Validation of *E.coli*-expressed P450 Enzymes for Inhibition Screening

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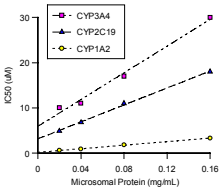
Introduction

GSK fluorescence substrates (FCA, BMC, MMC, DEF) and ethoxyresorufin (ER) have been extensively validated for cytochrome P450 inhibition using lymphoblast-expressed enzymes from BD Gentest, MA, USA (1-4). A limitation of these assays is the variable microsomal protein concentrations required for each P450 (Table 1). The effect of microsomal protein concentrations and non-specific binding of lipophilic compounds on *in vitro* IC50 values has been demonstrated.

Effect of microsomal protein on IC50 values



- CYP Inhibition with a lipophilic compound (clogP = 7.1)

Escherichia coli-expressed P450s from the LINK consortium have been characterised as surrogates for human liver enzymes (5). These P450s have high expression and stable plasmid constructs and are commercially available from Cypex Ltd, Dundee, UK.

The aim of the study was to validate the *E.coli*-expressed P450 with GSK substrates for P450 inhibition screening and to standardise the assay protein concentrations to 0.1 mg/mL.

Material and Methods

Validation Approach

- Compare P450 enzyme activities of Cypex *E.coli* bactosomes and BD Gentest microsomes using GSK substrates
- define amount of expressed P450 required for production of fluorescent metabolite (AFU)
- add control protein (no expressed P450), if required, to give 0.1 mg/mL
- Confirm time and protein linearity of metabolism
- Investigate enzyme kinetics with 0.1 mg/mL protein
- Determine IC50s for test compounds using the defined incubation conditions for Cypex *E.coli* and compare with BD Gentest IC50 values.

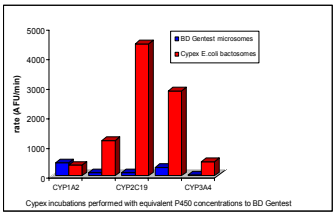
Experimental Conditions

Each substrate was incubated for 10 minutes at 37C with protein (*E.coli* bactosomes expressing human CYP450 cDNA ± control *E.coli* bactosomes, Cypex Ltd) and 0.4 mM NADPH regenerating system. The production of fluorescent metabolite was monitored at the appropriate excitation and emission wavelengths.

Results

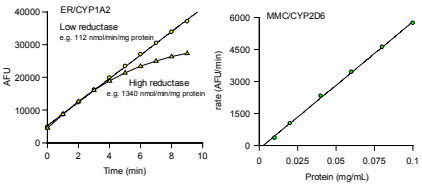
Comparison of Cypex *E.coli* and BD Gentest enzyme activities

- Enzyme activities with Cypex *E.coli* were generally greater than BD Gentest



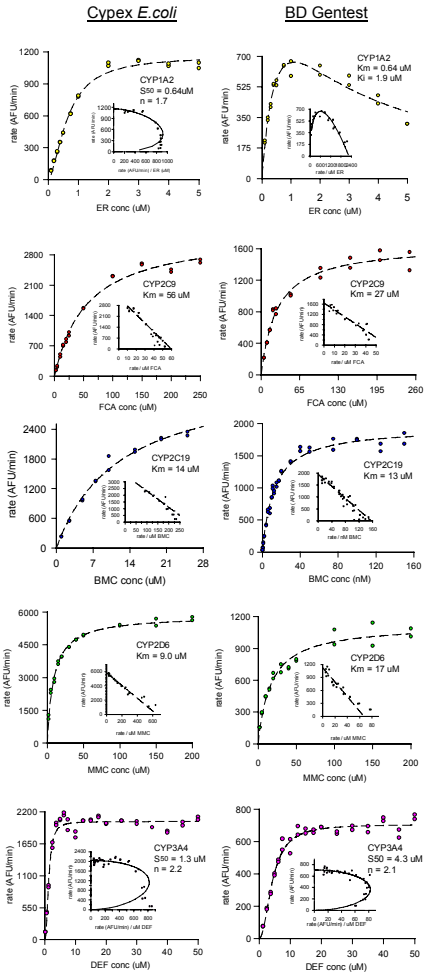
Linearity with time and protein

- ER/CYP1A2 was not linear in batches with high reductase c activity therefore low reductase c batches were used.
- All assays were linear over a 10 minute incubation
- All assays were linear up to 0.1 mg/mL protein



Comparison of enzyme kinetics for Cypex *E.coli* and BD Gentest

- Michaelis-Menten profiles were comparable for FCA/CYP2C9, BMC/CYP2C19 and MMC/CYP2D6
- Complex enzyme kinetics were observed for ER/CYP1A2 and DEF/CYP3A4
  - Atypical ER/CYP1A2 kinetics, dependent on enzyme source, have been reported (6)
- The effect of assay protein concentrations on apparent Km has previously been reported (7)



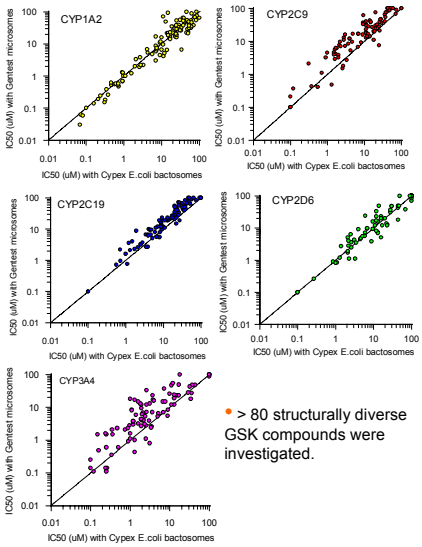
Defined incubation conditions for Cypex *E.coli* (Table 1)

Enzyme	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4
Substrate	ER	FCA	BMC	MMC	DEF
[S] (uM)	0.5	50	10	10	1
(approx. Km/S50)		(25)	(15)	(20)	(5)
Protein expressing specific CYP450 (mg/mL)	0.01	0.1	0.0006	0.06	0.06
	(0.1)	(0.4)	(0.2)	(0.2)	(0.16)
Control protein (mg/mL)	0.09	N/A	0.0994	0.04	0.04
Total potein (mg/mL)	0.1	0.1	0.1	0.1	0.1
		(0.4)	(0.2)	(0.2)	(0.16)
Incubation time (min)	10	10	10	10	10
Excitation λ (nm)	530	409	409	409	485
Emission λ (nm)	590	508	460	485	530

- Red indicates deviation from BD Gentest conditions (shown in brackets)

Correlation of Cypex *E.coli* and BD Gentest IC50 values

- Comparable (within 2-fold) IC50 values were obtained for ER/CYP1A2 (irrespective of different kinetic profiles), BMC/CYP2C19 and MMC/CYP2D6
- IC50 values for Cypex *E.coli* were on average 2.2 ± 1.3 and 2.6 ± 2.6 fold lower than BD Gentest for FCA/CYP2C9 and DEF/CYP3A4 respectively. This is a result of lower assay protein concentrations and less non-specific binding of lipophilic compounds



- > 80 structurally diverse GSK compounds were investigated.

Conclusions

- Appropriate assay conditions with GSK substrates have been successfully defined for *E.coli* P450 enzymes
- In general lower IC50 values are anticipated for FCA/CYP2C9 and DEF/CYP3A4 with *E.coli* P450 enzymes

References

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