

# 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<sub>2</sub> 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<sub>2</sub>) and reduces O<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> uptake by *C. albicans*. We show that both the growth of and O<sub>2</sub> 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<sub>2</sub> 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<sub>600</sub> of 0.4 and grown for 15 h, unless otherwise specified, at (37 ± 1)°C in a shaking incubator (at

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200 min<sup>-1</sup>). 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),  $\mu_m$  is the maximum growth rate (h<sup>-1</sup>),  $\lambda$  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  $\mu\text{mol}$  KCN or 7.3 – 183  $\mu\text{mol}$  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)°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].

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

where  $\alpha = 4Dt$  and  $\beta = \alpha \ln(4\pi DtdC)$ , 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  $\alpha$  (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  $\alpha$  has the same significance as in (2), and  $\beta$  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 Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub> (pH 6.5) and then resuspended to 5 mL in the same buffer. The O<sub>2</sub> uptake by cell suspensions was measured polarographically with a Clark-type O<sub>2</sub> electrode (Hansatech Instruments Ltd, King's Lynn) at (37  $\pm$  1)°C. The cell suspension in the cuvette (5 mg wet weight mL<sup>-1</sup>) contained 2 mM glucose in 50 mM Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub> (pH 6.5). The O<sub>2</sub> 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  $\mu_m$  and  $A_{\max}$  by 42% and 6%, respectively, and increased  $\lambda$  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  $\mu_m$  and increasing  $\lambda$ . 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 ( $17.1 \pm 0.1$ ) with increasing concentrations of SHAM and  $\lambda$  was 22 – 213% longer than the control ( $3.19 \pm 0.05$  h).

Cyanide inhibited the growth of *C. albicans* (Fig 2), reducing both the growth rate ( $\mu_m$ ) and the overall growth ( $A_{max}$ ), and increasing the lag time ( $\lambda$ ). 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 ( $4.24 \pm 0.09$  h<sup>-1</sup>). The  $A_{max}$  was 7 – 96% lower than the control ( $17.1 \pm 0.1$ ) with increasing concentrations of KCN, while  $\lambda$  was approximately 16 – 79% longer than the control ( $3.19 \pm 0.05$  h).

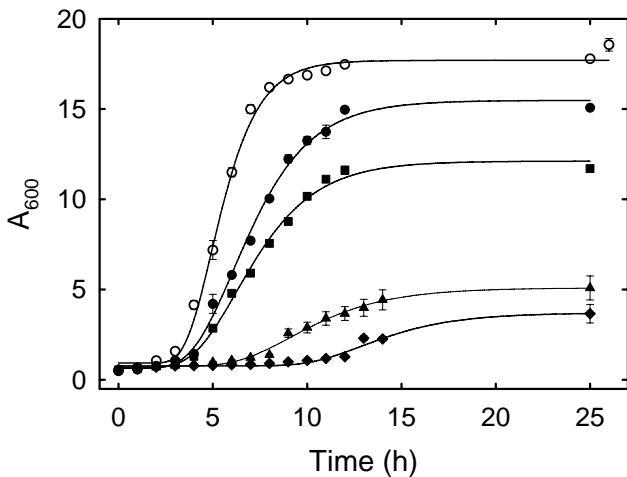


Fig. 1 Effect of varying concentration of SHAM on the growth of *C. albicans*. Cells were grown in YPD in a shaking incubator at  $200 \text{ min}^{-1}$  at  $(37 \pm 1)^\circ\text{C}$ . Error bars are  $\pm$  SE for 3 – 15 separate cultures. Curves are fits of the Gompertz function (1) to all the data 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  $\mu_m$  were  $4.1 \pm 0.2$  mM and  $1.7 \pm 0.6$  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.

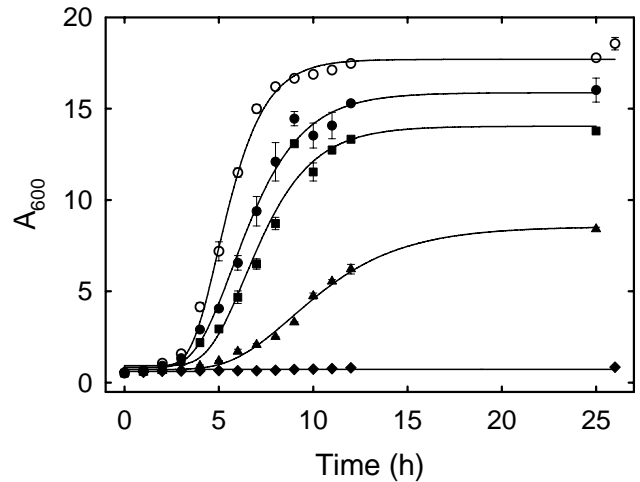


Fig. 2 Effect of varying concentrations of KCN on the growth of *C. albicans*. Cells were grown in YPD in a shaking incubator at  $200 \text{ min}^{-1}$  at  $(37 \pm 1)^\circ\text{C}$ . Error bars are  $\pm$  SE (standard error) for 3 – 15 separate cultures. Curves are fits of the Gompertz function (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 ( $17.1 \pm 0.1$ ). The lag time was approximately 79% longer than the control ( $3.19 \pm 0.05$  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  $\mu_m$ s 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).

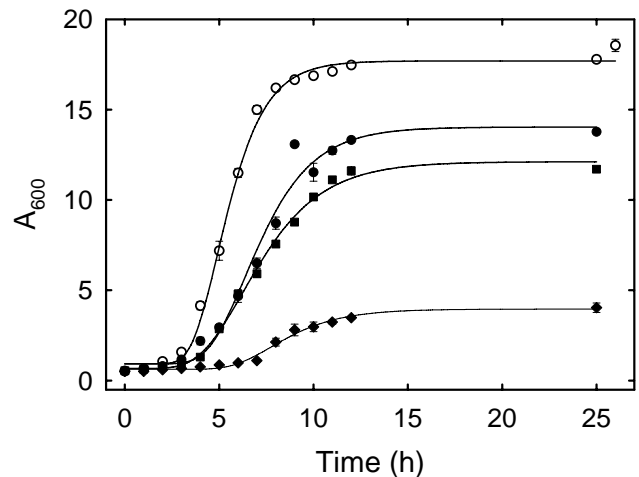


Fig. 3 Synergistic effect of 1 mM KCN and  $0.1 \text{ g (50 mL)}^{-1}$  SHAM. Cells were grown in YPD in a shaking incubator at  $200 \text{ min}^{-1}$  at  $(37 \pm 1)^\circ\text{C}$ . Error bars are  $\pm$  SE for 3 – 15 separate cultures. Curves are fits of the Gompertz function (1) of all the data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

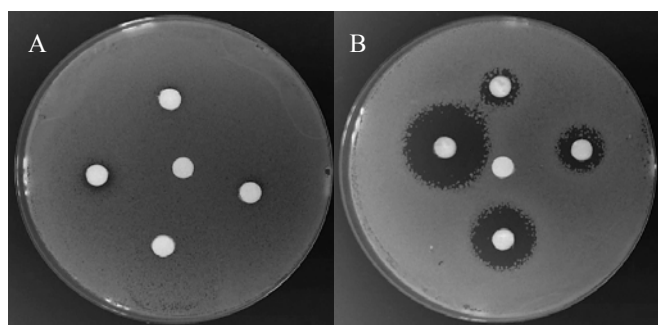
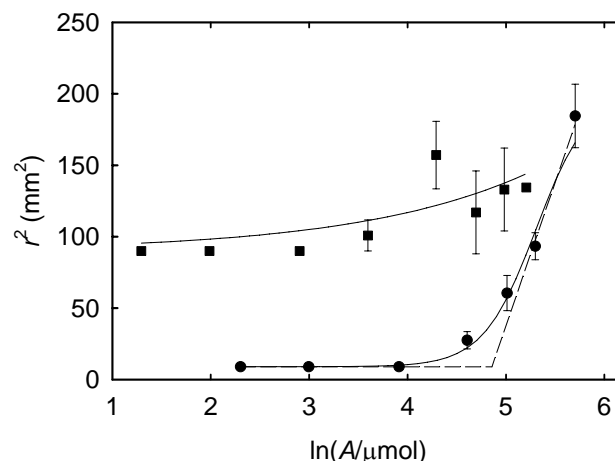


Fig. 4. Effect of SHAM (A) and KCN (B) on the growth of *C. albicans*. Amounts of SHAM on the each disk were (clockwise from top) 73  $\mu\text{mol}$ , 36.5  $\mu\text{mol}$ , 18.3  $\mu\text{mol}$  and 7.3  $\mu\text{mol}$  while amounts of KCN were (clockwise from top) 20  $\mu\text{mol}$ , 40  $\mu\text{mol}$ , 100  $\mu\text{mol}$  and 200  $\mu\text{mol}$ . 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<sup>-</sup> 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  $4.8 \pm 0.1$  for KCN and  $7 \pm 1$  for SHAM, corresponding to about 120  $\mu\text{mol}$  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  $\text{O}_2$  uptake was measured.

A suspension of freshly harvested *C. albicans* consumed  $\text{O}_2$  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  $\text{O}_2$  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  $\text{O}_2$  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  $\text{O}_2$  uptake of a range of KCN concentrations (0 – 500  $\mu\text{M}$ ) was determined with *C. albicans* (Fig. 7). In the presence of 250  $\mu\text{M}$  KCN, the rate of  $\text{O}_2$  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  $13 \pm 1 \mu\text{M}$ .

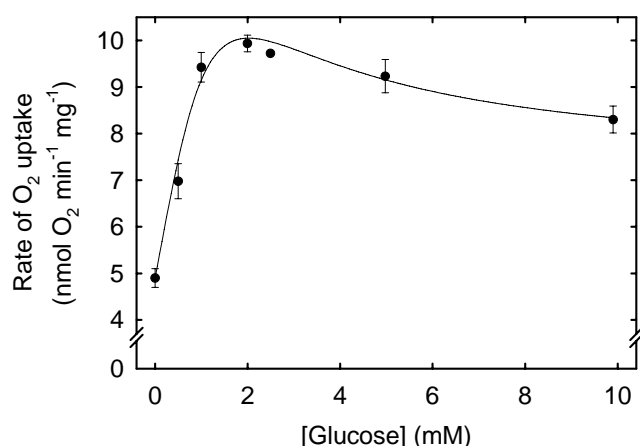


Fig. 6. Rate of  $O_2$  uptake by *C. albicans* in the presence of 0 – 10 mM glucose. Assays were carried out in 50 mM  $Na_2HPO_4$ - $NaH_2PO_4$  (pH 6.5) at  $(37 \pm 1)^\circ C$  using 5 mg wet weight  $mL^{-1}$ . Error bars are  $\pm$  SE for at least 3 separate cultures and rates were determined 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.

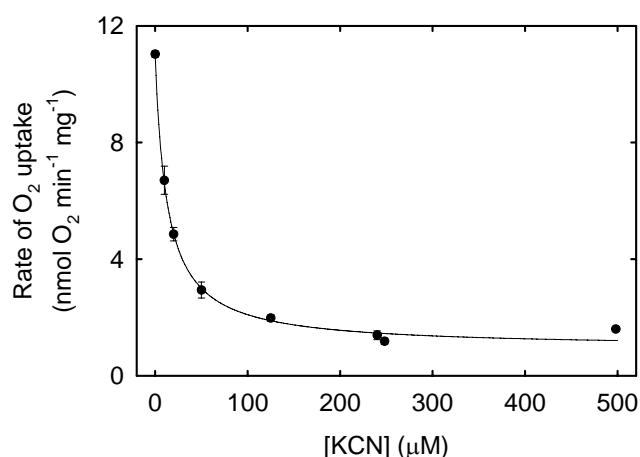


Fig. 7. Rate of  $O_2$  uptake by *C. albicans* in the presence of 0 - 500  $\mu M$  KCN and 2 mM glucose. Assays were carried out at  $(37 \pm 1)^\circ C$  in 50 mM phosphate (pH 6.5) containing 2 mM glucose and 5 mg wet weight  $mL^{-1}$ . Error bars are  $\pm$  SE for at least 3 separate cultures and rates were obtained after the addition of KCN. The curve is a fit of  $v = K_i v_0 / (K_i + [KCN])$  [32] to all the data obtained at a given KCN concentration. Here,  $K_i$  is the apparent inhibition constant and  $v_0 = 11.5 \pm 0.2$   $nmol O_2 min^{-1} mg^{-1}$  wet weight.

In order to assess the SHAM-sensitivity of  $O_2$  uptake, the effect on the rate of  $O_2$  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  $O_2$  uptake by *C. albicans* (Fig. 8A) and also artefactually increased the oxygen concentration in the cuvette because  $O_2$  is about 25 times more soluble in ethanol than it is in water [30]. Therefore dimethylsulphoxide (DMSO), an alternative solvent in which  $O_2$  is much less soluble [30], was tested to see whether the solvent effects could be minimised (Fig. 8B). Ethanol

affected the rate of  $O_2$  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  $O_2$  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  $O_2$  uptake by about 5%, which is approximately half of the residual activity observed when cytochrome oxidase was inhibited by cyanide (Fig. 7).

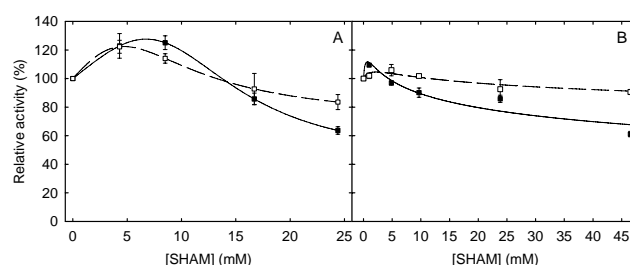


Fig. 8. Effect of (A) SHAM in ethanol ( $\blacksquare$ ) and the corresponding volumes of ethanol ( $\square$ ) and of (B) SHAM in DMSO ( $\blacksquare$ ) and the corresponding volumes of DMSO ( $\square$ ) on the rate of  $O_2$  uptake by *C. albicans*. The assay was carried out at  $37^\circ C$  in 50 mM  $Na_2HPO_4$ - $NaH_2PO_4$  (pH 6.5) containing 2 mM glucose and 5 mg wet weight  $mL^{-1}$ . The control activity (100%) represents a rate in of  $11.5 \pm 0.2$   $nmol O_2 min^{-1} mg^{-1}$  wet weight. Error bars are  $\pm$  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  $O_2$  uptake by *C. albicans* by 90%, although the  $K_i$  was only 13  $\mu M$  (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  $O_2$  by *C. albicans* is  $(9 \pm 1)\%$  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 O<sub>2</sub> uptake 5%, which accounts for about 50% of the activity of AOX. Although the *K<sub>s</sub>* are approximately the same, the residual growth (about 20%) and the residual rate of O<sub>2</sub> 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], Δ<sup>6</sup>-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 O<sub>2</sub> 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.

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