % (w/v) bacteriological peptone, 1 % (w/v) yeast extract) at 30 °C and 200 rpm.

Effect of SBC3 on growth of *S. aureus* and *C. albicans*

To each well of a 96–well plate (Sarsdedt), 100 μl of fresh nutrient broth medium (for *S. aureus* growth) or minimal medium (2 % (w/v) glucose, 0.17 % (w/v) yeast nitrogen base and 0.5 % (w/v) ammonium sulphate) (for *C. albicans* growth) were added. A serial dilution of SBC3 was perfomed on the plate to give a concentration range of 0.39–100 μg/ml. Cell suspensions (100 μl) of *S. aureus* (4×10^7 cells/ml) or *C. albicans* (1×10^6 cells/ml) were added to each well and the plates were incubated at 37 or 30 °C, respectively, for 24 h. The OD_{570nm} of the cultures was determined using a microplate reader (Bio-Tek, Synergy HT) and all growth was expressed as a percentage of that in the control. All susceptibility assays were performed on three separate occasions.

In vivo toxicity assay

Larvae were injected with 20 μl of SBC3 solution (10, 100, 250, 500 or 1,000 μg/ml) or 5 % (v/v) DMSO

through the last left pro-leg as described. Larvae were incubated at 30 °C for 24 h prior to quantifying survival.

Effect of SBC3 on survival of larvae infected with *S. aureus* or *C. albicans*

Larvae were injected with 20 µl of SBC3 solution (10, 100 or 250 µg/ml) 4 h post-inoculation with *S. aureus* or *C. albicans*. The control consisted of larvae inoculated with the *C. albicans* or *S. aureus.* Larvae were incubated at 30 °C and survival was assessed at 24, 48 and 72 h.

Determination of larval haemocyte density

The density of circulating haemocytes in larvae was assessed as described previously (Rowan et al. 2009). Experiments were performed on three independent occasions and the mean ± SE were determined.

2 D SDS-PAGE analysis of protein expression in larvae administered SBC3

Haemolymph (100 µl) was collected from larvae inoculated with 20 µl SBC3 (100 µg/ml) or 5 % (v/v) DMSO following incubation at 30 °C for 24 h. Protein concentration was determined by Bradford assay and protein was diluted to 200 µg per sample. Isoelectric focusing of protein samples on a pH 4-7 strip and 2D electrophoresis was performed as described previously (Bergin et al. 2006). Each 2D gel was scanned on a Hewlett Packard scanjet 5100c scanner and the images were analyzed using Progenesis SameSpot Software. The Progenesis software enabled the analysis of protein expression changes between gel replicates with significance determined using ANOVA. A table of protein spots was built and every protein was linked to the matching proteins between the gels creating a list of proteins that can be cross referenced as a final check to ensure correct alignment.

LC/MS analysis of peptides

In-gel digestion was performed on 2-Dimensional gel spots of interest from a reference gel. The gel pieces were excised, trypsin digested (Shevchenko et al. 2006) and fragmented protein samples were eluted through the LC/MS (Aglient 6340 Ion Trap) which determines the relative charge to mass ratio from detected ionized particles. The data were analysed using the mascot search engine to identify the protein (<http://www.matrixscience.com) or via (*) Spectrum Mill MS Proteomics. MASCOT scores above 67 are deemed to have a significant match ($p < 0.05$). The mass error tolerance was 1 Da allowing for a maximum of no more than two missed cleavages. Verification of protein sequences was confirmed by blasting the protein sequence on the Uniprot (www.uniprot.org) and NCBI (www.ncbi.nlm.nih.gov) websites. Progenesis was used to determine the protein fold changes between 2-Dimensional gels.

Statistical analysis

All experiments were performed on three independent occasions and results are expressed as the mean ± SE. Changes in larval survival were analysed with the log rank (Mantel-Cox) method using GraphPad Prism version 5.00. Analysis of changes in haemocyte density, in vitro toxicity and protein expression were performed by One-way ANOVA using GraphPad Prism version 5.00 for Windows 8, GraphPad Software, San Diego, California USA, (www.graphpad.com).

Results

Determination of in vitro efficacy of SBC3 against *S. aureus* and *C. albicans*

The effect of SBC3 on the growth of *S. aureus* and *C. albicans* was measured as described. In the case of *S. aureus* a concentration of 25 µg/ml resulted in 71.2 ± 1.28 % ($p < 0.001$) inhibition of growth (Fig. 2) while the same concentration resulted in 86.2 ± 1.42 % ($p < 0.001$) inhibition in the growth of *C. albicans* (Fig. 2). Both microbes appear susceptible to SBC3 but there appears to be greater inhibition of the growth of *C. albicans* than *S. aureus* at the higher concentrations (>25 µg/ml).

Determination of effect of SBC3 on larval viability

The effect of SBC3 on the viability of *G. mellonella* larvae was assessed as described. The results (Fig. 3)

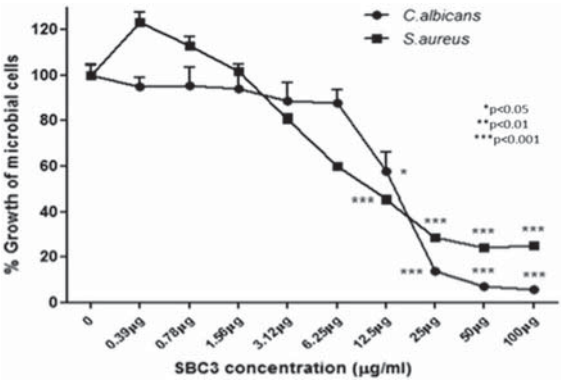

Fig. 2 In vitro toxicity of SBC3 to *S. aureus* and *C. albicans*

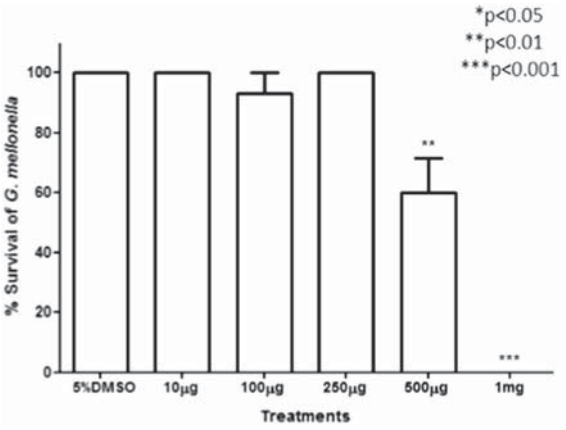

Fig. 3 Larval survival 24 h after administration with 20 µl of control solution (5 % DMSO) or SBC3 (10, 100, 250, 500, 1,000 µg/ml)

indicate no toxic effect up to a concentration of 250 µg/ml however larvae inoculated with a dose of 500 µg/ml showed a 40.0 ± 11.54 % ($p < 0.01$) reduction in viability after 24 h. Larvae inoculated with SBC3 (10, 100, 250 µg/ml) did not show signs of cuticular darkening (melanisation) which would be an indicator of acute toxicity.

Effect of SBC3 on survival of larvae infected with *S. aureus* or *C. albicans*

In order to ascertain the in vivo activity of SBC3, larvae were infected with each pathogen as described and subsequently administered 20 µl of SBC3 (10, 100 or 250 µg/ml) 4 h post infection. Those larvae infected with *S. aureus* showed 15 ± 5.0 % survival at 72 h however the larvae administered a dose of 10, 100 or 250 µg/ml SBC3 showed 46.7 ± 12.01 % ($p < 0.05$), 36.7 ± 3.33 % and 40.0 ± 5.77 % survival, respectively, at the same time point (Fig. 4a). Larvae inoculated with *C. albicans* alone showed 100 % mortality at 48 h. However those larvae inoculated with a dose of 10, 100 or 250 µg/ml SBC3 subsequent to infection showed 30 ± 5.77 % ($p < 0.01$), 16.7 ± 6.66 % ($p < 0.05$) and 13.3 ± 3.33 % ($p < 0.05$) survival at the same time point, respectively (Fig. 4b).

Assessment of the effect of SBC3 on the immune response *G. mellonella* larvae

The cellular immune response of insects is mediated by immune cells (haemocytes) which phagocytose and kill invading pathogens (Lavine and Strand 2002) and by the production of antimicrobial peptides (Boman and Hultmark 1987). Previous work has demonstrated that introduction of selected agents (e.g. silver nitrate, caspofungin, β-glucan) into the haemocoel of *G. mellonella* larvae can prime the immune response and lead to an increase in the density of circulating haemocytes and in the expression of antimicrobial and immune related peptides (Rowan et al. 2009; Kelly and Kavanagh 2011; Mowlds et al. 2010). In order to exclude the possibility that the observed in vivo antimicrobial activity of SBC3 was due to the increased density of immune cells in the larvae, the density of circulating haemocytes in larvae was assessed. The results indicate that there was no significant change in the haemocyte density in those larvae that received doses of 10 and 100 µg/ml SBC3 (Fig. 5). Those larvae that received 20 µl doses of 500 or 1,000 µg/ml demonstrated a 76.7 ± 1.0 % or 55.9 ± 4.0 % reduction, respectively, in haemocyte density relative to the control. This may have arisen due to lysis of the haemocytes by the SBC3.

The humoral immune response of insects is mediated by a series of antimicrobial peptides and immune related peptides (Kavanagh and Reeves 2004). The proteomic profile of larvae that had received SBC3 was analysed in order to determine whether exposure to the compound increased this element of the larval immune response (Fig. 6). The results indicate that the abundance of a number of proteins is reduced in larvae that received SBC3 (Table 1). Proteins such as larval

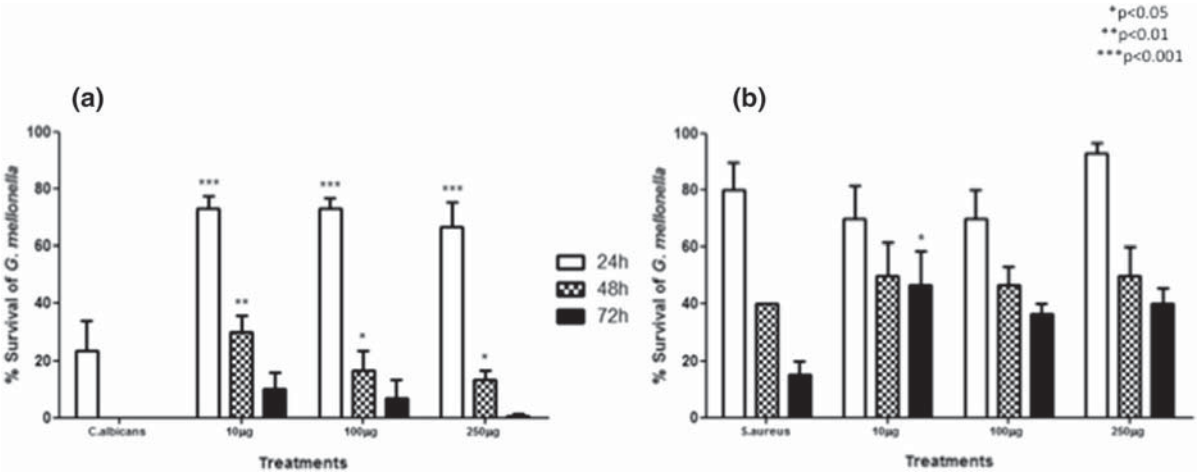

Fig. 4 Survival of larvae infected with (**a**) *S. aureus* or (**b**) *C. albicans* following administration with SBC3 solution (20 µl of 10, 100 or 250 µg/ml) 4 h after infection

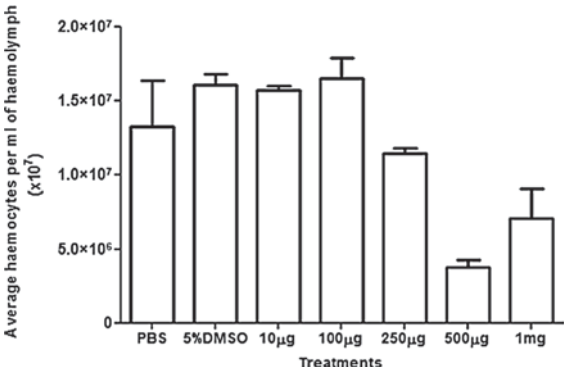

Fig. 5 Haemocyte density of larvae administered PBS, 5 % DMSO or SBC3 (10, 100, 250, 500, 1,000 µg/ml) at 24 h

haemolymph protein, 27 kDa haemolymph protein and apolipophorin-3 were decreased in intensity. The abundance of arylphorin was increased by 2-fold.

Discussion

Due to the many similarities between the immune system of insects and the innate immune system of mammals, insects have become popular choices for measuring the virulence of microbial pathogens and give results comparable to those that can be obtained using mammals (Brennan et al. 2002; Jander et al. 2000). Insects have the benefit of being inexpensive to purchase and house, and can give results in 24–48 h. Insect can also be used to evaluate the in vivo activity of antimicrobial drugs (Hamamoto et al. 2004; Lionakis et al. 2005) but caution must be exercised in their use as the introduction of foreign material into the insect haemoceol can provoke a non-specific immune response (Mowlds et al. 2010; Kelly and Kavanagh 2011).

The antimicrobial activity of silver is well established (Percival et al. 2005) and silver has been

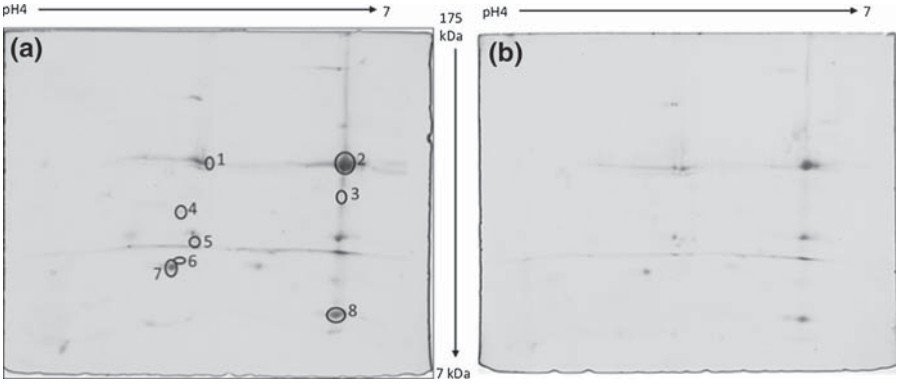

Fig. 6 2D SDS-PAGE of separated haemolymph proteins from *G. mellonella* treated with (**a**) 5 % DMSO or (**b**) SBC3 (100 µg/ml). Proteins of interest (1–8) were excised and identified by LC/MS

Table 1 Identity of proteins showing alteration in abundance in larvae that received a 20 µl dose of SBC3 (100 µg/ml). (* Spectrum Mill MS Proteomics analysis)

Spot no.	Protein identity	Organism	Mr	PI	Score	Sequence coverage (%)	Accession no.	Relative fold change	
								DMSO (Control)	100 µg SBC3
1	Arylphorin	*G. mellonella*	83,651	5.23	1,280	40	AAA74229	1	2.01
2	Larval hemolymph protein	*G. mellonella*	13,783	9.08	74	10	ABG91580	1	0.80
3	Carboxylesterase	*G. mellonella*	58,669	8.34	85	15	*	1	0.68
4	Masquerade-like serine proteinase	*P. rapae*	46,063	5.54	173	7	ACZ68116	1	0.58
5	Actin-4	*B. mori*	42,200	5.47	560	48	AGR44814	1	0.85
6	Hypothetical protein YQE_03765, partial	*D. ponderosae*	54,682	5.44	79	3	ENN79708	1	1.27
7	27 kDa hemolymph protein	*G. mellonella*	26,387	5.17	597	57	P83632	1	0.57
8	Apolipophorin-3	*G. mellonella*	20,499	8.59	659	54	P80703	1	0.79

incorporated into many medical products (e.g. catheters, plasters) where it can limit the growth of bacteria and the development of biofilms (Adams et al. 1999). While the mode of action of silver is not fully elucidated it has been shown to inactivate NADH and succinate dehydrogenase and so interfere with electron transfer in respiration (Park et al. 2009). Exposure to silver may also lead to the generation of reactive oxygen species (ROS) which induces protein and DNA degradation (Park et al. 2009; Atiyeh et al. 2007; Castellano et al. 2007). Previous work demonstrated the induction of an oxidative stress response in *C. albicans* when exposed to Ag(I) ions (Rowan et al. 2010).

The experimental drug SBC3 was demonstrated to be of low toxicity to *G. mellonella* larvae and the results presented here indicated that larvae infected with *C. albicans* or *S. aureus* and subsequently administered SBC3 demonstrated increased survival. SBC3 does not provoke an enhanced immune response as indicated by the lack of an increase in the haemocyte density and the expression of antimicrobial and immune related peptides. A number of proteins were shown to decrease in abundance in larvae that received SBC3 and these included carboxylesterase (spot 3, Table 1) which has physiological functions in neurotransmitter degradation, hormone and pheromones specific metabolism, detoxification, defense and behavior (Vogt et al. 1985; Taylor and Radic 1994), 27 kDa haemolymph protein (spot 2) which is secreted into the haemolymph as a signal molecule (Kelly and Kavanagh 2011) and apolipophorin-3 (spot 8) which functions in the innate immune response (Gupta et al. 2010). Spot 6, hypothetical protein YQE_03765 partial, was increased in abundance in larvae that received SBC3 and functions as a death domain protein with roles in apoptosis, immune defense, and immune signalling (Lahm et al. 2003). The abundance of arylphorin (spot 1), which is involved in storage of amino acids and in aiding the immune defence of insects (Beresford et al. 1997), was increased in abundance by 2-fold. In contrast, administration of silver nitrate to *G. mellonella* larvae stimulated the immune response by increasing the density of circulating haemocytes (Rowan et al. 2009). One possibility for this difference in the response of *G. mellonella* larvae to silver in silver nitrate and in SBC3, is that silver nitrate releases ionic Ag + and NO3-in biological media, whereas SBC3 is covalently bonded L-Ag-OAc (L = NHC) and no free Ag + is formed. The lipid nature of SBC3 may not provoke an immune response as it may be better tolerated by the insect's immune system. As a consequence it can be concluded that the increased survival of larvae that received SBC3 is due to the anti-microbial properties of the compound and not to a non-specific immune response induced by the introduction of the compound.

This is the first demonstration of the in vivo activity of SBC3 against *S. aureus* and *C. albicans*. Future studies will assess the mode of action of the compound in insect larvae and mice followed by survival as well

as PK/PD studies in rodents. It is hoped that SBC3 may be developed into an intravenous antibiotic for use against pathogenic bacteria and fungi that show resistance to conventional antimicrobial drugs.

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References

Adams AP, Santschi EM, Mellencamp MA (1999) Antibacterial properties of a silver chloride-coated nylon wound dressing. Vet Surg 28:219–225

Atiyeh BS, Costagliola M, Hayek SN, Dibo SA (2007) Effect of silver on burn wound infection control and healing: review of the literature. Burns 33:139–148

Beresford P, Basinski-Gray JM, Chiu JKC, Chadwick JS, Aston WP (1997). Characterisation of hemolytic and cytotoxic gallysins: A relationship with arylophorins. Dev Comp Immunol 21:253–266.

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. Microbe Infect 8:2105–2112

Boman HG, Hultmark D (1987) Cell-free immunity in insects. Ann Rev Microbiol 41:103–126

Brennan M, Thomas DY, 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

Browne N, Heelan M, Kavanagh K (2013) An analysis of the structural and functional similarities of insect hemocytes and mammalian phagocytes. Virulence 4:1–7

Castellano JJ, Shafii SM, Ko F, Donate G, Wright TE, Mannari RJ, Payne WG, Smith DJ, Robson MC (2007) Comparative evaluation of silver-containing antimicrobial dressings and drugs. Int Wound J 4:114–122

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

Desbois AP, Coote PJ (2012) Utility of greater wax moth larva (*Galleria mellonella*) for evaluating the toxicity and efficacy of new antimicrobial agents. Adv Appl Microbiol 78:25–53

Fuchs B, Mylonakis E (2006) Using non-mammalian host to study fungal virulence and host defense. Curr Opin Microbiol 9:346–351

Gupta L, Noh JY, Jo YH, Oh SH, Kumar S, Noh MY, Lee YS, Cha SJ, Seo SJ, Kim I, Han YS, Barillas-Mury C (2010) Apolipophorin-III mediates anti-plasmodial epithelial responses in *Anopheles gambiae* (G3) mosquitoes. PLoS ONE 5:e15410

Hackenberg F, Tacke M (2014) Benzyl-substituted metallocarbene antibiotics and anticancer drugs. Dalton transactions. (in print). http://dx.doi.org/10.1039/C4DT00624K

Hamamoto H, Kurokawa K, Kaito C, Kamura K, Manitra Razanajatovo I, 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

Hindi KM, Siciliano TJ, Durmus S, Panzner MJ, Medvetz DA, Reddy DV, Hogue LA, Hovis CE, Hilliard JK, Mallet RJ, Tessier CA, Cannon CL, Youngs WJ (2008) Synthesis, stability, and antimicrobial studies of electronically tuned silver acetate N-heterocyclic carbenes. J Med Chem 51:1577–1583

Hornsey M, Wareham DW (2011) In vivo efficacy of glycopeptide-colistin combination therapies in a *Galleria mellonella* model of *Acinetobacter baumannii* infection. Antimicrob Agents Chemother 55:3534–3537

Jander G, Rahme L, Ausbel F (2000) Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. J Bacteriol 182:3843–3845

Kascatan-Nebioglu A, Melaiye A, Hindi K, Durmus S, Panzner MJ, Hogue LA, Mallett RJ, Hovis CE, Coughenour M, Crosby SD, Milsted A, Ely DL, Tessier CA, Cannon CL, Youngs WJ (2006) Synthesis from caffeine of a mixed N-heterocyclic carbene-silver acetate complex active against resistant respiratory pathogens. J Med Chem 49:6811–6818

Kavanagh K, Fallon J (2010) *Galleria mellonella* larvae as models for studying fungal virulence. Fungal Biol Rev 24:79–83

Kavanagh K, Reeves EP (2004) Exploiting the potential of Insects for in vivo pathogenicity testing of Microbial pathogens. FEMS Microbiol Rev 28:101–112

Kelly J, Kavanagh K (2011) Caspofungin primes the immune response of the larvae of Galleria mellonella and induces a non-specific antimicrobial response. J Med Microbiol 60:189–196

Lahm A, Paradisi A, Green DR, Melino G (2003) Death fold domain interaction in apoptosis. Cell Death Differ 10:10–12

Lavine MD, Strand MR (2002) Insect haemocytes and their role in immunity. Insect Biochem Mol Biol 32:1295–1309

Lionakis MS, Kontoyiannis DP (2005) Fruit flies as a mini-host model for studying drug activity and virulence in *Aspergillus*. Med Mycol 43:111–114

Lionakis MS, Lewis RE, May GS, Wiederhold NP, Albert ND, Halder G, Kontoyiannis DP (2005) Toll-deficient *Drosophila* flies as a fast, high-throughput model for the study of antifungal drug efficacy against invasive aspergillosis and *Aspergillus* virulence. J Infect Dis 191:1188–1195

Maple PAC, Hamilton-Miller JMT, Brumfitt W (1989) Worldwide antibiotic resistance in methicillin-resistant *Staphylococcus aureus*. Lancet 333:537–540

Mijnendonckx K, Leys N, Mahillon J, Silver S, Van Houdt R (2013) Antimicrobial silver: uses, toxicity and potential for resistance. Biometals 26:609–621

Mowlds P, Coates C, Renwick J, Kavanagh K (2010) Dose-dependent cellular and humoral responses in *Galleria mellonella* larvae following β-Glucan inoculation. Microb Infect 12:146–153

Oehninger L, Rubbiani R, Ott I (2013) N-Heterocyclic carbene metal complexes in medicinal chemistry. Dalton Trans 42:3269–3284

Park HJ, Kim JY, Kim J, Lee JH, Hahn JS, Gu MB, Yoon J (2009) Silver-ion-mediated reactive oxygen species generation affecting bactericidal activity. Water Res 43:1027–1032

Patil S, Tacke M (2011) NHC-Silver(I) Acetates as bioorganometallic anticancer and antibacterial drugs. In: Melník M, Segľa P, Tatarko M (eds) Insights into coordination, bioinorganic and applied inorganic chemistry. Press of Slovak University of Technology, Bratislava, pp 555–566

Patil S, Deally A, Gleeson B, Müller-Bunz H, Paradisi F, Tacke M (2011) Novel benzyl-substituted N-heterocyclic carbene-silver acetate complexes: synthesis, cytotoxicity and antibacterial studies. Metallomics 3:74–88

Percival SL, Bowler PG, Russell D (2005) Bacterial resistance to silver in wound care. J Hosp Infect 60:1–7

Rex JH (2014) ND4BB: addressing the antimicrobial resistance crisis. Nature Rev Microbiol 12:231–232

Rowan R, Moran C, McCann M, Kavanagh K (2009) Use of *Galleria mellonella* larvae to evaluate the in vivo anti-fungal activity of $[Ag_2(mal)(phen)_3]$. Biometals 22:461–467

Rowan R, McCann M, Kavanagh K (2010) Analysis of the response of *Candida albicans* cells to Silver (I). Med Mycol 48:498–505

Sharkey MA, O'Gara JP, Gordon SV, Hackenberg F, Healy C, Paradisi F, Patil S, Schaible B, Tacke M (2012) Investigations into the antibacterial activity of the silver-based antibiotic drug candidate SBC3. Antibiotics 1:25–28

Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1:2856–2860

Taylor JL, Radic Z (1994) The cholinesterases: from genes to proteins. Annu Rev Pharmacol Toxicol 34:281–320

Vogt RG, Riddiford LM, Prestwich GD (1985) Kinetic properties of a sex pheromone-degrading enzyme: the sensillar esterase of *Antheraea Polyphemus*. Proc Natl Acad Sci USA 82:8827–8831