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Inhibition of the Growth of Pathogenic *Candida* spp. by Salicylhydroxamic Acid

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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 each of six *Candida* spp. was inhibited significantly by ~13 mM SHAM or 2 mM cyanide, albeit to differing extents. In *C. dubliniensis*, *C. krusei* and *C. tropicalis* the rate of O_2 uptake was inhibited by 18-36% by 25 mM SHAM, but this had little or no effect on *C. glabrata*, *C. guilliermondii* or *C. parapsilosis*. Although SHAM substantially inhibited the growth of *Candida* spp., 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 *Candida* spp. infection.

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

I. Introduction

WE have reported previously that salicylhydroxamic acid (SHAM) is an effective inhibitor of the growth of *Candida albicans* [1]. While *C. albicans* is often isolated from healthy individuals, other *Candida* spp. are increasingly considered to be aggressive pathogens. Several large-scale studies of candidaemia [2, 3] 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.* [4] 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 [2, 3].

Candida spp. are becoming increasingly resistant to the commonly used antifungals which, combined with their pathogenicity, necessitates the identification of novel potential drug targets. Ideally, such targets should be found in *Candida* spp., but not in humans or other flora. One possibility that we have previously investigated in *C. albicans* is the alternative oxidase (AOX), which is an electron transfer chain enzyme that accepts electrons directly from ubiquinol and reduces O_2 to water. The enzyme is sensitive the salicylhydroxamic acids (SHAM), but insensitive to cyanide. It has been suggested that AOX is a viable target in the treatment of trypanosomes [5] and that it plays an important role in the stress response in various fungi [6] and in plants [7]. Morover, hydroxamic acids (such as SHAM) inhibit the growth of some bacteria, some of which also have an AOX homologue [8], and other fungi [9, 10]. Inhibition of AOX has been reported to potentiate the inhibition of the growth of *C. albicans* by fluconazole [11], but we have shown that while SHAM inhibits the AOX of *C. albicans*, it does not explain how SHAM inhibits growth [1]. This prompts the question as to whether SHAM has similar effects on the growth of other common pathogenic *Candida* spp.

Here, we report on the effects of SHAM and KCN on the growth of six pathogenic species of *Candida* in liquid culture and on plates, using a disk diffusion protocol [12]. We also report on the effect of SHAM and KCN on the rate of O_2 uptake by *Candida* spp. We show that while the growth of each of the *Candida* spp. is inhibited by SHAM or KCN, the same is not necessarily true of O_2 uptake. Nevertheless, SHAM has some potential in the treatment of *Candida* spp. infection and is used in the treatment of urinary tract infections and urolithiasis [13, 14].

II. Materials and Methods

A. Culture growth

Candida dubliniensis, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* were obtained from the University of Tasmania and Launceston General Hospital culture collections and the identity of each was confirmed using the Remel RapID™ Yeast Plus System® (Remel Laboratories, Lenexa, Kansas, USA). Two previously reported ambiguities arose from the use of this test. First, although there were two tests (α-D-glucosidase and *p*-nitrophenyl phosphatase) that could differentiate between *C. albicans* and *C. dubliniensis*, the product database did not include data for *C. dubliniensis* and an unambiguous confirmation of the identity of this species was not possible

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[15]. Second, *C. parapsilosis* was identified as *Rhodotorula rubra* (a mis-identification also previously reported for this kit [16]), but it was unlikely as *R. rubra* is pigmented and no pigmentation was observed. However, the identity of both *C. dubliniensis* and *C. parapsilosis* was supported by their morphology when grown on CHROMagar Candida (Oxoid) [17, 18].

Candida spp. were 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 KCN or SHAM, as previously reported for *C. albicans* [1]. Briefly, cultures were inoculated from an overnight culture to an initial A_{600} of 0.4 and grown for 15 h, unless otherwise specified, at (37 ± 1)°C in a shaking incubator (at 200 min^{-1}). Where the cultures were monitored repeatedly, exit cultures on MacConkey agar, blood 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.

As described previously [1], the growth of liquid cultures was monitored using the absorbance of the culture at 600 nm (A_{600}) and the Gompertz function

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

was fitted directly to the data 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.

B. Disk diffusion analysis

As described previously [1], 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 ± 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 (see the Appendix). As described previously [1], the data were analysed using the logistic model

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

where A_0 is the inflection point of the logistic function, and α $4Dt$, t is the time, and β is related to the upper limit of the radius of the zone of clearing. 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. 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 × *g* for 15 min at 4°C (Sigma Laboratory Centrifuge 4K15). The cells were washed with 50 mM Na_2HPO_4-NaH_2PO_4 (pH 6.5) and then resuspended to 5 mL in the same buffer. The O_2 uptake by cell suspensions was measured polarographically with a Clark-type O_2 electrode (Hansatech Instruments Ltd, King's Lynn) at (37 ± 1)°C. The cell suspension in the cuvette (5 mg wet weight mL^{-1}) contained 2 mM glucose in 50 mM Na_2HPO_4-NaH_2PO_4 (pH 6.5). The O_2 concentration in the cuvette was digitised each second (each datum represented the average of 1000 determinations) using hardware and software developed in-house [20].

III. Results

A. Measurement of growth

The effects on the growth of each of the *Candida* spp. of 2 mM KCN alone or 0.1 g $(50\ mL)^{-1}$ SHAM alone were determined (Fig 1). Of the six *Candida* spp. examined, *C. krusei* was the fastest growing species ($\mu_m = 4.9 \pm 0.2\ h^{-1}$) and *C. parapsilosis* was the slowest growing species ($\mu_m = 1.65 \pm 0.08\ h^{-1}$). However, both *C. glabrata* and *C. tropicalis* also grew relatively rapidly ($\mu_m > 3\ h^{-1}$), whereas *C. guilliermondii* grew relatively slowly ($\mu_m < 2\ h^{-1}$). The growth of each species was inhibited by 2 mM KCN and by 0.1 g $(50\ mL)^{-1}$ SHAM, although *C. tropicalis* appeared to be the most sensitive to both compounds (μ_m was reduced 88% by 2 mM KCN or 87% by 0.1 g $(50\ mL)^{-1}$ SHAM). As is apparent from Fig 1, *C. glabrata* and *C. krusei* were relatively unaffected by these concentrations of KCN and SHAM, similar to *C. albicans* [1].

It will also be apparent from Fig 1, B and F, that (1) did not fit the initial lag phase of the growth of *C. glabrata* or *C. tropicalis* especially well. We attempted to overcome this problem by fitting to the data (i) a Richards function [21] and (ii) an empirical function, based on the logistic function, given by Damoglou *et al.* [22]. Each of these expressions has one more parameter than (1), but the improvement in the fit using

either of the expressions did not justify the extra parameter (p = 0.19). In order to minimise the influence of this failure to fit the lag phase data, some early data points (t = 0–2 h) were selectively omitted in fitting the growth curves shown in Fig 1.

In the absence of any inhibitor, *C. glabrata* reached the highest A_{max} of 17.5 ± 0.3 (Fig 1B), while *C. guilliermondii* and *C. tropicalis* reached the lowest A_{max} of 12.1 ± 0.9 and 12.2 ± 0.6, respectively (Fig 1, C and F). The A_{max} of each species was decreased in the presence of 2 mM KCN or 0.1 g (50 mL)$^{-1}$ SHAM, although *C. dubliniensis* was the most affected by KCN and SHAM (A_{max} was decreased by 49% and 52%, respectively (Fig 1A)). *Candida glabrata* and *C. krusei* were relatively unaffected by these concentrations of KCN and SHAM (Fig 1, B and D), similar to *C. albicans* [1]. It should be noted that KCN alone and SHAM alone inhibited the growth of *C. guilliermondii*, *C. parapsilosis* and *C. tropicalis* so efficiently that A_{max} was not especially well defined (Fig 1, C, E and F, respectively). However, we were able to limit the impact of this because μ_m, λ and A_{max} are interdependent [23].

In the absence of any inhibitor, *C. glabrata* had the shortest lag time (2.37 ± 0.09 h), while *C. parapsilosis* had the longest lag time (6.2 ± 0.3 h). The lag time of each species was increased in the presence of 2 mM KCN or 0.1 g (50 mL)$^{-1}$ SHAM, although that of *C. parapsilosis* was the most affected by KCN and SHAM (20 ± 11 h and 8.4 ± 0.4 h, respectively). The lag times of *C. dubliniensis* and *C. tropicalis* were relatively unaffected by KCN and SHAM, similar to *C. albicans* [1].

B. Disk diffusion assays

Disk diffusion assays were carried out to estimate the relative sensitivity of the *Candida* spp. to KCN and SHAM. As detailed previously [1], the data were analysed using the logistic model (2), from which the 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 each of the *Candida* spp. shown in Fig 2, 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 was apparent around the disk at the higher amounts. This implies that A_c should be at or above the upper limit of the range used, which, as discussed previously, could not be attained because of the insolubility of the compound. In contrast, KCN clearly inhibited growth (Fig 2). As we have previously shown [1],

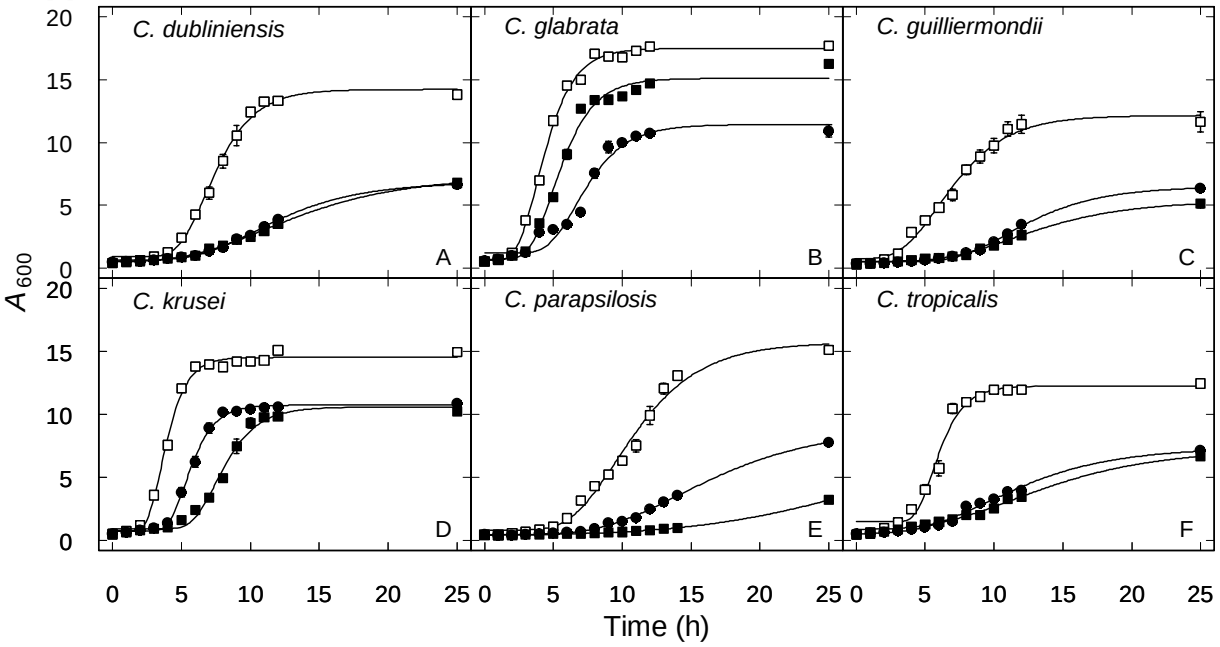

Fig. 1. Growth of *C. dubliniensis* (A), *C. glabrata* (B), *C. guilliermondii* (C), *C. krusei* (D), *C. parapsilosis* (E) and *C. tropicalis* (F). Cells were grown in a shaking incubator at 200 min^{-1} at (37 ± 1)°C in YPD in the absence (○) or presence of either 0.1 g (50 mL)$^{-1}$ SHAM (●) or 2 mM KCN (■). Error bars are ± SE for at least 3 separate cultures, and where not visible are hidden by the symbol. Curves are fits of the Gompertz function (1) of all the data obtained from cultures at the given KCN or SHAM concentration with selective omission of some early data points (t = 0–2 h) as discussed in the text.

the apparent diffusion coefficient for KCN in these conditions was 5-6 × 10^{-6} cm^2 s^{-1}, from which the critical concentration (C) was estimated for each species and the values ranged from 0.04 mM to 1 mM (Table I).

The corresponding critical concentration values for SHAM were not determined directly (Fig 2). However, the crude estimates shown in Table I were derived from ln(A_c) and the corresponding cyanide data for each species using

$$C_{\mathrm{SHAM}} \approx C_{\mathrm{CN}} \frac{A_c^{\mathrm{SHAM}}}{A_c^{\mathrm{CN}}}, \quad (3)$$

where C_x and ln(A_c^x) are the critical concentration and the ln(A_c) estimate, respectively, for compound x (see the Appendix for a derivation of this expression). Based on this approximation, the values of C for SHAM ranged from about 1 mM to 16 mM for the six species (Table I). Of course, the numerical estimates of ln(A_c) and C for SHAM represent extrapolations from the data and must be regarded with some scepticism.

TABLE I
ESTIMATES FROM THE DIFFUSION ANALYSIS FOR SIX *CANDIDA* SPP.

Species	ln(A_c/μmol)		C (mM)	
	KCN	SHAM	KCN	SHAM[a]
C. dubliniensis	4.2 ± 0.1	6 ± 1	1 ± 1	6
C. glabrata	4.48 ± 0.05	7 ± 1	0.16 ± 0.04	2
C. guilliermondii	4.2 ± 0.2	5 ± 1	0.5 ± 0.3	1
C. krusei	3.5 ± 0.3	8 ± 1	0.04	4
C. parapsilosis	4.7 ± 0.2	8 ± 1	0.6 ± 0.3	16
C. tropicalis	3.5 ± 0.3	7 ± 1	0.04	1

The errors represent ± SE of the parameter estimate obtained from the data shown in Fig 2.
[a]The values of C for SHAM were obtained using (3).

C. Oxygen uptake assays

To determine whether the inhibitory effect on the growth of *Candida* spp. 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.

Freshly harvested cell suspensions consumed O_2 at rates ranging from 21 ± 1 nmol O_2 min^{-1} mg^{-1} wet weight for *C. glabrata* to 1.7 ± 0.2 nmol O_2 min^{-1} mg^{-1} wet weight for *C. parapsilosis*, and the other four species took up O_2 at rates of 5-11 nmol O_2 min^{-1} mg^{-1} wet weight (Table II). The addition of 250 μM KCN inhibited O_2 uptake by *C. dubliniensis* (by 84%), *C. glabrata* (by 94%) and *C. tropicalis* (by 66%), but the same concentration of KCN stimulated O_2 uptake by *C. guilliermondii* and *C. krusei* by 46% and 80%, respectively. The addition of KCN had no significant effect on the rate of O_2 uptake by *C. parapsilosis*. The addition of SHAM inhibited the rate of O_2 uptake by *C. dubliniensis* (by 36%), *C. krusei* (by 25%) and *C. tropicalis* (by 18%), but had little or no effect on the rate of O_2 uptake by *C. glabrata*, *C. guilliermondii* or *C. parapsilosis* (Table II). Since SHAM was dissolved in DMSO (for reasons discussed previously [1]), which had a small effect on some of the rates of O_2 uptake, the effect of SHAM should be compared to the rate in the presence of DMSO specified in Table II.

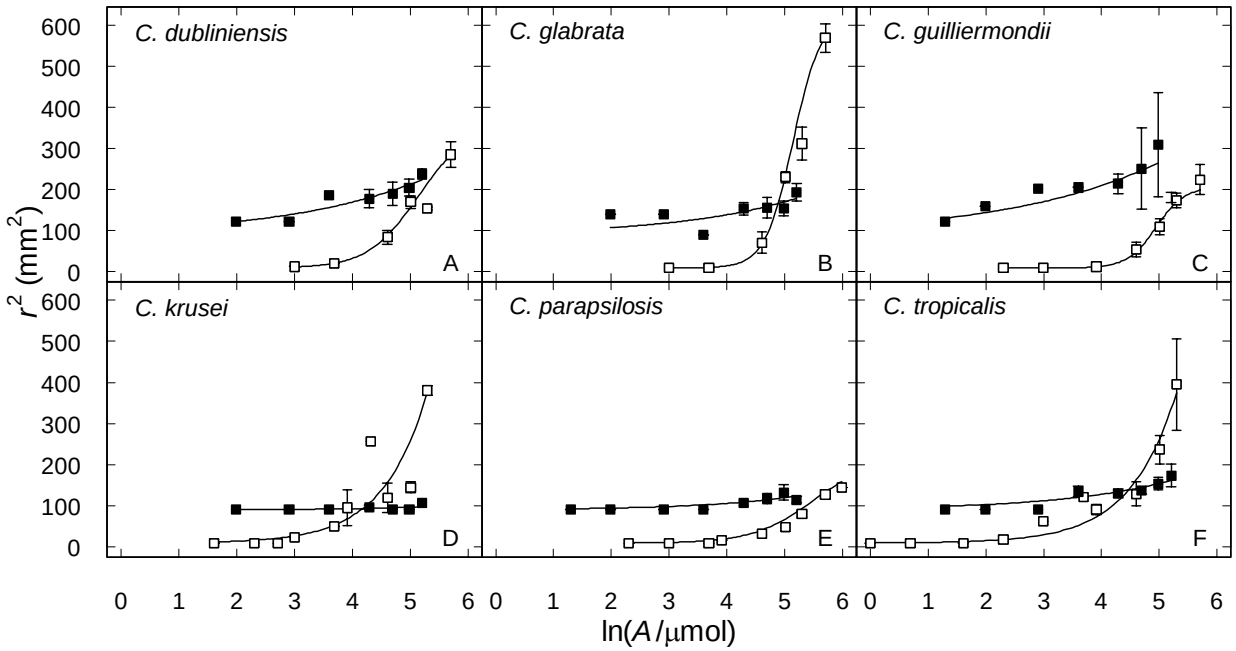

Fig. 2. Radii of inhibition of the growth of *C. dubliniensis* (A), *C. glabrata* (B), *C. guilliermondii* (C), *C. krusei* (D), *C. parapsilosis* (E) and *C. tropicalis* (F) in the presence of various amounts of KCN (●) or SHAM (■). The SHAM data (and the corresponding errors) have been multiplied by 10 to allow them to be easily seen. The solid curves represent fits of the logisitic model (2) to the data. Errors bars are ± SE of at least 3 measurements.

IV. Discussion

The data reported here show that the growth of each of six pathogenic species of *Candida* was inhibited by KCN and by SHAM (Fig 1). From the disk diffusion experiments the critical concentrations for the onset of growth inhibition were 0.04-1 mM KCN and 1-16 mM SHAM (Table I). Of course, a significant limitation was imposed on the SHAM disk diffusion experiments by the relative insolubility of SHAM. Nevertheless, these values are similar to those previously reported for *C. albicans* [1].

Cyanide inhibited the rate of O_2 uptake by some *Candida* spp. by about 90%, but stimulated the rate of others (Table II). This implies that the inhibition of respiration by cyanide was not responsible for the inhibition of the growth of *Candida* spp., as was the case for *C. albicans* [1].

The cyanide-induced stimulation of O_2 uptake by *C. guilliermondii* and *C. krusei* (Table II) probably reflects a more reduced quinone pool in the mitochondrial electron transfer chain, arising from the inhibition of cytochrome oxidase by cyanide. The more reduced quinone pool would increase the driving force for electron transfer through AOX [24, 25]. This hypothesis is based on the idea that the activity of AOX increases when the capacity of the cytochrome-dependent pathway is exceeded [26], because (i) the apparent K_m for ubiquinol of the cytochrome bc_1 complex is much smaller than that of AOX and (ii) the maximal activity of AOX is high. Normally, the quinone pool is oxidised by cytochrome bc_1, but the electrons are accepted by AOX when the quinone pool is largely reduced [27].

A similar argument prompts the conclusion that inhibition by SHAM of AOX did not make a significant contribution to the inhibition by SHAM of the growth of *Candida* spp. in these conditions. From Fig 1 it is apparent that 0.1 g (50 mL)$^{-1}$ SHAM (equivalent to about 13 mM SHAM) inhibited the growth of each of the six *Candida* spp., but 25 mM SHAM inhibited the rate of O_2 uptake of some species and had little or no effect on the rate of O_2 uptake of other species (Table II). The same conclusion was reached for *C. albicans* [1].

The most significant result of this work is that SHAM is an effective inhibitor of several pathogenic *Candida* spp. (Fig 1). The critical concentration estimates (*C*) given in Table II were based on the assumption that the diffusion coefficients of SHAM and KCN were similar. We have previously reported that the diffusion coefficient for KCN is about 5-6 × 10^{-6} cm^2 s^{-1} in these conditions [1]. This value is about 25% that of CN^- in an aqueous medium (2 × 10^{-5} cm^2 s^{-1}) at 25°C [28]. While this is lower than would be expected from literature data for other compounds [29, 30] or from models of diffusion in similar media [31, 32], the values were of the right order of magnitude (about 10^{-5} cm^2 s^{-1}), providing some support for the plausibility of the other parameter estimates. The value for the diffusion coefficient of SHAM estimated from the data in Fig 2 was 1-6 × 10^{-7} cm^2 s^{-1}. This is probably an underestimate because of the insolubility of SHAM, but it is likely that SHAM has a smaller diffusion coefficient than KCN, if only because of the difference in the size the molecules [33]. If it is the case that $D_{CN} > D_{SHAM}$, then the values of *C* reported in Table II would represent underestimates, as is demonstrated in the Appendix. However, 0.1 g (50 mL)$^{-1}$ SHAM (equivalent to about 13 mM SHAM) inhibited the growth of all six *Candida* spp. (Fig 1) and so the values of *C* reported in Table I, which ranged from 1 mM to 16 mM, are reasonable, except for the estimate for *C. parapsilosis*.

TABLE II
Rates of O_2 uptake in the presence or absence of KCN or SHAM

Species	Rate of O_2 uptake (nmol min^{-1} mg^{-1})			
	control	+ KCN	+ DMSO	+SHAM
C. dubliniensis	5.7±0.5	0.91±0.07	6.0±0.9	3.8 ±0.5
C. glabrata	21± 1	1.08±0.06	19.3±0.8	19 ±1
C. guilliermondii	6.6±0.2	9.7 ±0.4	6.6±0.2	7.0 ±0.3
C. krusei	8.4±0.1	15.2 ±0.5	10.3±0.4	7.7 ±0.1
C. parapsilosis	1.7±0.2	1.6 ±0.3	1.8±0.3	1.6 ±0.3
C. tropicalis	10.1±0.3	3.42±0.08	10.5±0.8	8.6 ±0.9

The errors represent ± SE for at least 6 different measurements.

The assays were carried out in 2 mM glucose, 50 mM Na_2HPO_4-NaH_2PO_4 pH 6.5 at 37°C using 5 mg mL^{-1} wet weight. The concentrations in the O_2 electrode cuvette of KCN and SHAM were 250 μM and 25 mM, respectively. The SHAM was dissolved in DMSO, which affected the rate of O_2 uptake, so the rate in the presence of 50 μL DMSO represents the solvent control for SHAM.

Given that SHAM did inhibit the growth of all six *Candida* spp. (Fig. 1), but that this is not explained by the inhibition of AOX, there must be some other target for SHAM. As we have previously indicated [1], SHAM and other hydroxamic acids also inhibit other enzymes and chelate cations. It is conceivable that the inhibition of growth in the presence of SHAM is due to one or more of these general hydroxamic acid effects. If the antifungal potential of SHAM and other hydroxamic is to be exploited, it is necessary to know how SHAM inhibits the growth of *Candida* spp.

V. Conclusions

The growth of each of the six *Candida* spp. examined 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_2 uptake was variable, indicating that SHAM had an inconsistent effect on energy transduction. Further work is required to establish how SHAM inhibits the growth of *Candida* spp.

Appendix

Equation (3) is based on the expression [34-36] for the radius of the clear zone (r)

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

where D is the diffusion coefficient of the compound on the disk, t is the time, d is the thickness of the agar and C is the effective concentration of the compound. From (4), r^2 should be a linear function of ln(A), but the radius is limited at the

lower end by the size of the disk ($r = r_0$), which prompts the idea that

$$r^2 = \begin{cases} r_0^2, & \ln(A) \le \ln(A_c) \\ r_0^2 + \alpha \ln\left(\frac{A}{A_c}\right), & \ln(A) > \ln(A_c) \end{cases} \quad (5)$$

where A_c is the critical amount of the compound on the disk above which clearing is observed and $\alpha = 4Dt$. We have shown previously that (5) is a reasonable representation of the data [1]. Equating (4) and (5) for $A > A_c$, substituting α as appropriate and solving for C gives

$$C = \frac{1}{\alpha \pi d} \exp\left[\ln(A_c) - \frac{r_0^2}{\alpha} \right]. \quad (6)$$

Using (6) for compounds i and j yields

$$\frac{C_i}{C_j} = \frac{\alpha_j}{\alpha_i} \exp\left(\frac{r_0^2}{\alpha_j} - \frac{r_0^2}{\alpha_i} \right) \left(\frac{A_c^i}{A_c^j} \right), \quad (7)$$

and assuming that the diffusion coefficients of the two compounds are similar, so that $a_i \approx a_j$, yields the approximation given by (3).

If the diffusion coefficients are not similar in magnitude, then, writing $\alpha_j \approx \alpha_i + \varepsilon$, then

$$\frac{C_i}{C_j} = \left(1 + \frac{\varepsilon}{\alpha_i} \right) \exp\left(\frac{\varepsilon r_0^2}{\alpha_i \alpha_j} \right) \left(\frac{A_c^i}{A_c^j} \right). \quad (8)$$

This means that if $D_j > D_i$ then $\varepsilon > 0$ and (3) would yield an underestimate of C_i and, conversely, if $D_j < D_i$ then $\varepsilon < 0$ and (3) would yield an overestimate of C_i.

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