Apoptosis in Candida biofilms exposed to amphotericin B

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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 micricularidal 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,
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 (Sterner & 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 (0.165 M MOPS, and incubated at 37 °C) 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. 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, washed twice in PBS (100 μg ml⁻¹), and then resuspended in more PBS (100 μl). 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 μg 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 (Immunoculture 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 10⁶ 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-(Asparyl)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 the SR-FLICA 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 %. Identical procedures were used to determine 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* SC5314. 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-Dhaferi & 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 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 biofilm cells.

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.
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.*, 2003; Shirtliff *et al.*, 2009).

**Fig. 1.** Live–dead staining of Candida biofilm cells with fluorescein diacetate. Biofilms (48 h) were incubated with amphotericin B (100 μg ml⁻¹) and fluorescein diacetate (100 μg 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 μm.

**Fig. 2.** Caspase detection using a polycaspase SR-FLICA reagent. Biofilms (48 h) were incubated with amphotericin B (50 μg 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 μm.
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 monosubstituted 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...
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 or C. parapsilosis (Δ) 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 ± s.e.s 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).

**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).

**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 μg ml⁻¹, or 2 to 32 mM (equivalent to 220 μg 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 μg ml⁻¹; Fig. 6a), and by even lower concentrations of butyrate (2 mM) at higher concentrations (50 μg 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 μg 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 μg 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 μg ml⁻¹ with amphotericin B at 50 μg ml⁻¹ (Fig. 6b). Biofilms of C. krusei were even more sensitive to this inhibitor and lost all viability after treatment with 8 μg valproate ml⁻¹ and 50 μg 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 yca1 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 μg ml⁻¹ (a) or 50 μg ml⁻¹ (b, c, d), and sodium butyrate (●; mM), sodium valproate (□; μg ml⁻¹), apicidin (▲; μg ml⁻¹) or trichostatin A (△; μg ml⁻¹). After 24 h at 37 °C, cell survival was determined by viable cell counts. Results are shown as means ± SEs of two independent experiments carried out in duplicate.](http://jmm.sgmjournals.org)
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.

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REFERENCES


Apoptosis and Candida biofilms


