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1 **Phenotypic microarray analysis of the effects of acetylcholine on *Candida albicans* metabolic rate**
2 **demonstrates its application for anti-fungal drug development**

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10 RUNNING TITLE: Phenotypic microarray analysis can be used for anti-fungal drug development

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13 resistance

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23 **Abstract**

24 *Candida albicans* metabolic activity in the presence and absence of acetylcholine was measured
25 using phenotypic microarray analysis. Acetylcholine inhibited *C. albicans* biofilm formation by
26 slowing metabolism independent of biofilm forming capabilities. Phenotypic microarray analysis can
27 therefore be used for screening compound libraries for novel anti-fungal drugs and measuring
28 antifungal resistance.

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43 **Main Body**

44 Phenotypic microarray (PM) technology has been applied to the field of drug discovery to gain a
45 better understanding of a compounds efficacy on specific cell types (2, 3); and can be used to
46 determine the metabolic fingerprint of microbes exposed to drugs (2). Metabolic fingerprinting
47 provides information on the metabolic pathways employed by a microbe in the presence of
48 exogenous compounds (2, 3). PM technology has improved knowledge of bacterial growth
49 inhibitors, strain phenotypes, phenotypes of pathway mutations, drug targets and sensitivity to
50 chemicals (1, 4, 7, 14, 15). To date, however, no study has applied PM analysis to investigate the
51 real-time effects of exogenous compounds on the growth characteristics of clinical fungal isolates.

52 Acetylcholine (ACh) has been demonstrated to modulate host inflammatory responses to infection
53 and has been shown to inhibit biofilm formation by a *C. albicans* laboratory type strain (SC5314),
54 both *in vitro* and *in vivo* (8). In this study, we utilised PM technology to investigate the effect of ACh
55 on 92 clinically relevant bloodstream isolates of *C. albicans*; collected as part of a candidaemia
56 epidemiology surveillance study (9, 11). In addition, as high biofilm forming (HBF) strains were found
57 to correlate with patient mortality (9), we aimed to investigate whether biofilm-forming strains
58 exhibited differences in susceptibility to ACh.

59 Bloodstream isolates of *C. albicans* were sub-cultured, propagated in yeast peptone dextrose, and
60 metabolic activity monitored by PM analysis during growth in RPMI \pm 25 mg/ml ACh, as previously
61 described (8). The OmniLog reader (Biolog, Hayward, USA) was used to photograph the plates every
62 15 min to measure dye conversion and the pixel intensity in each well converted to a signal value
63 reflecting cell metabolic output. The following information was gathered from the four parameter
64 logistic fit of average metabolic measurements for each *C. albicans* isolates (+/- ACh) at each time
65 point (M_t); the maximum and minimum metabolic values (M_{max} and M_{min}), the maximum growth rate
66 (μ_{max}), the lag time (h), the time at the inflection point (t_i) and the time taken to reach the half
67 maximum metabolic rate (μ_{max}) as shown below and in FIG 1 (5):

68 Lag time (h) = $t_i - 1/\mu_{\max} \times \ln((M_{\max} + M_{\max} \cdot \exp(\mu_{\max} \cdot t_i))/(M_{\max} + M_{\min} \cdot \exp(\mu_{\max} \cdot t_i)) - 1)$

69 Maximum metabolic rates (μ_{\max}) were derived from the equation above as follows:

70 $(M_t) = M_{\min} + (M_{\max} - M_{\min}) / (1 + \exp[-\mu_{\max} (t - t_i)])$

71 When $M_{\min} = 0$, $\mu_{\max} = [-\ln(M_{\max} - M_t - 1)] / (t - t_i)$

72 The time to reach half max absorbance ($t_{0.5}$) = time (h) that 0.699. $\log(M_{\max})$ is reached.

73 Natural log (Ln) transformations of data were performed where necessary. Paired sample t-tests
74 and Bonferroni correction were used to test for significant differences in metabolic characteristics
75 between untreated and ACh treated *C. albicans* bloodstream isolates. To determine the relationship
76 between biofilm formation and ACh sensitivity; *C. albicans* isolates were split into quartiles based on
77 biofilm forming ability, as determined previously by crystal violet biomass staining and Syto 9
78 fluorescence (9). Isolates within the first quartile (Q1; n=19) were classed as low biofilm formers
79 (LBFs) and isolates above the third quartile (Q3; n=22) were classed as HBFs. Statistical analysis was
80 performed by ANOVA with a Tukey post-test.

81 In the presence of ACh, the metabolic activity of 92 *C. albicans* bloodstream isolates demonstrated
82 that ACh prolonged the lag phase ($p < 0.0001$), lengthened $t_{0.5}$ ($p < 0.001$), slowed down the rate of
83 change in metabolic activity (indicated by a reduction of the gradient [$p < 0.0001$]) and reduced μ_{\max}
84 ($p < 0.0001$) (FIG 2). In addition, ACh also reduced the peak level of metabolic activity ($p < 0.0001$) and
85 the area under the curve (AUC [$p < 0.0001$]).

86 In the absence of ACh, ANOVA analysis revealed that there was no significant difference in the lag
87 phase, the $t_{0.5}$, the gradient, and the μ_{\max} between HBF and LBF isolates. However, a significant
88 difference was observed for the AUC ($p < 0.001$) (data not shown). In the presence of ACh, the
89 prolonged lag phase, the increased $t_{0.5}$, the reduction in peak metabolic activity and reduced AUC

90 were all significantly lower (all $p < 0.001$); but comparable for both HBF and LBF isolates (data not
91 shown).

92 Collectively, PM analysis indicates that *C. albicans* ACh susceptibility appears independent of biofilm
93 forming ability. Therefore, the data suggests that ACh inhibits biofilm formation, principally by
94 reducing yeast metabolism. Furthermore, the data shows that this phenomenon is not restricted to
95 laboratory strains based on the observations with a panel of clinically relevant strains. Previous
96 studies have revealed biofilm-forming ability of clinical isolates to be associated with *C. albicans*
97 mortality (9). In addition, HBF isolates showed greater resistance to conventional antifungals, such
98 as azoles and echinocandins (13). Interestingly, this study demonstrates that the effect of ACh on *C.*
99 *albicans* metabolism is homogenous and independent of biofilm forming ability. However, our
100 recent data indicate that differences in amino acid metabolic pathways drives the biofilm phenotype
101 (12), thus suggesting that the metabolic PM approach, which is based on reduction of tetrazolium
102 salts, is unable to detect these differences. Nevertheless, in terms of drug discovery, our data
103 indicates that *C. albicans* has the general ability to respond pharmacologically to this ubiquitous
104 molecule, suggesting a conserved, yet unrecognised, ACh target. Given the uniform response then it
105 is plausible that ACh and analogous molecules could be harnessed for therapeutic benefit.

106 Although yeast growth has been modelled previously using PM analysis (6), to our knowledge this is
107 the first report demonstrating the use of the technology to investigate the effects of
108 pharmacological intervention on clinically relevant *C. albicans* isolates in real time. Therefore, it
109 opens up the possibility of utilising PM technology for rapid high throughput screening of compound
110 libraries for the development of novel anti-fungal drugs, as well as investigating the resistance to
111 traditional antifungals in real time. This technology offers advantages over subjective endpoint CLSI
112 based sensitivity testing and creates opportunities to supersede existing metabolic based screening
113 tools, such as XTT, which are not without their limitations (10). Though the initial start-up expense
114 for this approach is not inconsequential, it lends itself to high throughput automation.

115 **Figure legends**

116 **FIG 1:** Diagrammatic representation of the parameters derived from the four parameter logistic fit of
117 average metabolic measurements for each *C. albicans* isolate. Values such as the M_{\min} , M_{\max} , time to
118 reach the inflection point (t_i), $t_{0.5}$ and the gradient and area under the curve (AUC) can be
119 determined directly from the charted metabolic data; whereas μ_{\max} was calculated from the
120 equation and all were utilised to determine the metabolic activity curve characteristics for strain or
121 drug treatments.

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123 **FIG 2:** Key differences in metabolic profiles between *C. albicans* bloodstream isolates grown in RPMI
124 in the presence (grey bars) and absence (white bars) of 25 mg/ml acetylcholine. The results
125 represent the mean and standard deviation of the lag time prior to the log phase of metabolic
126 output (hours), the time ($t_{0.5}$) to reach 50% of the peak level of metabolic activity (hours) and the
127 gradient of the log phase representing the exponential increase in *C. albicans* metabolic rate (Ln
128 M/h). *** Significant difference ($p < 0.001$) as assessed by a paired sample t-test with a Bonferroni
129 correction between control and acetylcholine treated *C. albicans*. Data is derived from triplicate
130 curves for each condition for 92 *C. albicans* bloodstream isolates.

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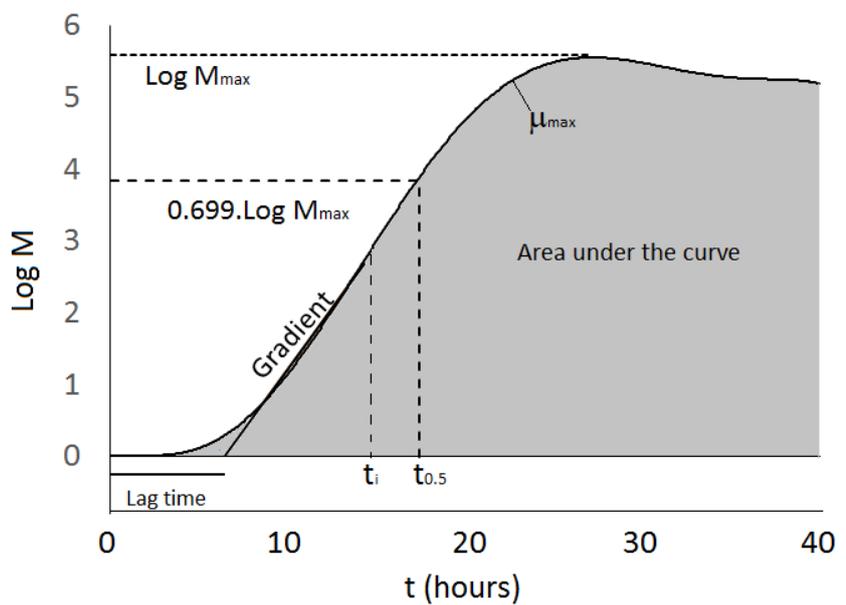
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187 Figure 1



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