A RAPID FLUORESCENCE BASED SCREEN FOR CYP51 INHIBITION.

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Drug treatments such as fungicides and anti-parasitics often target the pathogen CYP51 (e.g. Lamb et al (1999) and Riley J. et al (2015)) and there is a risk with new drugs using the same strategy that they will exhibit crossover inhibition of human CYP51, leading to unwanted side effects. Here we have developed a quick, easy to run, screen for human CYP51 inhibition using the fluorogenic substrate BOMCC.

Results

Human CYP51 was co-expressed in *E. coli* in a 10 litre fermenter with human CYP reductase. Bacterial membranes were prepared and supplemented with partially purified human cytochrome b_5 at a 5 fold molar excess to the CYP. The yield of CYP51 in the membranes was 92 ± 8 pmol/mg (3 fermenter runs).

The membrane preparations were used to set up an assay for CYP51 activity using BOMCC as substrate as follows: the CYP51 preparations were incubated in 200 µl total volume in black flat bottomed 96 well plates with BOMCC, after the addition of NADPH generating system the fluorescence was monitored over time and then plotted in Excel. Initially, the reaction mix was incubated at 37°C for 5 minutes before the reaction was initiated by the addition of NADPH generating system however there was a significant (10 - 20 min) lag before the reaction became linear after the addition of NADPH (Fig. 1). The assay was then changed so that the NADPH generating system was added at the start of the incubation and the fluorescence monitoring was started 20 min after the addition of the NADPH. Kinetic analysis of BOMCC metabolism showed that, over the substrate concentration range tested the enzyme showed Michaelis Menten kinetics (Hanes plot and direct fit Fig. 2)

The Km for BOMCC metabolism to CHC was 14.7 ± 3.8 µM (n=3 different CYP51 preparations) so a final concentration of 10 µM BOMCC was chosen for the inhibition assays.

The CYP51 inhibition screen was run with compounds known to inhibit either human CYP51 or CYP51 orthologs from other species. Azalanstat and testosterone were run alongside four azole antifungal / antiparasite drugs, ketoconazole, itraconazole, fluconazole and miconazole. Miconazole is routinely used in our mammalian CYP inhibition screening assays as a positive inhibition control. The IC_{50} values for CYP51 inhibition for these compounds are shown in Table 1. As expected, fluconazole didn't inhibit human CYP51 (it has been shown to inhibit CYP51 from other species). The other compounds all showed inhibition of CYP51 within the published range although the IC_{50} values published for some of the compounds tested can cover a 10 fold or more range.
Azalanstat was chosen for use as the positive inhibition control for future studies.

Inhibition data collected from studies using azalanstat as the positive control gave the following IC_{50} values;

CYP51 Batch 1 0.020 µM ± 0.006 n=22 assays
CYP51 Batch 2 0.023 µM ± 0.005 n=29 assays
CYP51 Batch 3 0.021 µM ± 0.006 n=20 assays
Pooled batches 0.019 µM ± 0.005 n=10 assays
Across all batches 0.021 µM ± 0.006 n=81 assays

Table 1. IC_{50} values for test inhibitors

Inhibitor	IC_{50} (µM)
Azalanstat	0.021 ± 0.006
Testosterone	18 ± 8
Miconazole	0.032 ± 0.005
Itraconazole	0.32 ± 0.09
Ketoconazole	0.056 ± 0.018
Fluconazole	>100

Conclusion

We have cloned and expressed active human CYP51 with human CYP reductase and developed a fast, robust CYP51 inhibition assay using BOMCC as substrate. Data gained using azalanstat as a positive inhibition control over an extended period has shown that the assay is robust and gives good reproducibility both within a single batch of enzyme and across different batches of enzyme.

Refs

Lamb, D. C., Kelly D. E., Waterman M. R., Stromstedt M., Rozman, D. & Kelly S. L. (1999) Yeast 15 (9), p 755-63
Characteristics of the heterologously expressed human lanosterol 14α-demethylase and inhibition of the purified human and C. albicans CYP51 with azole antifungal agents.

Riley J., Brand S., Voice M., Caballero I., Calvo D. & Read K. (2015) PLoS Negl Trop Dis 9(9)
Development of a Fluorescence-based Trypanosoma cruzi CYP51 Inhibition Assay for Effective Compound Triaging in Drug Discovery Programmes for Chagas Disease.

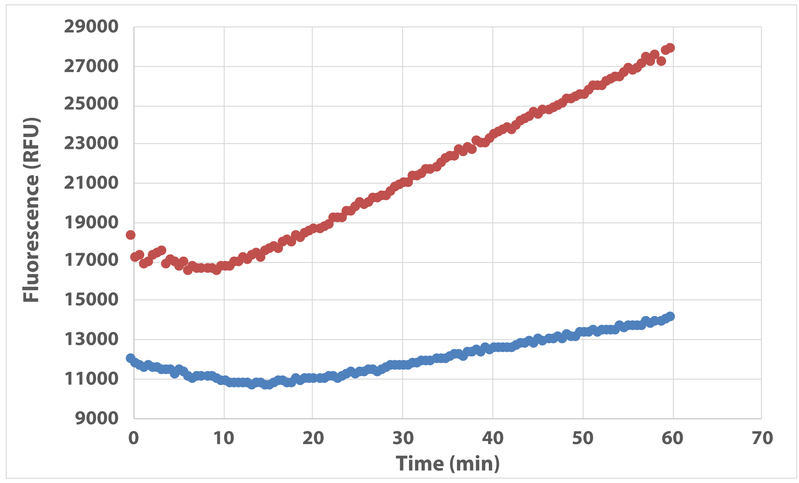
Methods

BOMCC was obtained from ThermoFisher, UK, NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Molekula UK, all other chemicals were obtained from either Sigma, VWR or Fisher Scientific, UK.

The cDNA coding for human CYP51 was cloned into pCW with an N terminal ompA leader together with the cDNA coding for human NADPH P450 reductase, each under the control of a *tac tac* promoter. *E. coli* JM109 transformed with the co-expression plasmid was used for protein expression in 10 litre fermenters using standard conditions. CYP levels were determined spectrophotometrically, the NADPH P450 reductase was quantified by determining the NADPH cytochrome *c* reductase activity.

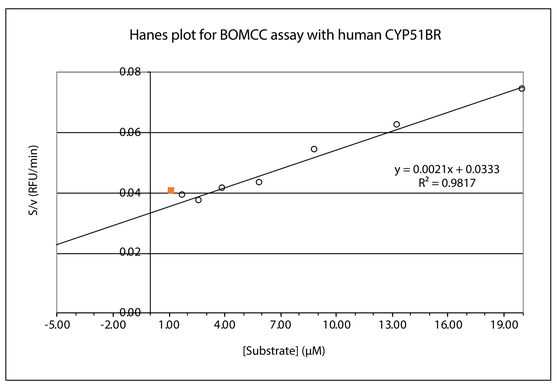
Plate reader assays were carried out at 37°C in 96 well plates in 42 mM potassium phosphate pH 7.4, 4.2 mM $MgCl_2$ with a final P450 concentration of 30 nmol/ml. The reaction was started by the addition of NADPH generating system to the wells. The plate was incubated at 37°C and, after a 20 min preincubation, the fluorescence was monitored approximately every 90 seconds over a period of 30 min. The rate of increase of fluorescence was plotted in Excel. Potential inhibitors were dissolved in 50% v/v DMSO / water (final concentration in the assay 1 % v/v). IC_{50} values were calculated in Excel using XLfit software (IDBS).

Fig. 1 Time linearity in duplicate of CHC generation by CYP51 at 37°C showing the lag after adding NADPH



20 pmol/ml CYP51 was incubated with 20 µM (red) and 9 µM (blue) BOMCC at 37°C. After a 5 minute preincubation at 37°C NADPH generating system was added and the fluorescence was measured

Fig. 2 Kinetic analysis of BOMCC metabolism by CYP51



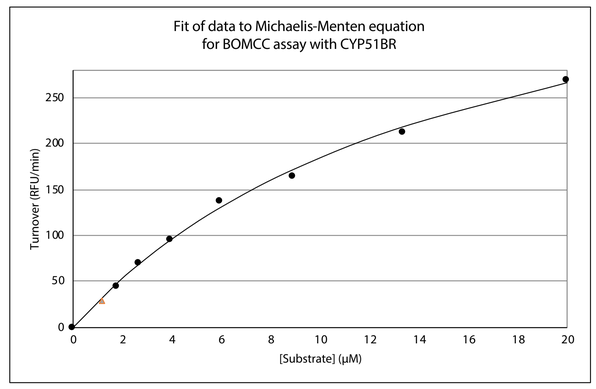
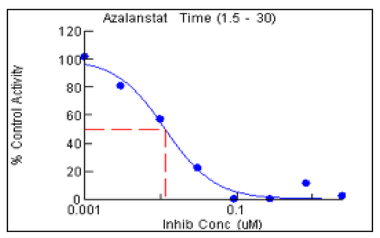
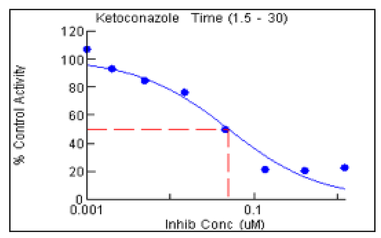


Fig. 3 Typical IC_{50} plots for CYP51 using BOMCC as substrate.





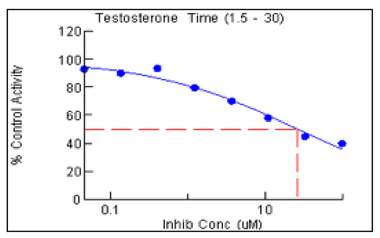
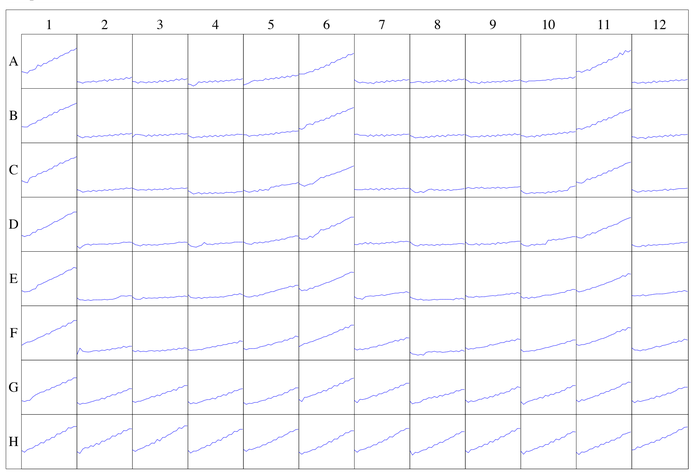


Fig. 3 Typical raw data from the screening assay - Fluorescence vs time



Row A 5 µM inhibitor diluted 1 in 3 down the plate to row G
Row H No inhibitor control specific to the column.
Column 1 No inhibitor control, the mean rate of all of the wells in this column is used as the 100% control activity.
Column 2 +ve control (Azalanstat)
Columns 3 & 8 Test compound 1
Columns 4 & 9 Test compound 2
Columns 5 & 10 Test compound 3
Columns 6 & 11 Test compound 4
Columns 7 & 12 Test compound 5