

Salicylhydroxamic Acid Inhibits the Growth of *Candida albicans*

Shu-Ying Marissa Pang, Stephen Tristram and Simon Brown

Abstract—*Candida* spp. are common and aggressive pathogens. Because of the growing resistance of *Candida* spp. to current antifungals, novel targets, found in *Candida* spp. but not in humans or other flora, have to be identified. The alternative oxidase (AOX) is one such possibility. This enzyme is insensitive to cyanide, but is sensitive to compounds such as salicylhydroxamic acid (SHAM), disulfiram and *n*-alkyl gallates. The growth *Candida albicans* was inhibited by SHAM ($K_i = 9\text{-}15$ mM) and cyanide ($K_i = 2\text{-}4$ mM), albeit to differing extents. The rate of O_2 uptake was inhibited by less than 10% by 25 mM SHAM and by about 90% by 250 μM KCN. Although SHAM substantially inhibited the growth of *C. albicans*, it is unlikely that the inhibition of AOX was the cause. Salicylhydroxamic acid is used therapeutically in the treatment of urinary tract infections and urolithiasis, but it also has some potential in the treatment of *C. albicans* infection.

Keywords—alternative oxidase, *Candida albicans*, growth, respiration, salicylhydroxamic acid.

I. INTRODUCTION

Candida albicans is isolated from 15 – 60% of healthy individuals. However, *Candida* spp. are increasingly considered highly prevalent and aggressive pathogens because of their growing resistance to the currently available antifungals. Several large-scale studies of candidaemia [1, 2] show that the probability of death within 30 days varied from about 40% for cases of infection with *C. albicans*, *C. glabrata* or *C. tropicalis* to about 20% with *C. parapsilopsis*. Gudlaugsson *et al.* [3] concluded that the *Candida* spp. infections, rather than any underlying disease, accounted for about 38% of deaths of patients with candidaemia. Although *C. albicans* and *C. glabrata* have been the most commonly isolated species, other species, such as *C. tropicalis* and *C. krusei*, are also associated with significant mortality [1, 2].

The growing antifungal resistance of *Candida* spp. and their pathogenicity necessitate the identification of novel potential drug targets. Ideally, such targets should be found in *Candida* spp., but not in humans or other flora. One such possibility is the alternative oxidase (AOX), which is an electron transfer

chain enzyme that accepts electrons directly from ubiquinol (UQH_2) and reduces O_2 to water.

Alternative oxidase activity is inhibited by compounds such as salicylhydroxamic acid (SHAM) [4], disulfiram and *n*-alkyl gallates [5, 6], none of which inhibit the activity of cytochrome oxidase. Conversely, cyanide inhibits cytochrome oxidase, but does not inhibit AOX. These two inhibitors can be used to distinguish between O_2 uptake catalysed by AOX and cytochrome oxidase. The disruption of electron transfer by cyanide and SHAM would affect ATP synthesis and the activity of metabolic pathways, either of which would inhibit growth. Inhibition of AOX has been reported to potentiate the inhibition of the growth of *C. albicans* by fluconazole [7]. It has been suggested that AOX is a viable target in the treatment of trypanosomes [8] and that it plays an important role in the stress response in various fungi [9] and in plants [10]. Moreover, hydroxamic acids (such as SHAM) inhibit the growth of some bacteria, some of which also have an AOX homologue [11], and other fungi [12, 13].

Here, we report on the effects of SHAM and KCN on the growth of *C. albicans* in liquid culture and on plates, using a disk diffusion protocol [14]. We also report on the effect of SHAM and KCN on the rate of O_2 uptake by *C. albicans*. We show that both the growth of and O_2 uptake by *C. albicans* are inhibited by SHAM or KCN. However, inhibition of AOX is not likely to be the cause of the effect of SHAM because the concentrations required (K_i) to inhibit growth and O_2 uptake differ considerably. Nevertheless, SHAM has some potential in the treatment of *C. albicans* infection and is used in the treatment of urinary tract infections and urolithiasis [15, 16].

II. MATERIALS AND METHODS

A. Culture growth

Candida albicans was obtained from the University of Tasmania culture collection and its identity was confirmed using the Remel RapID™ Yeast Plus System® (Remel Laboratories, Lenexa, Kansas, USA).

Candida albicans was grown in liquid YPD (1% (w/v) yeast extract, 2% (w/v) bacteriological peptone, 2% (w/v) glucose) in the presence or absence of either or both of KCN and SHAM. Routinely, cultures (50 mL YPD in a 250 mL conical flask) were inoculated with sufficient cells from an overnight culture to get an initial A_{600} of 0.4 and grown for 15 h, unless otherwise specified, at $(37 \pm 1)^\circ\text{C}$ in a shaking incubator (at

S. -Y. M. Pang was with the School of Human Life Sciences, University of Tasmania, Launceston, Tasmania 7250, Australia (e-mail: sympang@utas.edu.au).

S. Tristram is with the School of Human Life Sciences, University of Tasmania, Launceston, Tasmania 7250, Australia (e-mail: Stephen.Tristram@utas.edu.au).

S. Brown is with the School of Human Life Sciences, University of Tasmania, Launceston, Tasmania 7250, Australia (phone: +61-3-6324-5467; fax: +61-3-6324-3995; e-mail: Simon.Brown@utas.edu.au).

200 min⁻¹). Where the cultures were monitored repeatedly, exit cultures on MacConkey agar (2% (w/v) peptone, 1% (w/v) lactose, 0.5% (w/v) bile salts, 0.5% (w/v) NaCl, 0.0075% (w/v) neutral red and 1.2% (w/v) agar), blood agar (5% (w/v) defibrinated horse blood and tryptone soya agar consisting of 1.5% (w/v) tryptone, 0.5% (w/v) soya peptone, 0.5% (w/v) NaCl and 1.5% (w/v) agar) and YPD agar (YPD supplemented with 1.5% (w/v) agar) plates were prepared to check for possible contamination. *Candida* spp. were maintained at 37°C on YPD agar and subcultured onto a fresh plate every three days.

The Gompertz function [17], as parameterized by Zweitering *et al.* [18], but with the addition of a parameter to estimate the initial inoculum ($A(0)$)

$$A(t) = A(0) + [A_{\max} - A(0)] \times \exp\left\{-\exp\left[\frac{\mu_m \exp(1)}{A_{\max} - A(0)}(\lambda - t) + 1\right]\right\} \quad (1)$$

was fitted directly to the growth data (A_{600}) by nonlinear regression using the `nlm` function in R [19]. In (1), $A(t)$ represents the absorbance at 600 nm at time t (h), μ_m is the maximum growth rate (h⁻¹), λ is the lag time (h) and A_{\max} is the maximum absorbance. Other models were tested, but the improvement in the fit did not justify the introduction of any extra parameters ($p = 0.187$).

B. Disk diffusion analysis

Disks (diameter = 6 mm) were prepared from Whatman's antibiotic assay filter paper and autoclaved. Individual disks were placed in the wells of a 96-well microtitre plate and volumes of KCN or SHAM (in absolute ethanol) were added to each well to give 5 – 400 μmol KCN or 7.3 – 183 μmol SHAM, respectively. The residual solvent was allowed to evaporate before the disks were used.

Diffusion assays were carried out on YPD plates to which was added 2.5 mL of a 0.5 McFarland standard suspension of *Candida* spp. prepared in 0.85% saline. The suspension was allowed to distribute evenly across the plate and the excess was discarded. The disks were put in place and the plates were incubated at $(37 \pm 1)^\circ\text{C}$ for 24 h at which time the diameter of each of the cleared zones was measured.

Generally, disk diffusion assays are analysed by reporting the radius of the zone of inhibition (r), but such experiments can also be used to estimate the critical concentration (C) at which inhibition of growth becomes apparent and the diffusion coefficient (D) in agar of the compound of interest. The estimation of these parameters is based on the linear relationship between r^2 and $\ln(A)$, where A is the amount of the compound on the disk [20-22].

$$r^2 = 4Dt \ln(A) - 4Dt \ln(4\pi d t d C) = \alpha \ln(A) - \beta, \quad (2)$$

where $\alpha = 4Dt$ and $\beta = \alpha \ln(4\pi d t d C)$, and D is the diffusion coefficient of the compound on the disk, t is the time, d is the thickness of the agar, C is the effective inhibitory concentration of the compound and A is the amount of the compound on the disk. Obviously, the radius is at least the size of the disk (r_0), as below a certain critical value of $\ln(A)$

(= $\ln(A_c)$) cell growth is not inhibited by the compound (so $r = r_0$), and no greater than the size of the plate (radius = R) on which the cells are cultured.

The direct application of (2) (the linear model) is only possible if growth is inhibited significantly over the range of $\ln(A)$. Even then it requires that a decision be made about the location of the range in which the relationship is linear.

An extension of (2) (the bilinear model) involves an objective estimation of $\ln(A_c)$

$$r^2 = \begin{cases} r_0^2, & \ln(A) \leq \ln(A_c) \\ r_0^2 + \alpha \ln\left(\frac{A}{A_c}\right), & \ln(A) > \ln(A_c) \end{cases} \quad (3)$$

which makes use of all the obtained data and eliminates any need to choose the linear data range. In (3), only α (which has the same significance as in (2)) and the critical amount (A_c) have to be estimated by nonlinear regression, since r_0 is the actual radius of the disk. The bilinear model also requires that growth is inhibited significantly over the range of $\ln(A)$.

A third model (the logistic model) is based on the logistic function and (2)

$$r^2 = r_0^2 + \frac{2(\beta - r_0^2)}{1 + \exp\left[-\frac{2\alpha}{\beta - r_0^2} \ln\left(\frac{A}{A_0}\right)\right]} \quad (4)$$

where A_0 is the inflection point of the logistic function, and α has the same significance as in (2), and β is related to the upper limit of the radius of the zone of clearing. This model facilitates the estimation of $\ln(A_c)$ even where there is only limited inhibition of growth.

C. Oxygen uptake measurements

Cells were harvested after 15 h growth in liquid YPD by centrifugation at $1335 \times g$ for 15 min at 4°C (Sigma Laboratory Centrifuge 4K15). The cells were washed with 50 mM Na₂HPO₄-NaH₂PO₄ (pH 6.5) and then resuspended to 5 mL in the same buffer. The O₂ uptake by cell suspensions was measured polarographically with a Clark-type O₂ electrode (Hansatech Instruments Ltd, King's Lynn) at $(37 \pm 1)^\circ\text{C}$. The cell suspension in the cuvette (5 mg wet weight mL⁻¹) contained 2 mM glucose in 50 mM Na₂HPO₄-NaH₂PO₄ (pH 6.5). The O₂ concentration in the cuvette was digitised each second (each datum represented the average of 1000 determinations) using hardware and software developed in-house [23].

III. RESULTS

A. Growth of *C. albicans*

The effect of SHAM on the growth of *Candida* spp. was assessed by adding solid SHAM to the culture. Generally, SHAM was dissolved in ethanol, which itself inhibited growth. In liquid culture, 1.37% (v/v) ethanol (the concentration that would have been associated with 0.1 g in 50 mL of culture or about 13 mM SHAM) reduced μ_m and A_{\max} by 42% and 6%, respectively, and increased λ by 22%,

and 2.67% (v/v) ethanol exerted an even more significant effect (data not shown). The effects of SHAM and solvent were confounded and so it was decided that solid SHAM should be added to the culture.

Salicylhydroxamic acid inhibited the growth of *C. albicans* (Fig 1) by reducing both A_{max} and μ_m and increasing λ . In the presence (in 50 mL) of 0.05 g, 0.1 g, 0.15 g and 0.2 g of SHAM (corresponding to approximately 6.5 mM, 13 mM, 19.5 mM and 26 mM SHAM, respectively), the growth rates were reduced by 43%, 57%, 87% and 91% respectively. The A_{max} was 9 – 83% of the control (17.1 ± 0.1) with increasing concentrations of SHAM and λ was 22 – 213% longer than the control (3.19 ± 0.05 h).

Cyanide inhibited the growth of *C. albicans* (Fig 2), reducing both the growth rate (μ_m) and the overall growth (A_{max}), and increasing the lag time (λ). The growth rate was reduced by approximately 36%, 41%, 79% and 76% in the presence of 1 mM, 2 mM, 5 mM and 10 mM KCN, respectively, compared with the control (4.24 ± 0.09 h⁻¹). The A_{max} was 7 – 96% lower than the control (17.1 ± 0.1) with increasing concentrations of KCN, while λ was approximately 16 – 79% longer than the control (3.19 ± 0.05 h).

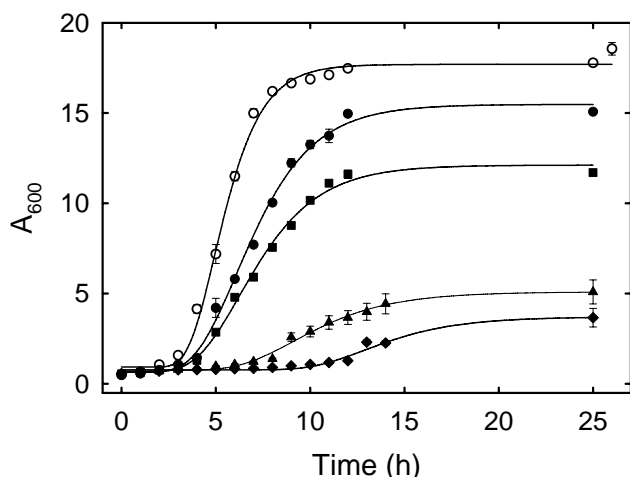


Fig. 1 Effect of varying concentration of SHAM on the growth of *C. albicans*. Cells were grown in YPD in a shaking incubator at 200 min^{-1} at $(37 \pm 1)^\circ\text{C}$. Error bars are \pm SE for 3 – 15 separate cultures. Curves are fits of the Gompertz function (1) to all the data obtained from cultures at a given SHAM concentration. \circ - control; \bullet - $0.05 \text{ g (50 mL)}^{-1}$ SHAM; \blacksquare - $0.1 \text{ g (50 mL)}^{-1}$ SHAM; \blacktriangle - $0.15 \text{ g (50 mL)}^{-1}$ SHAM; \blacklozenge - $0.2 \text{ g (50 mL)}^{-1}$ SHAM.

The apparent inhibition constants (K_i) for the effect of KCN on $A_{max} - A(0)$ and μ_m were 4.1 ± 0.2 mM and 1.7 ± 0.6 mM, respectively, and the corresponding values for the effect of SHAM were $0.11 \pm 0.01 \text{ g (50 mL)}^{-1}$ SHAM (approximately 15 \pm 2 mM) and $0.07 \pm 0.05 \text{ g (50 mL)}^{-1}$ SHAM (approximately 9 \pm 7 mM), respectively.

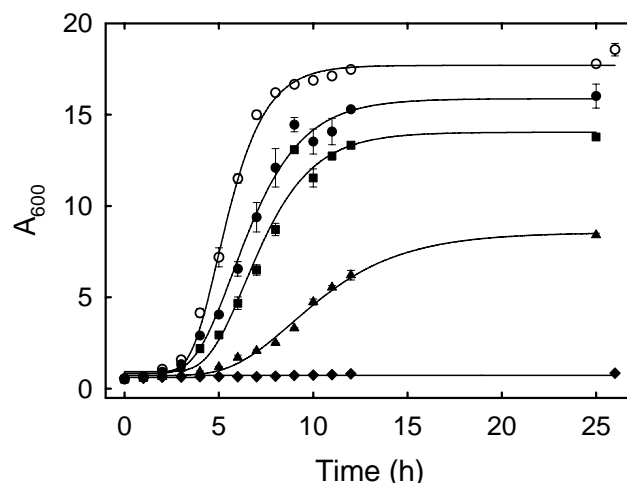


Fig. 2 Effect of varying concentrations of KCN on the growth of *C. albicans*. Cells were grown in YPD in a shaking incubator at 200 min^{-1} at $(37 \pm 1)^\circ\text{C}$. Error bars are \pm SE for 3 – 15 separate cultures. Curves are fits of the Gompertz function (1) of all the data obtained from cultures at a given KCN concentration. \circ - control; \bullet - 1 mM KCN; \blacksquare - 2 mM KCN; \blacktriangle - 5 mM KCN; \blacklozenge - 10 mM KCN.

In the presence of both 1 mM KCN and $0.1 \text{ g (50 mL)}^{-1}$ SHAM, a synergistic effect was observed (Fig 3). The growth rate was reduced by 86% and A_{max} was 77% lower than the control (17.1 ± 0.1). The lag time was approximately 79% longer than the control (3.19 ± 0.05 h). This synergistic effect was slightly greater than would be expected from the effects of 1 mM KCN and SHAM alone (for example, the residual μ_{ms} in the presence of KCN, SHAM or both were 64%, 43% and 14% of the control, respectively, and multiplying the first two values yields 27% of the control, which is about twice the 14% observed).

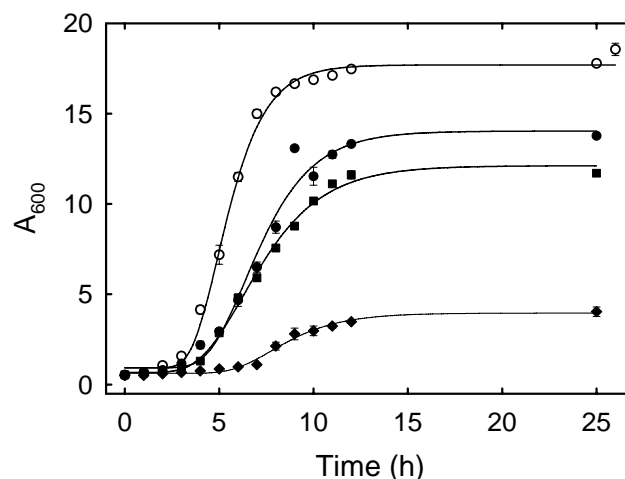


Fig. 3 Synergistic effect of 1 mM KCN and $0.1 \text{ g (50 mL)}^{-1}$ SHAM. Cells were grown in YPD in a shaking incubator at 200 min^{-1} at $(37 \pm 1)^\circ\text{C}$. Error bars are \pm SE for 3 – 15 separate cultures. Curves are fits of the Gompertz function (1) of all the data obtained from cultures at the given KCN/SHAM concentration(s). \circ - control; \bullet - 1 mM KCN; \blacksquare - $0.1 \text{ g (50 mL)}^{-1}$ SHAM; \blacklozenge - 1 mM KCN + $0.1 \text{ g (50 mL)}^{-1}$ SHAM.

B. Disk diffusion assays

Disk diffusion assays were carried out to estimate the relative sensitivity of the *C. albicans* to KCN and SHAM. The KCN data were analysed using the linear, bilinear and logistic models (1-3) where possible, whereas the SHAM data were analysed using only the logistic model (4) because it was not possible to apply high amounts to the disk. From these data, the diffusion coefficient (D), critical amount ($\ln(A_c)$) and concentration (C) of the compound were estimated.

As SHAM is relatively insoluble in ethanol or water, it was not possible to apply very high amounts to the disk. Preliminary experiments using dimethylsulphoxide (DMSO) as a solvent for SHAM rather than ethanol were unsuccessful because of the high boiling point of DMSO, which made it difficult to evaporate. Moreover, the amount of SHAM on the disk was overestimated, because the solid tended to accumulate on the surface of the disk and it was difficult to move the disk without dislodging some of the SHAM.

As is illustrated by the data for *C. albicans* shown in Fig. 4, at low amounts on the disk, below the estimated A_c , no inhibition was observed and r was constant ($r = r_0$). As the amount on the disk was increased, r increased. If sufficient inhibitor was present on the disk, no growth was observed as the concentration of the compound in the agar became too high (data not shown). The effect of SHAM on growth was limited, although a small zone of clearing is apparent in Fig. 4A around the disk at the highest amount (Fig. 5). This implies that A_c should be above the upper limit of the range used. In contrast, KCN clearly inhibited growth (Fig. 4B).

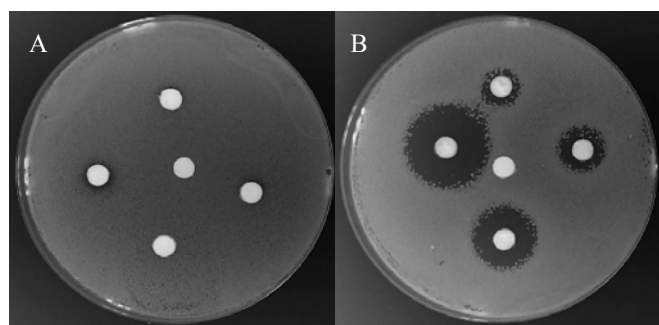
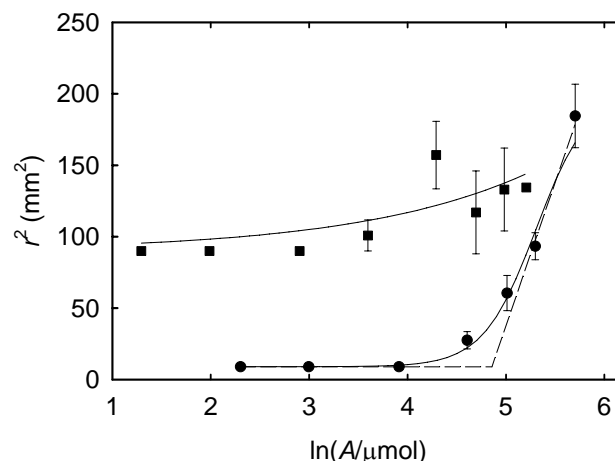


Fig. 4. Effect of SHAM (A) and KCN (B) on the growth of *C. albicans*. Amounts of SHAM on the each disk were (clockwise from top) 73 μmol , 36.5 μmol , 18.3 μmol and 7.3 μmol while amounts of KCN were (clockwise from top) 20 μmol , 40 μmol , 100 μmol and 200 μmol . The disk in the middle of each plate served as a control to which solvent (but neither SHAM nor KCN) was added.

From the data in Fig. 5 the diffusion coefficient for KCN was estimated to be $5\text{-}6 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, about 25% that of CN⁻ in an aqueous medium ($2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$) at 25°C [24]. While this is lower than would be expected from literature data for other compounds [25, 26] or from models of diffusion in similar media [27, 28], the values were of the right order of magnitude (about $10^{-5} \text{ cm}^2 \text{ s}^{-1}$), providing some support for the plausibility of the other parameter estimates. Based on the

logistic model (4), the estimated values of $\ln(A_c)$ were 4.8 ± 0.1 for KCN and 7 ± 1 for SHAM, corresponding to about 120 μmol KCN and 1.1 mmol SHAM, respectively. Of course, the estimate for SHAM represents an extrapolation from the data and must be regarded with some scepticism. Based on these estimates, critical concentrations (C) of $0.6 \pm 0.2 \text{ mM}$ KCN



and about 6 mM SHAM were derived.

Fig. 5. Radii of inhibition of the growth of *C. albicans* in the presence of various amounts of KCN or SHAM. The SHAM data (and the corresponding errors) have been multiplied by 10 to allow them to be easily seen. Errors bars are $\pm \text{SE}$ (or $\pm 10 \times \text{SE}$ for SHAM) of at least 3 measurements. The solid lines are fits of the logistic model (4), while the dashed line is a fit of the bilinear model (3) to the data. ● - KCN; ■ - SHAM.

C. Oxygen uptake assays

To determine whether the inhibitory effect on the growth of *C. albicans* of SHAM was related to an inhibition of the alternative oxidase of the mitochondrial electron transfer chain the rate of O_2 uptake was measured.

A suspension of freshly harvested *C. albicans* consumed O_2 at a rate of $4.9 \pm 0.2 \text{ nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ wet weight (based on measurements of five separate cultures), but the addition of glucose to the cuvette increased the rate of O_2 uptake. In order to determine the optimum concentration of glucose to be added to the cuvette prior to the initiation of the assay, a range of glucose concentrations (0 – 10 mM) was tested with *C. albicans* (Fig. 6). The greatest rate of uptake of O_2 by *C. albicans* was observed with 2 mM glucose and so 2 mM glucose was included in subsequent assays.

In order to determine the apparent inhibition constant for cyanide (K_i), the effect on the rate of O_2 uptake of a range of KCN concentrations (0 – 500 μM) was determined with *C. albicans* (Fig. 7). In the presence of 250 μM KCN, the rate of O_2 uptake by *C. albicans* was inhibited by about 90%, the asymptotic residual activity was $(9 \pm 1)\%$ of the control activity, and the estimated K_i was $13 \pm 1 \mu\text{M}$.

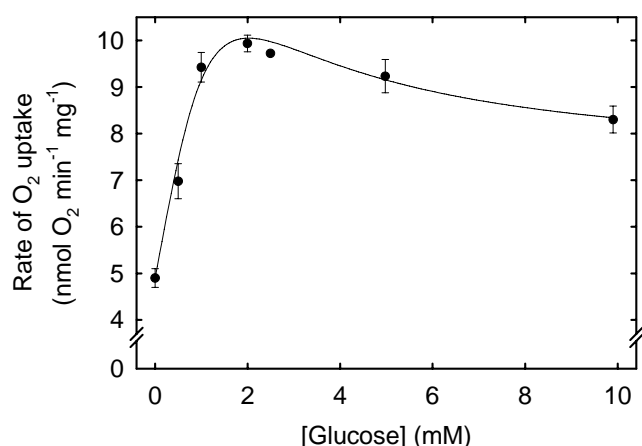


Fig. 6. Rate of O₂ uptake by *C. albicans* in the presence of 0 – 10 mM glucose. Assays were carried out in 50 mM Na₂HPO₄-NaH₂PO₄ (pH 6.5) at (37 ± 1)°C using 5 mg wet weight mL⁻¹. Error bars are ± SE for at least 3 separate cultures and rates were determined before and after the addition of glucose. The curve is a fit of a generalised rate expression [29] to all of the data obtained at each glucose concentration and is only intended to guide the eye.

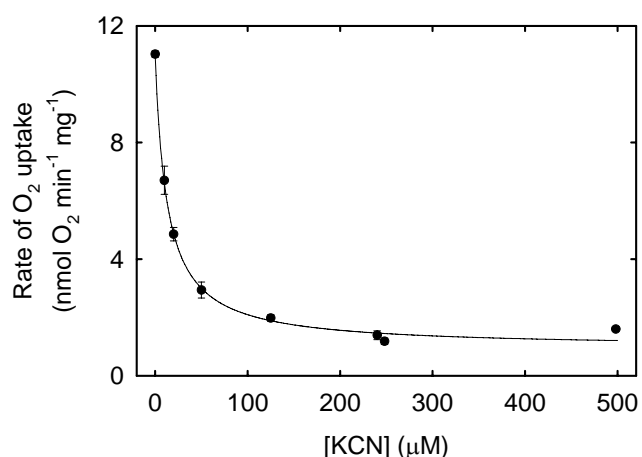


Fig. 7. Rate of O₂ uptake by *C. albicans* in the presence of 0 - 500 μM KCN and 2 mM glucose. Assays were carried out at (37 ± 1)°C in 50 mM phosphate (pH 6.5) containing 2 mM glucose and 5 mg wet weight mL⁻¹. Error bars are ± SE for at least 3 separate cultures and rates were obtained after the addition of KCN. The curve is a fit of $v = K_i v_0 / (K_i + [\text{KCN}])$ [32] to all the data obtained at a given KCN concentration. Here, K_i is the apparent inhibition constant and $v_0 = 11.5 \pm 0.2$ nmol O₂ min⁻¹ mg⁻¹ wet weight.

In order to assess the SHAM-sensitivity of O₂ uptake, the effect on the rate of O₂ uptake of a range of SHAM concentrations (0 – 50 mM) was determined (Fig. 8). Salicylhydroxamic acid was dissolved in ethanol, but ethanol alone had a significant effect on the rate of O₂ uptake by *C. albicans* (Fig. 8A) and also artefactually increased the oxygen concentration in the cuvette because O₂ is about 25 times more soluble in ethanol than it is in water [30]. Therefore dimethylsulphoxide (DMSO), an alternative solvent in which O₂ is much less soluble [30], was tested to see whether the solvent effects could be minimised (Fig. 8B). Ethanol

affected the rate of O₂ uptake more significantly than did DMSO (Fig. 8, A and B) and so SHAM dissolved in DMSO was used in subsequent experiments. However, low concentrations of SHAM dissolved in ethanol stimulated O₂ uptake, as has been reported previously [31], although no explanation for this phenomenon has been proposed. Based on the fitted lines (Fig. 8), at 24.4 mM SHAM the relative activity was estimated to be 76% or 81% in ethanol or DMSO, respectively. The K_i was estimated to be approximately 20 mM SHAM because this inhibited the rate of O₂ uptake by about 5%, which is approximately half of the residual activity observed when cytochrome oxidase was inhibited by cyanide (Fig. 7).

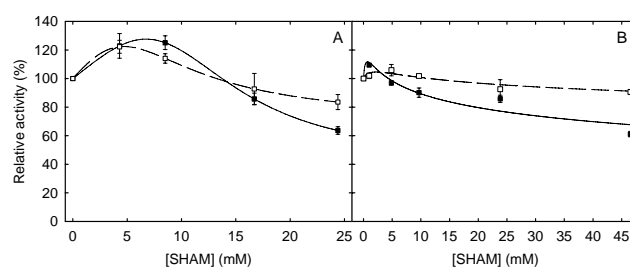


Fig. 8. Effect of (A) SHAM in ethanol (■) and the corresponding volumes of ethanol (□) and of (B) SHAM in DMSO (■) and the corresponding volumes of DMSO (□) on the rate of O₂ uptake by *C. albicans*. The assay was carried out at 37°C in 50 mM Na₂HPO₄-NaH₂PO₄ (pH 6.5) containing 2 mM glucose and 5 mg wet weight mL⁻¹. The control activity (100%) represents a rate in of 11.5 ± 0.2 nmol O₂ min⁻¹ mg⁻¹ wet weight. Error bars are ± SE of at least 3 measurements. The dashed and solid lines are fits of an empirical equation [33] to the raw data and are only intended to guide the eye.

IV. DISCUSSION

The data reported here show that the growth of *C. albicans* was inhibited by KCN and by SHAM. The apparent K_i s for the inhibition of the growth of *C. albicans* by KCN or SHAM were 2 – 4 mM or 9 – 15 mM (Figs. 1 and 2), respectively, and from the disk diffusion experiments the critical concentrations for the onset of growth inhibition were 0.6 mM KCN and 6 mM SHAM (Fig. 5). Of course, a significant limitation was imposed on the SHAM disk diffusion experiments by the relative insolubility of SHAM.

Cyanide inhibited the rate of O₂ uptake by *C. albicans* by 90%, although the K_i was only 13 μM (Fig. 7) which is much less than the K_i for the inhibition of growth and is also less than the critical concentration (about 0.6 mM) estimated from the disk diffusion assays (Fig. 5). This implies that the inhibition of respiration by cyanide was not responsible for the inhibition of the growth of *C. albicans*.

A similar argument prompts the conclusion that inhibition by SHAM of the alternative oxidase did not make a significant contribution to the inhibition by SHAM of the growth of *Candida* spp. in these conditions. The residual activity seen with the uptake of O₂ by *C. albicans* is (9 ± 1)% and the majority of this is accounted for by the activity of AOX.

While 9 – 15 mM SHAM inhibited the growth of *C. albicans* by 50%, approximately 20 mM SHAM inhibited the rate of O₂ uptake 5%, which accounts for about 50% of the activity of AOX. Although the *K_s* are approximately the same, the residual growth (about 20%) and the residual rate of O₂ uptake (approximately 90%) were not.

Given that SHAM did inhibit the growth of *C. albicans* (Fig. 1), there must be some other target for SHAM. Salicylhydroxamic acid also inhibits tyrosinases [34], ureases [35-37] and peroxidases [34, 38], but no homologues of urease or tyrosinase could be found in the complete genome or the derived proteome of *C. albicans* [39]. Moreover, no tyrosinase activity could be detected in *C. albicans* (data not shown). While these observations do not eliminate the possibility that either or both of these enzymes is present in *C. albicans*, the SHAM-sensitive peroxidases appear to be more likely targets.

Other hydroxamic acids (RCONR'OH) also inhibit urease [35-37], peroxidases [34, 38] and tyrosinase [34], but they also inhibit β-lactamase [40], DNA replication [41], Δ⁶-desaturase [42], various metalloproteases [43] and 5-lipoxygenase [44], among other enzymes. Hydroxamic acids also chelate cations [45, 46], including iron [45, 47]. While chelators inhibit the transfer of electrons [48-50] and probably other processes, supplementing the culture medium with cations may alleviate the effect of chelators on growth [51]. It is of course conceivable that the inhibition of growth in the presence of SHAM is due to one or more of these general hydroxamic acid effects.

V. CONCLUSIONS

The growth of *C. albicans* was affected substantially by SHAM at concentrations comparable to the therapeutic dose of fluconazole. For this reason, SHAM has some potential as an antifungal. However, AOX is not likely to be a potential target for antifungals in the conditions in which these experiments were performed. This was because the effect of SHAM on O₂ uptake was limited, indicating that SHAM had a limited effect on energy transduction. These experiments should be repeated with other *Candida* spp. to determine whether or not *C. albicans* is especially sensitive to SHAM.

ACKNOWLEDGMENT

We thank Patrizia Carr for her invaluable assistance while this work was being carried out.

REFERENCES

- [1] A. M. Tortorano, J. Peman, H. Bernhardt, L. Klingspor, C. C. Kibbler, O. Faure, E. Biraghi, E. Canton, K. Zimmermann, S. Seaton, and R. Grillot, "Epidemiology of candidaemia in Europe: results of 28-month European Confederation of Medical Mycology (ECMM) hospital-based surveillance study," *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 23, pp. 317-322, 2004.
- [2] M. A. Pfaller, R. N. Jones, G. V. Doern, H. S. Sader, S. A. Messer, A. Houston, S. Coffman, and R. J. Hollis, "Bloodstream infections due to *Candida* species: SENTRY Antimicrobial Surveillance Program in North America and Latin America, 1997-1998," *Antimicrobial Agents and Chemotherapy*, vol. 44, pp. 747-751, 2000.
- [3] O. Gudlaugsson, S. Gillespie, K. Lee, J. Vande Berg, J. Hu, S. Messer, L. Herwaldt, M. Pfaller, and D. Diekema, "Attributable mortality of nosocomial candidemia, revisited," *Clinical Infectious Diseases*, vol. 37, pp. 1172-1177, 2003.
- [4] G. R. Schonbaum, W. D. Bonner, Jr., B. T. Storey, and J. T. Bahr, "Specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids," *Plant Physiology*, vol. 47, pp. 124-128, 1971.
- [5] J. N. Siedow and M. E. Girvin, "Alternative Respiratory Pathway: Its role in seed respiration and its inhibition by propyl gallate," *Plant Physiology*, vol. 65, pp. 669-674, 1980.
- [6] J. N. Siedow and D. M. Bickett, "Structural features required for inhibition of cyanide-insensitive electron transfer by propyl gallate," *Archive of Biochemistry and Biophysics*, vol. 207, pp. 32-39, 1981.
- [7] L. Yan, M. Li, Y. Cao, P. Gao, Y. Cao, Y. Wang, and Y. Jiang, "The alternative oxidase of *Candida albicans* causes reduced fluconazole susceptibility," *Journal of Antimicrobial Chemotherapy*, vol. to be published, 2009.
- [8] N. Sen and H. K. Majumder, "Mitochondrion of protozoan parasite emerges as potent therapeutic target: exciting drugs are on the horizon," *Current Pharmaceutical Design*, vol. 14, pp. 839-846, 2008.
- [9] A. Veiga, J. D. Arrabaca, and M. C. Loureiro-Dias, "Stress situations induce cyanide-resistant respiration in spoilage yeasts," *Journal of Applied Microbiology*, vol. 95, pp. 364-371, 2003.
- [10] V. N. Popov, R. A. Simonian, V. P. Skulachev, and A. A. Starkov, "Inhibition of the alternative oxidase stimulates H₂O₂ production in plant mitochondria," *FEBS Letters*, vol. 415, pp. 87-90, 1997.
- [11] S.-Y. M. Pang, S. Tristram, and S. Brown, "An *in silico* model of the alternative oxidase," *International Journal of Biosciences and Technology*, vol. submitted for publication, 2009.
- [12] R. M. Nervig and S. Kadis, "Effect of hydroxamic acids on growth and urease activity in *Corynebacterium renale*," *Canadian Journal of Microbiology*, vol. 22, pp. 544-551, 1976.
- [13] C. Y. Wang and L. H. Lee, "Mutagenicity and antibacterial activity of hydroxamic acids," *Antimicrobial Agents and Chemotherapy*, vol. 11, pp. 753-755, 1977.
- [14] J. J. Gavin, "Analytical microbiology. II. The diffusion methods," *Applied Microbiology*, vol. 5, pp. 25-33, 1957.
- [15] S. Budavari, "The Merck Index," 12 ed. Whitehouse Station: Merck & Co., Inc., 1996.
- [16] A.-E. A. Salem and M. M. Omar, "Atomic absorption and spectrophotometric determinations of salicylhydroxamic acid in its pure and pharmaceutical dosage forms," *Turkish Journal of Chemistry*, vol. 27, pp. 383-393, 2003.
- [17] B. Gompertz, "On the nature of the function expressive of the law of human mortality, and on a new mode of determining the value of life contingencies," *Philosophical Transactions of the Royal Society of London*, vol. 115, pp. 513-585, 1825.
- [18] M. H. Zweitering, I. Jongenburger, F. M. Rombouts, and K. van't Riet, "Modeling of the bacterial growth curve," *Applied and Environmental Microbiology*, vol. 56, pp. 1875-1881, 1990.
- [19] R Development Core Team, "R: A language and environment for statistical computing." Vienna, Austria: R Foundation for Statistical Computing, 2006.
- [20] R. K. Finn, "Theory of agar diffusion methods of assay," *Analytical Chemistry*, vol. 31, pp. 975-977, 1959.
- [21] M. L. Delignette-Muller and J. P. Flandrois, "An accurate diffusion method for determining bacterial sensitivity to antibiotics," *Journal of Antimicrobial Chemotherapy*, vol. 34, pp. 73-81, 1994.
- [22] A. L. Koch, "Diffusion through agar blocks of finite dimensions: a theoretical analysis of three systems of practical significance in microbiology," *Microbiology*, vol. 145, pp. 643-654, 1999.
- [23] S. Brown and N. L. Taylor, "Inhibition of mitochondrial electron transfer by antipsychotic medication," *Human and Veterinary Toxicology*, vol. 42, pp. 209-211, 2000.
- [24] J. B. Hiskey and V. M. Sanchez, "Mechanistic and kinetic aspects of silver dissolution in cyanide solutions," *Journal of Applied Electrochemistry*, vol. 20, pp. 479-487, 1990.
- [25] B. K. Davis, "Diffusion in polymer gel implants," *Proceedings of the National Academy of Sciences of the USA*, vol. 71, pp. 3120-3123, 1974.
- [26] L. Friedman, "Structure of agar gels from studies of diffusion," *Journal of the American Chemical Society*, vol. 52, pp. 1311-1314, 1930.

- [27] N. Fatin-Rogue, K. Starchev, and J. Buffle, "Size effects on diffusion processes within agarose gels," *Biophysical Journal*, vol. 86, pp. 2710-2719, 2004.
- [28] E. J. Schantz and M. A. Lauffer, "Diffusion measurements in agar gel.," *Biochemistry*, vol. 1, pp. 658-663, 1962.
- [29] W. G. Bardsley, P. Leff, J. Kavanagh, and R. D. Waight, "Deviations from Michaelis-Menten kinetics. The possibility of complicated curves for simple kinetic schemes and the computer fitting of experimental data for acetylcholinesterase, acid phosphatase, adenosine deaminase, arylsulphatase, benzylamine oxidase, chymotrypsin, fumarase, galactose dehydrogenase, β -galactosidase, lactate dehydrogenase, peroxidase and xanthine oxidase.," *Biochemical Journal*, vol. 187, pp. 739-765, 1980.
- [30] R. Battino, T. R. Rettich, and T. Tominaga, "The solubility of oxygen and ozone in liquids," *Journal of Physical and Chemical Reference Data*, vol. 12, pp. 163-178, 1983.
- [31] S. Aoki and S. Ito-Kuwa, "Respiration of *Candida albicans* in relation to its morphogenesis," *Plant and Cell Physiology*, vol. 23, pp. 721-726, 1982.
- [32] Nomenclature Committee of the International Union of Biochemistry, "Symbolism and terminology in enzyme kinetics.," *European Journal of Biochemistry*, vol. 128, pp. 281-291, 1982.
- [33] O. Schabenberger, B. E. Tharp, J. J. Kells, and D. Penner, "Statistical tests for hormesis and effective dosages in herbicide dose response.," *Agronomy Journal*, vol. 91, pp. 713-721, 1999.
- [34] P. R. Rich, N. K. Wiegand, H. Blum, A. L. Moore, and W. D. Bonner, Jr., "Studies on the mechanism of inhibition of redox enzymes by substituted hydroxamic acids," *Biochimica et Biophysica Acta*, vol. 525, pp. 325-337, 1978.
- [35] J. Hase and K. Kobashi, "Inhibition of *Proteus vulgaris* urease by hydroxamic acids.," *Journal of Biochemistry*, vol. 62, pp. 293-299, 1967.
- [36] K. Kobashi, J. Hase, and K. Uehara, "Specific inhibition of urease by hydroxamic acids," *Biochimica et Biophysica Acta*, vol. 65, pp. 380-383, 1962.
- [37] W. N. Fishbein and P. P. Carbone, "Urease Catalysis. Ii. Inhibition of the Enzyme by Hydroxyurea, Hydroxylamine, and Acetohydroxamic Acid," *Journal of Biological Chemistry*, vol. 240, pp. 2407-2414, 1965.
- [38] B. Davies and D. W. Edwards, "Inhibition of myeloperoxidase by salicylhydroxamic acid.," *Biochemical Journal*, vol. 258, pp. 801-806, 1989.
- [39] T. Jones, N. A. Federspiel, H. Chibana, J. Dungan, S. Kalman, B. B. Magee, G. Newport, Y. R. Thorstenson, N. Agabian, P. T. Magee, R. W. Davis, and S. Scherer, "The diploid genome sequence of *Candida albicans*," *Proceedings of the National Academy of Sciences of the USA*, vol. 101, pp. 7329-7334, 2004.
- [40] J. H. Bell and R. F. Pratt, "Mechanism of inhibition of the beta-lactamase of *Enterobacter cloacae* P99 by 1:1 complexes of vanadate with hydroxamic acids," *Biochemistry*, vol. 41, pp. 4329-4338, 2002.
- [41] G. R. Gale, "Selective inhibition of deoxyribonucleic acid synthesis by salicylhydroxamic acid.," *Proceedings of the Society for Experimental Biology and Medicine.*, vol. 122, pp. 1236-1240, 1966.
- [42] I. Khozin-Goldberg, C. Bigogno, and Z. Cohen, "Salicylhydroxamic acid inhibits D6 desaturation in the microalga *Porphyridium cruentum*," *Biochimica et Biophysica Acta*, vol. 1439, pp. 384-394, 1999.
- [43] D. Leung, G. Abbenante, and D. P. Fairlie, "Protease inhibitors: current status and future prospects," *Journal of Medicinal Chemistry*, vol. 43, pp. 305-341, 2000.
- [44] J. B. Summers, K. H. Kim, H. Mazdiyasi, J. H. Holms, J. D. Ratajczyk, A. O. Stewart, R. D. Dyer, and G. W. Carter, "Hydroxamic acid inhibitors of 5-lipoxygenase: quantitative structure-activity relationships," *Journal of Medicinal Chemistry*, vol. 33, pp. 992-998, 1990.
- [45] E. C. O'Brien, S. Le Roy, J. Levailain, D. J. Fitzgerald, and K. B. Nolan, "Metal complexes of salicylhydroxamic acid and O-acetylsalicylhydroxamic acid," *Inorganica Chimica Acta*, vol. 266, pp. 117-120, 1997.
- [46] C. J. Marmion, D. Griffith, and K. B. Nolan, "Hydroxamic acids - an intriguing family of enzyme inhibitors and biomedical ligands," *European Journal of Inorganic Chemistry*, vol. 2004, pp. 3003-3017, 2004.
- [47] V. Špringer, M. Hornáková, R. Karlíček, and B. Kopecká, "Salicylhydroxamic acids and its iron(III) complexes.," *Collection of Czechoslovak Chemical Communications*, vol. 52, pp. 602-608, 1987.
- [48] B. Coyle, K. Kavanagh, M. McCann, M. Devereux, and M. Geraghty, "Mode of anti-fungal activity of 1,10-phenanthroline and its Cu(II), Mn(II) and Ag(I) complexes," *Biomaterials*, vol. 16, pp. 321-329, 2003.
- [49] P. R. Rich, A. L. Moore, and W. D. Bonner, Jr., "The effects of bathophenanthroline, bathophenanthrolinesulphonate and 2-thienoyltrifluoroacetone on mung-bean mitochondria and submitochondrial particles," *Biochemical Journal*, vol. 162, pp. 205-208, 1977.
- [50] H. J. Harmon and F. L. Crane, "Inhibition of mitochondrial electron transport by hydrophilic metal chelators. Determination of dehydrogenase topography," *Biochimica et Biophysica Acta*, vol. 440, pp. 45-58, 1976.
- [51] N. Schnell and K. D. Entian, "Identification and characterization of a *Saccharomyces cerevisiae* gene (PAR1) conferring resistance to iron chelators," *European Journal of Biochemistry*, vol. 200, pp. 487-493, 1991.