Apoptosis in *Candida* biofilms exposed to amphotericin B

Rawya S. Al-Dhaheri and L. Julia Douglas

Correspondence

L. Julia Douglas

j.douglas@bio.gla.ac.uk

Division of Infection and Immunity, Faculty of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK

Candida biofilms are resistant to a range of antifungal agents in current clinical use. The basis of this drug resistance is not clear, but in some cases it could be due to the presence of a small number of drug-tolerant or persister cells. In this study, specific staining methods were used to investigate the existence of persisters and apoptosis in *Candida* biofilms subjected to different concentrations of amphotericin B. Fluorescein diacetate staining revealed the presence of persisters in biofilms of one of two strains of *Candida albicans* tested, and in biofilms of *Candida krusei* and *Candida parapsilosis*. Caspase activity, indicative of apoptosis, was detected with SR-FLICA and (aspartyl)₂-rhodamine 110 fluorochrome-based staining reagents in all of these biofilms. The general inhibitor of mammalian caspases, Z-VAD-FMK, when used at a low concentration (2.5 µM), increased the viability of drug-treated biofilms up to 11.5-fold ($P < 0.001$ %). Seven specific caspase inhibitors had different effects on *C. albicans* biofilm viability, but inhibitors of caspases-1, -9, -5, -3 and -2 all significantly increased cell survival (40-fold, 8-fold, 3.5-fold, 1.9-fold and 1.7-fold, respectively). However, histone deacetylase (HDA) inhibitors enhanced the activity of amphotericin B for biofilms of all three *Candida* species. Sodium butyrate and sodium valproate, for example, when added concurrently with amphotericin B, completely eliminated biofilm populations of *C. albicans*. Overall, our results demonstrate an apoptotic process in amphotericin-treated biofilms of three *Candida* species. They also indicate that HDA inhibitors can enhance the action of the drug and in some cases even eradicate persister subpopulations, suggesting that histone acetylation might activate apoptosis in these cells.

Received 4 September 2009

Accepted 3 November 2009

INTRODUCTION

Opportunistic fungal pathogens of the genus *Candida* are known to be important agents of hospital-acquired infection. Many of these infections involve the formation of adherent biofilms on the surfaces of catheters, prosthetic heart valves and other medical devices (Kojic & Darouiche, 2004). Biofilm organisms are phenotypically different from planktonic cells and, characteristically, are significantly less susceptible to antimicrobial agents, making the management of implant infections difficult. Although recent evidence suggests that some *Candida* biofilms show decreased viability when treated with caspofungin and related echinocandins *in vitro* (Choi *et al.*, 2007; Katragkou *et al.*, 2008), they are resistant to most other clinically important antifungal agents, such as amphotericin B, fluconazole and voriconazole (Douglas, 2003; Hawser & Douglas, 1995; Ramage *et al.*, 2005).

The mechanisms that protect micro-organisms in biofilms from antimicrobial agents are poorly understood (Douglas,

2003; Ramage *et al.*, 2005). One proposal that has gained considerable support in recent years involves the existence of a small number of drug-tolerant or persister cells – usually 1 % or less of the overall population – that neither grow nor die in the presence of microbicidal agents (Keren *et al.*, 2004; Lewis, 2000, 2001, 2007). Persisters can withstand drug concentrations substantially above the MIC and represent specialized survivor cells that are phenotypic variants of the wild-type rather than mutants. It has been suggested that if micro-organisms exposed to antibiotics or other antimicrobial agents undergo a type of programmed cell death or apoptosis, persisters would be variants in which this process has been disabled (Lewis, 2000). Persisters have been identified in a variety of bacterial species (Lewis, 2007) and their existence in biofilms of *Candida albicans* has also been reported recently (Khot *et al.*, 2006; LaFleur *et al.*, 2006). However, it appears that not all strains of *C. albicans* produce persisters (Al-Dhaheri & Douglas, 2008). Moreover, although persisters have been found in biofilms of *Candida krusei* and *Candida parapsilosis*, they were absent from biofilms formed by *Candida glabrata* and *Candida tropicalis* (Al-Dhaheri & Douglas, 2008). In contrast to bacterial species tested,

Abbreviations: D₂R, (aspartyl)₂-rhodamine 110; DIC, differential interference contrast; HDA, histone deacetylase.

planktonic cultures (exponential or stationary phase) of *Candida* species seem to be devoid of persisters (Al-Dhaheri & Douglas, 2008; LaFleur *et al.*, 2006).

Programmed cell death responses have been described in a variety of fungi after exposure of the organisms to a range of conditions, such as weak acid stress, oxidative stress or UV irradiation. Fungal cells dying in this way display a number of markers characteristic of apoptosis, including the exposure of phosphatidylserine at the plasma membrane and the degradation of DNA (Hamann *et al.*, 2008; Mazzoni & Falcone, 2008; Ramsdale, 2008). Recent studies have demonstrated that planktonically grown cells of *C. albicans* undergo apoptosis when subjected not only to weak acid stress but also to amphotericin B (Phillips *et al.*, 2003). In mammalian cells, acquisition of an apoptotic morphology depends on the activation of cysteine-dependent aspartate-specific proteases (caspases). Functional analyses of genes in *Saccharomyces cerevisiae* have revealed similarities between fungal apoptosis and apoptosis in mammalian cells, and a homologue of mammalian caspases, YCA1 (Yeast Caspase 1), has been identified (Madeo *et al.*, 2002). The caspase superfamily consists of three families; YCA1 belongs to the metacaspase family of enzymes, which are found in plants, fungi and protozoa (Uren *et al.*, 2000). Overexpression of YCA1 (also known as MCA1) increases hydrogen-peroxide-induced caspase-like activity and apoptosis in *S. cerevisiae* (Madeo *et al.*, 2002). Recently, a homologue of YCA1, CaMCA1, has been identified in *C. albicans*; deletion of the CaMCA1 gene results in decreased caspase activity (Cao *et al.*, 2009).

Histone deacetylase (HDA) inhibitors, such as valproic acid, trichostatin A and butyric acid, are known to induce apoptosis in mammalian cells (Kawagoe *et al.*, 2002; Medina *et al.*, 1997). Valproic acid also induces YCA1-dependent apoptosis in *S. cerevisiae* (Mitsui *et al.*, 2005; Sun *et al.*, 2007). In *C. albicans*, HDA inhibitors have been reported to enhance sensitivity to azoles and other antifungal agents (Mai *et al.*, 2007; Smith & Edlind, 2002), to inhibit adhesion and serum-induced germ-tube formation (Noverr & Huffnagle, 2004; Simonetti *et al.*, 2007), and to promote colony-type switching (Klar *et al.*, 2001; Srikantha *et al.*, 2001). Histone acetylation and deacetylation play important roles in eukaryotic gene regulation. Acetylation is generally associated with activation, whereas lack of acetylation tends to correlate with repression; these two processes work together to achieve appropriate levels of transcription (Stern & Berger, 2000).

In this study, we have used specific staining methods to investigate the existence of persisters and apoptosis in biofilm cells of *C. albicans*, *C. krusei* and *C. parapsilosis* subjected to different concentrations of amphotericin B. We have also determined the effect of apoptosis-inhibiting compounds (general and specific caspase inhibitors) and apoptosis-inducing compounds (HDA inhibitors) on the survival of biofilm cells exposed to the drug.

METHODS

Organisms. Three *Candida* species were used in this study. *C. albicans* GDH 2346, *C. parapsilosis* AAHB 4479 and *C. krusei* (Glasgow strain) are clinical isolates whose origins are described elsewhere (Hawser & Douglas, 1994). *C. albicans* SC5314 was kindly provided by N. A. R. Gow, University of Aberdeen, Aberdeen, Scotland. All organisms were maintained on slopes of Sabouraud dextrose agar (Difco). Fresh slopes were prepared at 2 month intervals from long-term stocks held in 50 % glycerol at -70°C .

Medium and culture conditions. Organisms were grown in yeast nitrogen base (YNB) medium (Difco) containing 50 mM glucose (YNB glucose). Batches of medium (50 ml in 250 ml Erlenmeyer flasks) were inoculated from culture slopes and incubated at 37°C in an orbital shaker at 60 r.p.m. Cells were harvested after 24 h and washed twice in 0.15 M PBS (pH 7.2). Before use in biofilm experiments, washed cell suspensions were standardized to an optical density of 0.8 at 520 nm.

Caspase inhibitors, HDA inhibitors, amphotericin B and pepstatin A. Stock solutions (14.7 mM) of the general caspase inhibitor, Z-VAD-FMK (Calbiochem), were prepared in DMSO. Seven specific caspase inhibitors were also used. They were supplied by the manufacturers (caspase inhibitor set III; Calbiochem) as 2 mM solutions in DMSO and consisted of: caspase-1 inhibitor VI (Z-YVAD-FMK); caspase-2 inhibitor I (Z-VDVAD-FMK); caspase-3 inhibitor II (Z-DEVD-FMK); caspase-5 inhibitor I (Z-WEHD-FMK); caspase-6 inhibitor I (Z-VEID-FMK); caspase-8 inhibitor II (Z-IETD-FMK); caspase-9 inhibitor I (Z-LEHD-FMK). Stock solutions of the HDA inhibitors trichostatin A and apicidin (Calbiochem) were prepared in DMSO; HDA inhibitors sodium butyrate and sodium valproate (Sigma-Aldrich) were dissolved in sterile water. Stock solutions (8 mg ml^{-1}) of amphotericin B (Sigma-Aldrich) were prepared in DMSO and stored at -70°C . These solutions were further diluted in YNB glucose medium buffered to pH 7 with 0.165 M MOPS (Sigma-Aldrich) to give the final concentration required. Pepstatin A (Sigma-Aldrich) was dissolved at a concentration of 1 mg ml^{-1} in methanol containing 10 % (v/v) acetic acid.

Growth and amphotericin treatment of biofilms. Biofilms were formed on small discs (diameter 0.8 cm) cut from polyvinyl chloride Faucher tubes (French gauge 36; Vygon) essentially as described previously (Baillie & Douglas, 1999). The discs were placed in the wells of 24-well Costar tissue culture plates, and a standardized cell suspension ($80\text{ }\mu\text{l}$) was applied to the surface of each one. Cells were allowed to adhere for 1 h at 37°C . Non-adherent organisms were removed by washing, and the discs were incubated in wells of a fresh plate for 48 h at 37°C , submerged in 1 ml YNB glucose medium to allow biofilm formation. The biofilms were then treated with amphotericin B at concentrations of 10, 50 or $100\text{ }\mu\text{g ml}^{-1}$ as follows. Mature (48 h) biofilms were transferred to fresh wells, submerged in YNB glucose medium (1 ml) containing the appropriate concentration of amphotericin and buffered to pH 7 with 0.165 M MOPS, and incubated at 37°C for 24 h. Control biofilms were incubated in buffered medium in the absence of amphotericin B. After incubation, biofilm cells were harvested from the discs by scraping and vigorous vortexing, washed twice in PBS, and then resuspended in more PBS ($100\text{ }\mu\text{l}$). Viable cell counts were determined by the standard procedure of serial dilution followed by plating on YNB agar containing 200 mM glucose.

Live-dead cell staining with fluorescein. Fluorescein diacetate (Sigma-Aldrich) was used to distinguish living yeast cells, which are unstained, from dead cells, which fluoresce green. Mature (48 h) biofilms on catheter discs were submerged in 1 ml buffered YNB glucose medium containing $100\text{ }\mu\text{g fluorescein diacetate ml}^{-1}$ alone

(control), or 100 µg fluorescein diacetate ml⁻¹ with 100 µg amphotericin B ml⁻¹, and incubated for 24 h at 37 °C. The biofilm cells were then harvested, washed three times in PBS, resuspended in PBS (300 µl), and viewed with a ×100 oil immersion lens using a fluorescence microscope (Zeiss Axioimager M1).

SR-FLICA apoptosis detection assay. An SR-FLICA kit (Immunochemistry Technologies) was used to detect active caspase enzymes within biofilm cells. The SR-FLICA reagent was reconstituted in 50 µl DMSO, as recommended by the manufacturers, to form a stock concentrate. The concentrate was further diluted with 200 µl PBS to produce the working solution. Mature (48 h) biofilms were submerged in buffered YNB glucose medium containing amphotericin B (50 µg ml⁻¹) and incubated for 5 h or 24 h at 37 °C. The biofilms were washed gently in PBS and the cells were resuspended to a concentration of approximately 10⁷ cells ml⁻¹. SR-FLICA working solution (10 µl) was then added to 200 µl biofilm cell suspension and the mixtures were incubated for 1 h at 37 °C in the dark. The cells were washed twice using a wash buffer provided in the kit, resuspended in wash buffer (300 µl) and examined under a ×100 oil immersion lens using a fluorescence microscope (Zeiss Axioimager M1) with a bandpass filter (excitation 550 nm, emission >580 nm). Apoptotic cells with active caspase enzymes fluoresced red.

(Aspartyl)₂-rhodamine 110 (D₂R) apoptosis detection assay. A CaspSCREEN kit (Biovision Research Products) was also used to detect caspase activity. Mature (48 h) biofilms were submerged in buffered YNB glucose medium containing amphotericin B (50 µg ml⁻¹) and incubated for 24 h at 37 °C. Biofilm cells were washed gently in PBS and centrifuged to give a pellet (10⁵ cells) that was resuspended in D₂R incubation buffer (0.3 ml). DTT (1 M, 3 µl) and D₂R reagent (1 µl) were then added and the mixture was incubated for 45 min at 37 °C in the dark. Resuspended cells were observed under a ×100 oil immersion lens using a fluorescence microscope (Zeiss Axioimager M1) with a bandpass filter (excitation 488 nm, emission 530 nm). Apoptotic cells with active caspase enzymes fluoresced green.

Effect of caspase inhibitors. To investigate the effect of Z-VAD-FMK on biofilm growth and viability, 48 h biofilms were washed gently with PBS and submerged in YNB glucose medium (1 ml) buffered with MOPS and containing different concentrations (2.5, 5, 10 and 20 µM) of Z-VAD-FMK. Control biofilms were transferred to buffered medium without Z-VAD-FMK. Biofilms were incubated for 24 h at 37 °C and then cells were harvested and their numbers determined by viable cell counts. To investigate the effect of Z-VAD-FMK on amphotericin B activity, the same procedure was followed except that 48 h biofilms were submerged in buffered YNB glucose medium containing Z-VAD-FMK (2.5–20 µM) and amphotericin B (50 µg ml⁻¹). The final concentration of DMSO in assay mixtures ranged from 0.12 to 0.26%. Identical procedures were used to determine the effect of specific caspase inhibitors (caspase inhibitor set III) on biofilm growth and amphotericin B activity, except that these inhibitors were used at a single concentration of 2.5 µM.

Effect of HDA inhibitors. To investigate the effect of HDA inhibitors on biofilm growth and viability, mature (48 h) biofilms were washed gently with PBS and submerged in YNB glucose medium (1 ml) buffered with MOPS and containing different concentrations (2, 8 or 32 µg ml⁻¹; 2, 8 or 32 mM for sodium butyrate) of HDA inhibitor. The biofilms were then incubated for a further 24 h at 37 °C. The final concentration of DMSO in all cases was less than 0.5%; this concentration had no effect on biofilm viability. Control biofilms were incubated in medium without inhibitor. After incubation, biofilm cells were harvested and washed, and viable counts were determined as described above. To investigate the effect of HDA inhibitors on amphotericin B activity, the same procedure

was followed except that 48 h biofilms were submerged in buffered YNB glucose medium containing amphotericin B (50 µg ml⁻¹) and HDA inhibitor (2, 8 or 32 µg ml⁻¹; 2, 8 or 32 mM for sodium butyrate). Again, the final concentration of DMSO in the medium was less than 0.5%. Control biofilms were incubated in medium without HDA inhibitor, or without both HDA inhibitor and amphotericin B.

RESULTS AND DISCUSSION

Live–dead staining of biofilm cells with fluorescein diacetate

Fluorescein diacetate was used to discriminate between living and dead biofilm cells; this dye specifically stains dead cells green. Biofilms (48 h) of *C. albicans* (both strains), *C. krusei* and *C. parapsilosis* were treated with a high concentration (100 µg ml⁻¹) of amphotericin B for 24 h at 37 °C, and the cells were then stained with fluorescein diacetate. With most of these biofilms, small numbers of unstained (live) cells were detected. These cells appeared to have a normal morphology and their numbers varied according to the *Candida* species under investigation (Fig. 1). *C. parapsilosis* biofilms contained more of the live cells than did biofilms of *C. krusei* or *C. albicans* GDH 2346. By contrast, no live cells were detected in biofilms of *C. albicans* SC5314 exposed to a high concentration of amphotericin B. Fluorescence was rarely observed in untreated, control cells (i.e. 72 h biofilm cells). On the assumption that the unstained cells represented drug-tolerant persisters, these results with fluorescein staining confirm earlier viability measurements (Al-Dhaheer & Douglas, 2008) which showed that persisters were present in biofilms of *C. albicans* GDH 2346, *C. krusei* and *C. parapsilosis*, but absent from biofilms of *C. albicans* SC5314.

Caspase detection using a polycaspase SR-FLICA reagent

The SR-FLICA reagent (fluorochrome-labelled inhibitor of caspase) was used to detect the presence of caspases in *Candida* biofilm cells. When the reagent enters a cell, any active caspases present will bind covalently to the peptide inhibitor sequence of SR-FLICA (VAD). As a result, the fluorescent label, sulforhodamine, is retained within the cell, which fluoresces red. Here, 48 h biofilms of *C. krusei*, *C. parapsilosis* and both strains of *C. albicans* were treated with amphotericin B (50 µg ml⁻¹) for 5 or 24 h. The cells were then labelled with the reagent and viewed under a fluorescence microscope.

Exposure of *C. albicans* biofilms to amphotericin B for 5 h resulted in relatively few cells that were stained with the SR-FLICA reagent (Fig. 2a, c). Considerably more stained cells (93%) were seen in biofilms that had been exposed to the drug for 24 h (Fig. 2b, d), suggesting an ongoing process of apoptosis induction. After such treatment, higher numbers of apoptotic cells appeared to be present

Fig. 1. Live–dead staining of *Candida* biofilm cells with fluorescein diacetate. Biofilms (48 h) were incubated with amphotericin B ($100 \mu\text{g ml}^{-1}$) and fluorescein diacetate ($100 \mu\text{g ml}^{-1}$) for 24 h at 37°C . Washed, resuspended biofilm cells were then examined by fluorescence microscopy. Differential interference contrast (DIC), fluorescence and overlaid images are shown of *C. albicans* SC5314 (a), *C. albicans* GDH 2346 (b), *C. krusei* (c) and *C. parapsilosis* (d). Dead cells show green fluorescence. Bars, $13 \mu\text{m}$.

in biofilms of *C. albicans* (both strains) (Fig. 2b, d) and *C. krusei* (Fig. 2e) than in biofilms of *C. parapsilosis* (Fig. 2f).

In *C. albicans* growing planktonically, exposure of the organism to a variety of environmental stresses, and to amphotericin B or farnesol, is known to produce characteristics typical of apoptosis. These include externalization of phosphatidylserine, chromatin condensation,

accumulation of reactive oxygen species, DNA degradation and caspase activation (Cao *et al.*, 2009; Phillips *et al.*, 2003; Shirtliff *et al.*, 2009). For example, a single-cell death assay was used to show that a majority of amphotericin-treated cells were nonviable and impermeable to propidium iodide. Apoptotic changes induced by the drug were confirmed by electron microscopy and by the production of intracellular reactive oxygen species (Phillips *et al.*,

Fig. 2. Caspase detection using a polycaspase SR-FLICA reagent. Biofilms (48 h) were incubated with amphotericin B ($50 \mu\text{g ml}^{-1}$) for 5 or 24 h at 37°C . Washed, resuspended biofilm cells were then labelled with the SR-FLICA reagent and examined by fluorescence microscopy. DIC and fluorescence images are shown of *C. albicans* GDH 2346 exposed to amphotericin B for 5 h (a) and 24 h (b); *C. albicans* SC5314 exposed to amphotericin B for 5 h (c) and 24 h (d); *C. krusei* exposed to amphotericin B for 24 h (e); and *C. parapsilosis* exposed to amphotericin B for 24 h (f). Caspase activity is indicated by orange/red fluorescence. Bars, $13 \mu\text{m}$.

2003). The benefits of such a suicide process to unicellular organisms like yeasts are not immediately obvious. However, apoptosis could be highly advantageous for a biofilm community that, in many ways, resembles a multicellular organism. The self-destruction of damaged cells that consume scarce nutrients in a vain attempt to repair themselves could enhance the viability and reproductive success of healthier members of the community (Buttner *et al.*, 2006; Lewis, 2000).

Although FLICAs have become widely employed for the detection of active caspases in yeasts as well as in mammalian cells, their use has sometimes been controversial (Pozarowski *et al.*, 2003; Vachova & Palkova, 2007). Madeo *et al.* (2002) reported FLICA binding to active caspase in intact (propidium iodide negative) cells of *S. cerevisiae*, and only in cells containing a functional *YCA1* gene; no binding was detected in a *yca1* mutant. However, other studies have indicated non-specific FLICA binding by heat-killed or ageing yeast cells (Vachova & Palkova, 2007; Wysocki & Kron, 2004). In the present investigation, fluorescent staining was rarely observed in control cells from biofilms not exposed to amphotericin B. Moreover, caspase activity, as detected by FLICA, increased with longer incubation times. However, to demonstrate unequivocally caspase activity in drug-treated biofilm cells, a second staining method, utilizing a D₂R reagent, was also used.

Caspase detection using a D₂R reagent

This assay is based on the cleavage of D₂R, which is a substrate for caspases. D₂R is non-fluorescent, but when cleaved by caspases it releases green fluorescent mono-substituted rhodamine 110 and free rhodamine. This method therefore allows only the detection of active caspases and so avoids the artefactual, non-specific staining that has been reported in some cases with the FLICA reagent (Vachova & Palkova, 2007). For biofilms exposed

to amphotericin B, the number of cells stained by D₂R was high (96 %; Fig. 3); cells from untreated biofilms, however, were rarely stained. These results confirm those obtained with the FLICA assay and indicate that drug-treated biofilm cells undergo apoptosis. Recently, two separate research groups have demonstrated caspase activity in planktonic cultures of *C. albicans* using the D₂R or FLICA reagent (Cao *et al.*, 2009; Shirtliff *et al.*, 2009).

General caspase inhibitor Z-VAD-FMK decreases the activity of amphotericin B against *Candida* biofilms

Mature (48 h) biofilms of *C. albicans* GDH 2346, *C. krusei* and *C. parapsilosis* were incubated for 24 h at 37 °C in fresh growth medium in the presence of different concentrations (2.5 to 20 µM) of Z-VAD-FMK, which is active against a range of caspases. Addition of this inhibitor had no significant effect on biofilm viability of any of the three species at any concentration tested (results not shown). However, when Z-VAD-FMK was added at low concentration (2.5 µM) together with amphotericin B (50 µg ml⁻¹), it significantly reduced the effect of the drug on *Candida* biofilms. Biofilm viability was increased 11.5-fold ($P < 0.001$) and 1.6-fold ($P < 0.05$) for *C. albicans* and *C. parapsilosis*, respectively, in the presence of both compounds (Fig. 4), although there was no significant increase in viability with *C. krusei* biofilms. In contrast, high concentrations (10 to 20 µM) of Z-VAD-FMK were ineffective at preventing cell death and instead appeared to enhance it (Fig. 4).

The effect of combined treatment with Z-VAD-FMK and amphotericin B was also investigated with biofilms of *C. albicans* SC5314. This strain appears to lack persisters, and biofilms lose all viability after exposure to amphotericin B at 30 µg ml⁻¹ (Al-Dhaheri & Douglas, 2008). When 48 h biofilms were treated with Z-VAD-FMK (2.5 µM) and

Fig. 3. Caspase detection using D₂R reagent. Biofilms (48 h) were incubated with amphotericin B (50 µg ml⁻¹) for 24 h at 37 °C. Washed, resuspended biofilm cells were then labelled with the D₂R reagent and examined by fluorescence microscopy. DIC and fluorescence images are shown of *C. albicans* GDH 2346 (a), *C. albicans* SC5314 (b), *C. krusei* (c) and *C. parapsilosis* (d). Caspase activity is indicated by green fluorescence. Bars, 5 µm.

Fig. 4. Effect of general caspase inhibitor Z-VAD-FMK on amphotericin B activity against biofilms of *C. albicans* GDH 2346 (●), *C. parapsilosis* (△) and *C. krusei* (■). Biofilms (48 h) were incubated with Z-VAD-FMK and amphotericin B (50 µg ml⁻¹) for 24 h at 37 °C and cell survival was determined by viable cell counts. Results are shown as means ± SES of two independent experiments carried out in duplicate.

amphotericin B (50 µg ml⁻¹) for 24 h at 37 °C, there was a complete loss of viability like that observed with control biofilms exposed only to the antifungal agent. However, biofilms treated with Z-VAD-FMK and a lower concentration (10 µg ml⁻¹) of amphotericin B showed an 11.9-fold increase in viability ($P < 0.001$).

The specificity of the effect noted with the general caspase inhibitor was investigated using pepstatin A, an inhibitor of aspartic proteinases, which are known to be produced by *Candida* species. Exposure of 48 h biofilms of either *C. albicans* strain to amphotericin B (50 µg ml⁻¹) and pepstatin A (2.5 µM) for 24 h at 37 °C did not significantly affect cell survival (results not shown).

Some specific caspase inhibitors decrease the activity of amphotericin B against *C. albicans* biofilms

A set of specific caspase inhibitors, active individually against caspases-1, -2, -3, -5, -6, -8 and -9, was also tested against *C. albicans* biofilms. Again, none of these inhibitors affected biofilm viability when incubated at 2.5 µM with 48 h biofilms of *C. albicans* GDH 2346 for 24 h at 37 °C (results not shown). However, when added to biofilms along with amphotericin B, some of the inhibitors significantly enhanced the survival of biofilm cells as compared with controls treated with only the antifungal drug. Caspase-1 inhibitor VI produced a 40-fold increase in biofilm cell survival ($P < 0.001$) compared with amphotericin-treated controls (Fig. 5). Similarly, inhibitors of caspases-9, -5, -3 and -2 increased cell survival 8-fold ($P < 0.001$), 3.5-fold ($P < 0.001$), 1.9-fold ($P < 0.001$) and 1.7-fold ($P < 0.01$), respectively. In contrast, caspase-6 and caspase-8 inhibitors decreased biofilm cell survival as compared with the amphotericin-treated controls (Fig. 5).

Fig. 5. Effects of specific inhibitors of caspases-1, -2, -3, -5, -6, -8 and -9 on amphotericin B activity against biofilms of *C. albicans* GDH 2346. Biofilms (48 h) were incubated with inhibitor (2.5 µM) and amphotericin B (50 µg ml⁻¹) for 24 h at 37 °C, and cell survival was determined by viable cell counts. Results are shown as means ± SES of two independent experiments carried out in duplicate.

Partial suppression of caspase activity in *C. albicans* by mammalian caspase inhibitors is not wholly unexpected. To date, only one enzyme with caspase-like activity, CaMCA1, has been identified in *C. albicans*. This, like its homologue in *S. cerevisiae*, YCA1/MCA1, is a metacaspase (Cao *et al.*, 2009). Mammalian caspases are cysteine proteases with a stringent specificity for cleaving protein substrates containing aspartic acid. Plant, protozoan and fungal metacaspases, however, display arginine and lysine protease specificity instead of the aspartic acid specificity characteristic of caspases. Nevertheless, Madeo *et al.* (2002) reported that extracts of a YCA1 overproducing strain of *S. cerevisiae*, which had been treated with H₂O₂ to induce apoptosis, showed a high proteolytic activity towards several substrates for mammalian caspases. Addition of the general caspase inhibitor, Z-VAD-FMK, to the extracts completely abrogated this catalytic activity. Mammalian caspase inhibitors, including Z-VAD-FMK, are also known to be remarkably efficient at blocking programmed cell death in plants (Bonneau *et al.*, 2008). However, there is some evidence that plant metacaspases do not cleave caspase substrates and are not inhibited by caspase inhibitors (Bonneau *et al.*, 2008). It is therefore possible that other caspase-like activities are present in both plants and yeasts. In *S. cerevisiae*, for example, an MCA1/YCA1-independent caspase-like activity has been detected in an *mca1* mutant using the D₂R staining procedure (Vachova & Palkova, 2005). There are several other reports of MCA1-independent caspase-like activities in *S. cerevisiae* detected by different approaches (Vachova & Palkova, 2007).

HDA inhibitors enhance the activity of amphotericin B against *Candida* biofilms

Mature (48 h) biofilms of *C. albicans* GDH 2346, *C. krusei* and *C. parapsilosis* were incubated for 24 h at 37 °C in fresh

growth medium in the presence of four different HDA inhibitors, namely sodium butyrate, sodium valproate, apicidin and trichostatin A. None of these compounds affected the viability of any of the species when tested at concentrations ranging from 2 to 32 $\mu\text{g ml}^{-1}$, or 2 to 32 mM (equivalent to 220 $\mu\text{g ml}^{-1}$ to 3.52 mg ml^{-1} , for sodium butyrate) (results not shown). However, when the inhibitors were added to biofilms concurrently with amphotericin B, there was a marked effect on viability (Fig. 6). Biofilm populations of *C. albicans* were completely eliminated by sodium butyrate (8 or 32 mM) at low concentrations of amphotericin B (10 $\mu\text{g ml}^{-1}$; Fig. 6a), and by even lower concentrations of butyrate (2 mM) at higher concentrations (50 $\mu\text{g ml}^{-1}$) of the drug (Fig. 6b). In the absence of butyrate, biofilms of this strain of *C. albicans* produce persisters that remain viable at amphotericin B concentrations of up to 100 $\mu\text{g ml}^{-1}$ (Al-Dhaheri & Douglas, 2008). Biofilms of *C. krusei* and *C. parapsilosis*, which also produce persisters, were rather less susceptible; combined treatment with butyrate (32 mM) and amphotericin B (50 $\mu\text{g ml}^{-1}$) reduced biofilm viability by 66 and 75 %, respectively (Fig. 6c, d).

A second HDA inhibitor, sodium valproate, also effectively eliminated biofilm populations of *C. albicans* when used at

a concentration of 32 $\mu\text{g ml}^{-1}$ with amphotericin B at 50 $\mu\text{g ml}^{-1}$ (Fig. 6b). Biofilms of *C. krusei* were even more sensitive to this inhibitor and lost all viability after treatment with 8 $\mu\text{g valproate ml}^{-1}$ and 50 $\mu\text{g amphotericin B ml}^{-1}$ (Fig. 6c). However, biofilms of *C. parapsilosis* lost only 43 % of their viability when treated in an identical manner (Fig. 6d).

The other HDA inhibitors, apicidin and trichostatin A, both significantly reduced biofilm viability of all species tested ($P < 0.002$) when used in conjunction with amphotericin B (Fig. 6), although neither inhibitor produced complete killing of the biofilm population. Trichostatin A was not tested against *C. albicans* since it was noted that this inhibitor used in combination with amphotericin B caused some detachment of *C. albicans* biofilms from polyvinyl chloride discs.

HDA inhibitors are known to induce apoptosis in mammalian cells (Kawagoe *et al.*, 2002; Medina *et al.*, 1997). Recently, valproate was reported to induce YCA1-dependent apoptosis in *S. cerevisiae*; a *ycal* mutant survived this treatment (Mitsui *et al.*, 2005). It was subsequently shown that valproate also stimulated the accumulation of neutral lipids, mainly triacylglycerol, in

Fig. 6. Effects of HDA inhibitors on amphotericin B activity against biofilms of *C. albicans* GDH 2346 (a, b), *C. krusei* (c) and *C. parapsilosis* (d). Biofilms (48 h) were incubated with amphotericin B at concentrations of 10 $\mu\text{g ml}^{-1}$ (a) or 50 $\mu\text{g ml}^{-1}$ (b, c, d), and sodium butyrate (●; mM), sodium valproate (□; $\mu\text{g ml}^{-1}$), apicidin (▲; $\mu\text{g ml}^{-1}$) or trichostatin A (△; $\mu\text{g ml}^{-1}$). After 24 h at 37 °C, cell survival was determined by viable cell counts. Results are shown as means $\pm$ SEs of two independent experiments carried out in duplicate.

the apoptotic wild-type cells (Sun *et al.*, 2007). Valproic acid is a short-chain fatty acid widely used in humans as an anticonvulsant, and has teratogenic and anti-tumour activities. Whether it induces lipid accumulation in *C. albicans* is not known. Sodium butyrate was even more effective than valproate at eradicating biofilms of *C. albicans* GDH 2346 when added to incubation mixtures together with amphotericin B. Butyrate inhibits germination in *C. albicans* (Noverr & Huffnagle, 2004); at concentrations of 4 to 8 mM, it also enhances the activity of fluconazole against planktonic *C. albicans* cells (Smith & Edlind, 2002).

Drug-tolerant persisters capable of withstanding high concentrations of amphotericin B have been detected in biofilms of many but not all strains of *C. albicans* tested. Biofilms of *C. albicans* SC5314, for example, appear to lack persisters, as demonstrated here by fluorescein staining, and previously by viable counts (Al-Dhaheri & Douglas, 2008). Similarly, while biofilms of *C. krusei* and *C. parapsilosis* produce persisters, biofilms of some strains of *C. glabrata* and *C. tropicalis* do not (Al-Dhaheri & Douglas, 2008). The reasons for these differences are not clear. Moreover, the mechanisms by which *Candida* persisters tolerate high drug concentrations are not understood. This investigation has demonstrated that persisters capable of surviving amphotericin B concentrations of 100 µg ml⁻¹ are nevertheless eradicated at lower drug concentrations when simultaneously subjected to an HDA inhibitor such as valproate or butyrate. On the basis of this finding it can be inferred that histone acetylation might somehow activate apoptosis in these cells. The HDA inhibitor, trichostatin A, is known to affect colony-type phenotypic switching in *C. albicans*. It causes a dramatic increase in the frequency of switching in the white-to-opaque transition but has no effect on the frequency of switching in the opaque-to-white transition, suggesting that deacetylation through a trichostatin-sensitive deacetylase selectively suppresses switching in one direction (Klar *et al.*, 2001). Targeted deletion of *HDA1*, which encodes a deacetylase sensitive to trichostatin A, had the same selective effect. Subsequent studies showed that a second HDA gene, *RPD3*, plays a role in suppressing the basic switch events in both directions (Srikantha *et al.*, 2001). Whether these or any other HDA genes are involved in the regulation of caspase-like activity in *C. albicans* biofilms remains to be demonstrated.

Trichostatin A and other HDA inhibitors have also been shown to enhance the sensitivity of planktonic *C. albicans* to the azoles fluconazole, itraconazole and miconazole. Smith & Edlind (2002) reported that expression of *ERG* genes (encoding azole targets) and *CDR/MDR1* genes (encoding multidrug transporters) was induced by fluconazole, but that trichostatin A reduced this upregulation by 50 to 100 %. The authors concluded that trichostatin A probably does not act directly on *ERG* and *CDR* gene promoters since decreased deacetylation (i.e. increased acetylation) should enhance, not inhibit, transcriptional upregulation. Rather, azole treatment could be associated

with histone deacetylation of the promoter region of a transcriptional repressor. The resulting downregulation of this repressor would lead to upregulation of *ERG* and *CDR*. However, HDA inhibition by trichostatin A would result in constitutive expression of this repressor, blocking *ERG/CDR* upregulation (Smith & Edlind, 2002).

Recent evidence suggests that the Ras-cAMP-PKA signalling pathway in *C. albicans* regulates programmed cell death induced by exposure to acetic acid or hydrogen peroxide, either by inhibiting antiapoptotic functions (such as stress responses) or by activating proapoptotic functions (Phillips *et al.*, 2006). Mutations that block Ras signalling were shown to suppress or delay the apoptotic response; in contrast, mutations that stimulate signalling accelerated the apoptotic response. The role of histone acetylation or deacetylation, if any, in this regulatory process is not known, nor is it clear whether the Ras pathway is involved in amphotericin-induced apoptosis either in planktonic or in biofilm cultures. However, treatment of *C. albicans* with trichostatin A has been reported to produce a significant reduction in transcription of *EFG1*, a gene that encodes a key regulatory protein in this pathway (Simonetti *et al.*, 2007). Studies with biofilms of Ras mutants of *C. albicans* could therefore be instructive and might help to elucidate the mechanism by which drug-tolerant persisters resist programmed cell death.

ACKNOWLEDGEMENTS

Rawya Al-Dhaheri is the recipient of a research studentship from the Health Authority, Abu Dhabi, United Arab Emirates. We are indebted to Andrew Roe for expert assistance with fluorescence microscopy.

REFERENCES

- Al-Dhaheri, R. S. & Douglas, L. J. (2008). Absence of amphotericin B-tolerant persister cells in biofilms of some *Candida* species. *Antimicrob Agents Chemother* **52**, 1884–1887.
- Baillie, G. S. & Douglas, L. J. (1999). *Candida* biofilms and their susceptibility to antifungal agents. *Methods Enzymol* **310**, 644–656.
- Bonneau, L., Ge, Y., Drury, G. E. & Gallois, P. (2008). What happened to plant caspases? *J Exp Bot* **59**, 491–499.
- Buttner, S., Eisenberg, T., Herker, E., Carmona-Gutierrez, D., Kroemer, G. & Madeo, F. (2006). Why yeast cells can undergo apoptosis: death in times of peace, love, and war. *J Cell Biol* **175**, 521–525.
- Cao, Y. Y., Huang, S., Dai, B. D., Zhu, Z. Y., Lu, H., Dong, L. L., Cao, Y. B., Wang, Y., Gao, P. H. & other authors (2009). *Candida albicans* cells lacking CaMCA1-encoded metacaspase show resistance to oxidative stress-induced death and change in energy metabolism. *Fungal Genet Biol* **46**, 183–189.
- Choi, H. W., Shin, J. H., Jung, S. I., Park, K. H., Cho, D., Kee, S. J., Shin, M. G., Suh, S. P. & Ryang, D. W. (2007). Species-specific differences in the susceptibilities of biofilms formed by *Candida* bloodstream isolates to echinocandin antifungals. *Antimicrob Agents Chemother* **51**, 1520–1523.
- Douglas, L. J. (2003). *Candida* biofilms and their role in infection. *Trends Microbiol* **11**, 30–36.

- Hamann, A., Brust, D. & Osiewacz, H. D. (2008). Apoptosis pathways in fungal growth, development and ageing. *Trends Microbiol* **16**, 276–283.
- Hawser, S. P. & Douglas, L. J. (1994). Biofilm formation by *Candida* species on the surface of catheter materials in vitro. *Infect Immun* **62**, 915–921.
- Hawser, S. P. & Douglas, L. J. (1995). Resistance of *Candida albicans* biofilms to antifungal agents in vitro. *Antimicrob Agents Chemother* **39**, 2128–2131.
- Katragkou, A., Chatzimoschou, A., Simitsopoulou, M., Dalakiouridou, M., Diza-Mataftsi, E., Tsantali, C. & Roilides, E. (2008). Differential activities of newer antifungal agents against *Candida albicans* and *Candida parapsilosis* biofilms. *Antimicrob Agents Chemother* **52**, 357–360.
- Kawagoe, R., Kawagoe, H. & Sano, K. (2002). Valproic acid induces apoptosis in human leukemia cells by stimulating both caspase-dependent and -independent apoptotic signaling pathways. *Leuk Res* **26**, 495–502.
- Keren, I., Kaldalu, N., Spoering, A., Wang, Y. & Lewis, K. (2004). Persister cells and tolerance to antimicrobials. *FEMS Microbiol Lett* **230**, 13–18.
- Khot, P. D., Suci, P. A., Miller, R. L., Nelson, R. D. & Tyler, B. J. (2006). A small subpopulation of blastospores in *Candida albicans* biofilms exhibit resistance to amphotericin B associated with differential regulation of ergosterol and β -1,6-glucan pathway genes. *Antimicrob Agents Chemother* **50**, 3708–3716.
- Klar, A. J., Srikantha, T. & Soll, D. R. (2001). A histone deacetylation inhibitor and mutant promote colony-type switching of the human pathogen *Candida albicans*. *Genetics* **158**, 919–924.
- Kojic, E. M. & Darouiche, R. O. (2004). *Candida* infections of medical devices. *Clin Microbiol Rev* **17**, 255–267.
- LaFleur, M. D., Kumamoto, C. A. & Lewis, K. (2006). *Candida albicans* biofilms produce antifungal-tolerant persister cells. *Antimicrob Agents Chemother* **50**, 3839–3846.
- Lewis, K. (2000). Programmed cell death in bacteria. *Microbiol Mol Biol Rev* **64**, 503–514.
- Lewis, K. (2001). Riddle of biofilm resistance. *Antimicrob Agents Chemother* **45**, 999–1007.
- Lewis, K. (2007). Persister cells, dormancy and infectious disease. *Nat Rev Microbiol* **5**, 48–56.
- Madeo, F., Herker, E., Maldener, C., Wissing, S., Lachelt, S., Herlan, M., Fehr, M., Lauber, K., Sigris, S. J. & other authors (2002). A caspase-related protease regulates apoptosis in yeast. *Mol Cell* **9**, 911–917.
- Mai, A., Rotili, D., Massa, S., Brosch, G., Simonetti, G., Passariello, C. & Palamara, A. T. (2007). Discovery of uracil-based histone deacetylase inhibitors able to reduce acquired antifungal resistance and trailing growth in *Candida albicans*. *Bioorg Med Chem Lett* **17**, 1221–1225.
- Mazzoni, C. & Falcone, C. (2008). Caspase-dependent apoptosis in yeast. *Biochim Biophys Acta* **1783**, 1320–1327.
- Medina, V., Edmonds, B., Young, G. P., James, R., Appleton, S. & Zalewski, P. D. (1997). Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin A (inhibitors of histone deacetylase): dependence on protein synthesis and synergy with a mitochondrial/cytochrome c-dependent pathway. *Cancer Res* **57**, 3697–3707.
- Mitsui, K., Nakagawa, D., Nakamura, M., Okamoto, T. & Tsurugi, K. (2005). Valproic acid induces apoptosis dependent of Yca1p at concentrations that mildly affect the proliferation of yeast. *FEBS Lett* **579**, 723–727.
- Noverr, M. C. & Huffnagle, G. B. (2004). Regulation of *Candida albicans* morphogenesis by fatty acid metabolites. *Infect Immun* **72**, 6206–6210.
- Phillips, A. J., Sudbery, I. & Ramsdale, M. (2003). Apoptosis induced by environmental stresses and amphotericin B in *Candida albicans*. *Proc Natl Acad Sci U S A* **100**, 14327–14332.
- Phillips, A. J., Crowe, J. D. & Ramsdale, M. (2006). Ras pathway signaling accelerates programmed cell death in the pathogenic fungus *Candida albicans*. *Proc Natl Acad Sci U S A* **103**, 726–731.
- Pozarowski, P., Huang, X., Halicka, D. H., Lee, B., Johnson, G. & Darzynkiewicz, Z. (2003). Interactions of fluorochrome-labeled caspase inhibitors with apoptotic cells: a caution in data interpretation. *Cytometry A* **55**, 50–60.
- Ramage, G., Saville, S. P., Thomas, D. P. & Lopez-Ribot, J. L. (2005). *Candida* biofilms: an update. *Eukaryot Cell* **4**, 633–638.
- Ramsdale, M. (2008). Programmed cell death in pathogenic fungi. *Biochim Biophys Acta* **1783**, 1369–1380.
- Shirtliff, M. E., Krom, B. P., Meijering, R. A., Peters, B. M., Zhu, J., Scheper, M. A., Harris, M. L. & Jabra-Rizk, M. A. (2009). Farnesol-induced apoptosis in *Candida albicans*. *Antimicrob Agents Chemother* **53**, 2392–2401.
- Simonetti, G., Passariello, C., Rotili, D., Mai, A., Garaci, E. & Palamara, A. T. (2007). Histone deacetylase inhibitors may reduce pathogenicity and virulence in *Candida albicans*. *FEMS Yeast Res* **7**, 1371–1380.
- Smith, W. L. & Edlind, T. D. (2002). Histone deacetylase inhibitors enhance *Candida albicans* sensitivity to azoles and related antifungals: correlation with reduction in *CDR* and *ERG* upregulation. *Antimicrob Agents Chemother* **46**, 3532–3539.
- Srikantha, T., Tsai, L., Daniels, K., Klar, A. J. S. & Soll, D. R. (2001). The histone deacetylase genes *HDA1* and *RPD3* play distinct roles in regulation of high-frequency phenotypic switching in *Candida albicans*. *J Bacteriol* **183**, 4614–4625.
- Sterner, D. E. & Berger, S. L. (2000). Acetylation of histones and transcription-related factors. *Microbiol Mol Biol Rev* **64**, 435–459.
- Sun, Q., Bi, L., Su, X., Tsurugi, K. & Mitsui, K. (2007). Valproate induces apoptosis by inducing accumulation of neutral lipids which was prevented by disruption of the *SIR2* gene in *Saccharomyces cerevisiae*. *FEBS Lett* **581**, 3991–3995.
- Uren, A. G., O'Rourke, K., Aravind, L., Pisabarro, M. T., Seshagiri, S., Koonin, E. V. & Dixit, V. M. (2000). Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. *Mol Cell* **6**, 961–967.
- Vachova, L. & Palkova, Z. (2005). Physiological regulation of yeast cell death in multicellular colonies is triggered by ammonia. *J Cell Biol* **169**, 711–717.
- Vachova, L. & Palkova, Z. (2007). Caspases in yeast apoptosis-like death: facts and artefacts. *FEMS Yeast Res* **7**, 12–21.
- Wysocki, R. & Kron, S. J. (2004). Yeast cell death during DNA damage arrest is independent of caspase or reactive oxygen species. *J Cell Biol* **166**, 311–316.