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

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

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Abstract

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

Main Body

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

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

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

68 Lag time (h) = t_i - 1/μ_{max} x ln((M_{max} + M_{max} . exp (μ_{max} . t_i))/(M_{max} + M_{min} . exp (μ_{max} . 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

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

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

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

Figure legends

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

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

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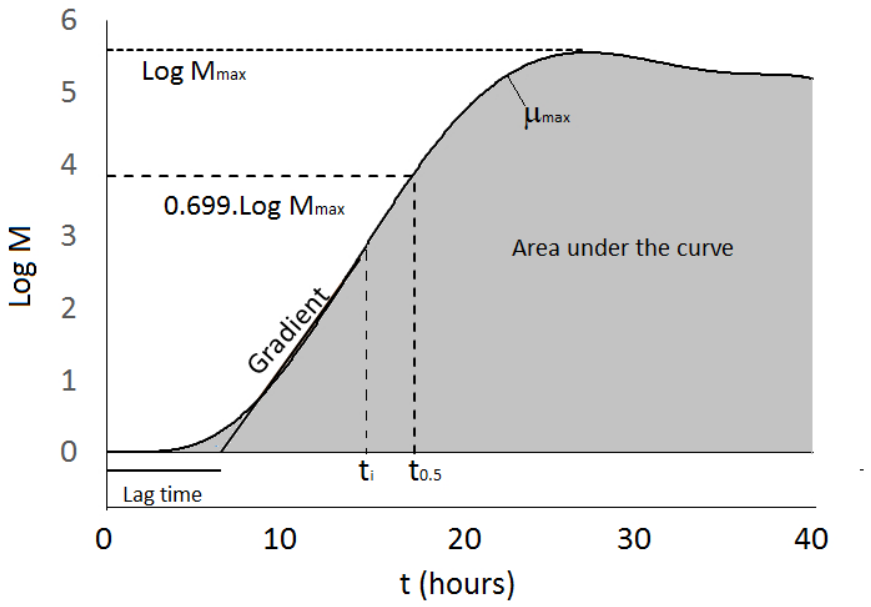
187 Figure 1



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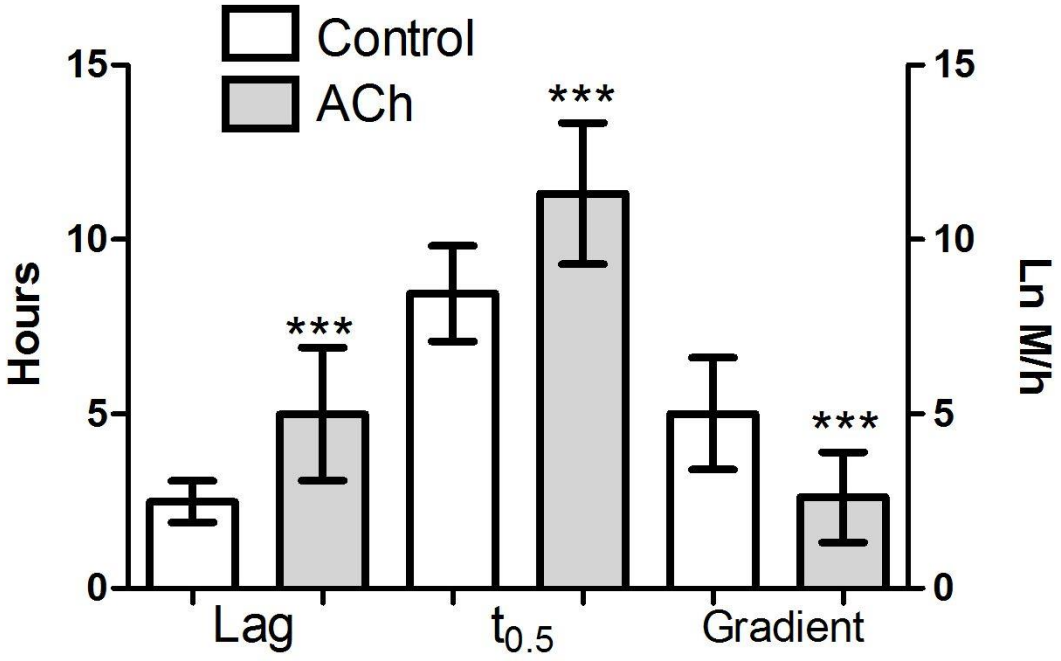
199 Figure 2



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